

Enhanced PKC β II translocation and PKC β II-RACK1 interactions in PKC ϵ -induced heart failure: a role for RACK1

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Received 25 June 2001; accepted in final form 21 August 2001

Pass, Jason M., Jiuming Gao, W. Keith Jones, William B. Wead, Xin Wu, Jun Zhang, Christopher P. Baines, Roberto Bolli, Yu-Ting Zheng, Irving G. Joshua, and Peipei Ping. Enhanced PKC β II translocation and PKC β II-RACK1 interactions in PKC ϵ -induced heart failure: a role for RACK1. *Am J Physiol Heart Circ Physiol* 281: H2500–H2510, 2001.—Recent investigations have established a role for the β II-isoform of protein kinase C (PKC β II) in the induction of cardiac hypertrophy and failure. Although receptors for activated C kinase (RACKs) have been shown to direct PKC signal transduction, the mechanism through which RACK1, a selective PKC β II RACK, participates in PKC β II-mediated cardiac hypertrophy and failure remains undefined. We have previously reported that PKC ϵ activation modulates the expression of RACKs, and that altered ϵ -isoform of PKC (PKC ϵ)-RACK interactions may facilitate the genesis of cardiac phenotypes in mice. Here, we present evidence that high levels of PKC ϵ activity are commensurate with impaired left ventricular function ($dP/dt = 6,074 \pm 248$ mmHg/s in control vs. $3,784 \pm 269$ mmHg/s in transgenic) and significant myocardial hypertrophy. More importantly, we demonstrate that high levels of PKC ϵ activation induce a significant colocalization of PKC β II with RACK1 ($154 \pm 7\%$ of control) and a marked redistribution of PKC β II to the particulate fraction ($17 \pm 2\%$ of total PKC β II in control mice vs. $49 \pm 5\%$ of total PKC β II in hypertrophied mice), without compensatory changes of the other eight PKC isoforms present in the mouse heart. This enhanced PKC β II activation is coupled with increased RACK1 expression and PKC β II-RACK1 interactions, demonstrating PKC ϵ -induced PKC β II signaling via a RACK1-dependent mechanism. Taken together with our previous findings regarding enhanced RACK1 expression and PKC ϵ -RACK1 interactions in the setting of cardiac hypertrophy and failure, these results suggest that RACK1 serves as a nexus for at least two isoforms of PKC, the ϵ -isoform and the β II-isoform, thus coordinating PKC-mediated hypertrophic signaling.

cardiac phenotype; protein-protein interactions; hypertrophy

THE DEVELOPMENT OF CARDIAC hypertrophy may occur as a compensatory mechanism to counter increases in he-

modynamic load, aberrations in contractile performance, and/or lesions in the myocardium. The increase in heart size that accompanies compensatory cardiac hypertrophy is associated with normal levels of ventricular wall stress and cardiomyocyte function (14, 16, 26, 28). However, if the pathological stimuli for cardiac hypertrophy persist, the compensatory hypertrophy can progress into a decompensated heart failure that is associated with a high level of morbidity and mortality in humans (14, 16, 26, 28). For this reason, much effort has been expended to elucidate the intracellular signaling elements involved in the transmission of hypertrophic signals within the myocardium.

One such signaling element that has garnered considerable attention is the β II-isoform of protein kinase C (PKC β II) (38). With the use of a constitutively active PKC β II mutant, Kariya and colleagues (17, 18) have demonstrated that PKC β II activity is sufficient to activate the promoters of β -myosin heavy chain (β -MHC) and α -skeletal muscle actin (α -actin) in cardiac myocytes, two genes commonly upregulated in cardiac hypertrophy. Stimulation of these promoters occurs through PKC β II-mediated binding of the transcription enhancer factor-1 to the DNA sequence M-CAT (17–19). More recently, other investigations (41, 44) have shown that cardiac-specific transgenic overexpression of a constitutively active PKC β II cDNA in the mouse heart engenders cardiac hypertrophy with decreased cardiac performance, corroborating a role for PKC β II in modulating cardiac function in vivo. This PKC β II-induced pathological cardiac phenotype is, at least in part, mediated by phosphorylation of troponin-I, and amelioration of this phenotype is achieved through the administration of the PKC β inhibitor LY-333531 (41, 44). Interestingly, failure of the human myocardium is also associated with increased expression and enzymatic activity of PKC β II protein (4).

In addition to a role for PKC β II, an ever-growing body of data also implicates PKC ϵ in the development of cardiac hypertrophy. For example, pressure overload

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hypertrophy induces particulate-associated PKC ϵ , in addition to PKC β I and PKC β II (12). In mice that over-express G α q, the resultant cardiac hypertrophy and failure is associated with selective translocation of PKC ϵ (7). Moreover, whereas mice that express low levels of cardiac-specific active PKC ϵ display a normal yet cardioprotected phenotype (5, 27, 30), mice with moderate-to-high levels of PKC ϵ activity exhibit myocardial hypertrophy (11, 27, 42). However, unlike that for the PKC β II-induced heart failure, phosphorylation of troponin-I in this setting is controversial (42). Furthermore, the signaling mechanisms underlying PKC ϵ -induced cardiac dysfunction and hypertrophy remain largely unknown.

One mechanism by which PKC isoforms initiate signaling events involves interactions between PKC isoforms and their selective intracellular receptors for activated C kinase (RACKs) (6). Recent studies (23, 27, 45) have demonstrated that PKC-RACK interactions and RACK expression modulate PKC-mediated manifestation of cardiac phenotype. With the use of transgenic mice that harbor constitutively active PKC ϵ , we (27) have previously shown that PKC ϵ -induced cardiac hypertrophy and failure are congruous with increased expression of both the PKC β II-selective RACK, RACK1, and the PKC ϵ -selective receptor, RACK2. Most strikingly, in addition to the commonly recognized PKC ϵ -RACK2 interaction (6), PKC ϵ was also found to bind to RACK1 in mice with the cardiac hypertrophied phenotype, demonstrating a novel and functional role for PKC ϵ -RACK1 interactions in the myocardium (27). These findings suggest that, by interacting with RACK1, PKC ϵ activity can be redirected through a PKC β II hypertrophic signaling pathway (27), a phenomenon hereafter referred to as RACK-mediated PKC isoform switching.

In concert with the concept of RACK-mediated PKC isoform switching, we hypothesize that the PKC ϵ -mediated cardiac hypertrophied phenotype may be conferred through the synergistic effects of both PKC ϵ -RACK1 (27) and PKC β II-RACK1 interactions. That is, in addition to enhanced interactions between PKC ϵ and RACK1 (which would redirect PKC ϵ function through a PKC β II signaling pathway) (27), there would also be an increased translocation of PKC β II as a result of increased RACK1 expression. Accordingly, we examined PKC β II-RACK1 interactions and the subcellular distribution of PKC β II in PKC ϵ mice displaying cardiac hypertrophy and failure. We hereby present evidence that PKC ϵ activity is correlated with cardiac hypertrophy and failure in a dose-dependent fashion. Furthermore, our data show that among the 10 PKC isoforms expressed in the mouse myocardium (FVB/N strain), PKC β II is the only isozyme exhibiting a subcellular redistribution in the PKC ϵ hypertrophied mice. More importantly, increased interactions between PKC β II and RACK1 are concomitant with the enhanced RACK1 expression, implicating RACK1 as a key signaling element in the genesis of cardiac hypertrophy.

MATERIALS AND METHODS

The experimental protocols described herein were performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Publication No. 86-23).

Generation and characterization of PKC ϵ transgenic mouse lines. Three transgenic mouse lines expressing cardiac-targeted PKC ϵ mutants were studied. Standard techniques were used for the generation of these mice (39). Briefly, a cardiac-specific α -MHC promoter (39) was used to drive the expression of PKC ϵ cDNA mutants in FVB/N mice. An HA tag was inserted into the 5' end of all constructs, which allowed differentiation of transgenic expression from that of the endogenous PKC ϵ . Two of the mouse lines express different levels of a constitutively active PKC ϵ (AE-PKC ϵ), which is created by an A-to-E point mutation at the pseudosubstrate domain [amino acid (aa) 159] (29): one mouse line expresses low levels of the PKC ϵ transgenic protein (AE-PKC ϵ -L) (5, 27), whereas the other line expresses high levels of protein (AE-PKC ϵ -H) (27, 29). As noted previously (27), mice expressing high levels of AE-PKC ϵ suffer from significant myocardial dysfunction and sudden death, and do not survive past 13 wk of age. The third mouse line expresses a dominant-negative mutant of PKC ϵ (DN-PKC ϵ), which is generated by mutations at both the pseudosubstrate domain (A to E, aa 159) and the ATP binding site (K to R, aa 436) (27, 29). As described previously (27), the level of DN-PKC ϵ protein expression (\sim 35-fold of control) is comparable to the level of AE-PKC ϵ protein found in AE-PKC ϵ -H mice (\sim 39-fold of control). The DN-PKC ϵ line is free of cardiac hypertrophy and does not show any phenotypic differences when compared with nontransgenic mice at 3, 10, and 20 wk of age. Transgenic positives were identified with the use of polymerase chain reaction (PCR) and Southern blotting analyses (27). Age-matched (9- to 12-wk old) transgenic negative littermates were used as controls (27).

Histology. After excision, hearts from control (nontransgenic), AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice were rinsed with 30 mM KCl and immediately immersion-fixed in 10% neutral buffered formalin. The hearts were then dehydrated through a graded series of alcohol and embedded in paraffin, and serial sections (5 μ m) were made every 75 μ m from apex to base. Adjacent sections were mounted onto slides and stained with hematoxylin-eosin for overall morphology and Masson's trichrome stain for collagen. Slides were then subjected to histopathological observation in a blinded fashion by a qualified pathologist.

Analysis of α -skeletal actin and MHC protein content. Expression of α -skeletal actin was determined using α -skeletal actin-specific antibodies (Sigma) and standard Western immunoblotting techniques (31). To determine the relative levels of α -MHC and β -MHC, equivalent amounts of myocardial homogenate were electrophoresed on 7% polyacrylamide gels (PAGE) containing 10% glycerol and 0.2% SDS to resolve the α -MHC and β -MHC isoforms (15). The gels were fixed in 30% methanol, 10% acetic acid for 20 min, and stained with brilliant blue G-colloidal protein stain (Sigma). Proteins corresponding to the α -MHC and β -MHC were quantified.

Assessment of cardiac contractile function. PKC ϵ transgenic mice and nontransgenic controls were anesthetized with intraperitoneal injections of pentobarbital sodium (40 μ g/g of body wt); additional doses were given during the protocol to maintain adequate anesthesia. The temperature of the anesthetized mice was maintained with a thermister-

regulated heating pad. Endotracheal intubation was performed via a cervical incision. The right carotid artery was isolated and a catheter was advanced into the left ventricle (LV). Aortic and left ventricular pressure was determined (Digi-Med HPA- τ and Digi-Med System Integrator). The right jugular vein was cannulated for delivery of either vehicle, isoproterenol (β -adrenergic receptor agonist), or angiotensin II. The mice were allowed to stabilize after the completion of the surgery and before the experimental protocol. To assess LV contractile function, progressive doses of either isoproterenol (50, 100, 500, 1,000, and 5,000 pg) or angiotensin II (0.2, 1, 5, and 10 ng) were administered. LV variables [heart rate, LV systolic pressure, LV end-diastolic pressure, LV diastolic pressure, dP/dt (rate of developed pressure), negative dP/dt ($-dP/dt$), time constant (τ), duration of contraction, duration of one-half relaxation, and duration of relaxation] were determined continuously and simultaneously. Animals were allowed to recover for at least 20 min after each dose. Baseline values before infusion of each dose of drug and peak value within 1–2 min after administration of each dose of drug were collected. At the completion of the experiments, mice were euthanized. The heart, lungs, and liver were immediately excised, weighed, and frozen for histological analyses.

Quantitative immunoblotting of PKC isoforms. Frozen myocardial tissue samples from FVB/N mice were processed as previously described and protein concentration was determined (31). For quantitative Western immunoblotting, increasing amounts of human recombinant PKC isoform protein (α , β I, β II, γ , ϵ , δ , θ , and ζ) (Calbiochem) were loaded onto the same SDS-PAGE gel along with a given amount of total myocardial tissue homogenate from five control hearts. Because basic local alignment search tool sequence alignment revealed that the antibody hybridization sequence is over 99% homologous between human and mouse PKC isoforms examined (except PKC δ , which shows \sim 80% homology), we anticipate that the antibody affinity is equivalent between human recombinant PKCs and the mouse myocardial PKCs (31).

To ensure equal loading of myocardial protein samples, Ponceau stain of nitrocellulose membranes was quantified by densitometric scanning (31). Antibodies against PKC isoforms α , γ , ϵ , θ , ζ , ι/λ , and μ (Transduction Laboratories); PKC isoforms β I, β II (Sigma); and PKC isoforms η and δ (Santa Cruz Biotechnology) were used, along with standard Western immunoblotting techniques to detect the PKC isoforms (31). The enhanced chemiluminescence signals generated by the recombinant proteins were used to construct dose-response curves for the various PKC isoforms. The dose-response curves were then used to determine the absolute protein amount of each PKC isoform in the mouse myocardium, which is reported as picograms of the PKC isoform per microgram of myocardial protein.

Coimmunoprecipitation. Immunoprecipitation experiments were carried out as described previously (27). Negative controls were conducted as follows. Samples were precleared with nonimmune agarose beads. IgG coupled to agarose beads was substituted for anti-PKC β II antibodies and was also used for negative controls (27, 30). For each reaction, 4 μ g of anti-PKC β II antibodies (Sigma) were incubated with 50 μ l of protein A/G-agarose beads (Santa Cruz) for 20–40 min at 4°C. The protein A/G-agarose-anti-PKC β II complex was washed three times with phosphate-buffered saline containing 0.1% Triton X-100. The protein A/G-anti-PKC β II complex was then incubated with 500- μ g protein of myocardial tissue homogenate overnight at 4°C, washed four times with phosphate-buffered saline containing 0.1% Triton X-100, and then

subjected to Western immunoblotting using RACK1 antibodies (Transduction Laboratories) or PKC β II antibodies (Sigma).

Statistical analyses. The data are expressed as means \pm SE. For the determination of α/β MHC, the relative levels of protein were compared using an unpaired two-tailed Student's *t*-test. For the analysis of cardiac function, all data were analyzed using one-factor or two-factor analysis of variance. When necessary, post hoc comparisons were performed with the use of a Newman-Keuls test. Differences were regarded as significant at the $P < 0.05$ probability level.

RESULTS

PKC ϵ expression and activity in PKC ϵ transgenic mice. To determine the direct effect of sustained PKC ϵ activity on the cardiac phenotype, we have developed two transgenic mouse lines that express different levels of active PKC ϵ (5, 27, 47). Two independently derived FVB/N transgenic founders were produced, containing either 8 (AE-PKC ϵ -L) or 35 (AE-PKC ϵ -H) copies of the AE-PKC ϵ transgene in addition to the endogenous PKC ϵ gene, as assessed by Southern blot analysis. As reported previously (27), AE-PKC ϵ -L are associated with a \sim 2.3-fold increase in total PKC ϵ activity whereas AE-PKC ϵ -H result in a \sim 4.5-fold increase in total PKC ϵ activity. Previous studies (27) have shown that changes in PKC ϵ expression in the transgenic hearts are localized in cardiac myocytes. A PKC ϵ transgenic mouse line that expresses a dominant negative mutant of PKC ϵ (27) was developed to discern 1) whether activity of PKC ϵ is necessary to confer alterations in cardiac phenotype, and 2) whether increased PKC ϵ protein expression by itself, without enhancing the kinase activity of this enzyme, is sufficient to modify cardiac phenotype (27). FVB/N founders were produced containing 73 copies of DN-PKC ϵ transgene. PKC ϵ activity in DN-PKC ϵ mice is significantly decreased by \sim 50% of control (27).

The effect of PKC ϵ activity on the expression of α -skelletal actin and MHCs. To characterize whether activation of PKC ϵ modulates the expression of the fetal gene program, we first examined the expression of proteins commonly modified in the development of cardiac hypertrophy (15), namely α -actin, α -MHC, and β -MHC.

In AE-PKC ϵ -L mice, we found that α -actin expression was unmodified when compared with controls (nontransgenic), and β -MHC expression was not detected. The heart weight-to-body weight ratio was also unaltered when compared with that of controls (4.5 ± 0.2 in control mice vs. 4.4 ± 0.2 in AE-PKC ϵ -L). Similarly, in DN-PKC ϵ mice, there was no change in α -actin expression, no detectable expression of β -MHC, and no difference in heart weight-to-body weight ratio (4.5 ± 0.4 in control mice vs. 4.8 ± 0.3 in DN-PKC ϵ mice).

Conversely, in AE-PKC ϵ -H mice, we found that α -actin expression was significantly elevated ($164 \pm 8\%$ of control; $P < 0.05$) (Fig. 1A). Additionally, AE-PKC ϵ -H mice were found to possess a reduced level of α -MHC expression ($41 \pm 4\%$ of controls; $P < 0.05$), and significant levels of β -MHC were detected (Fig. 1B). The alterations in protein expression observed in AE-

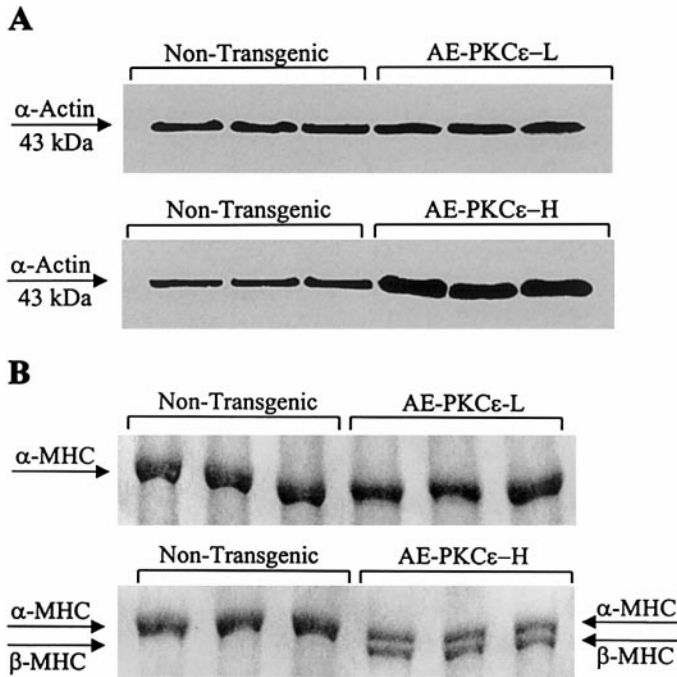


Fig. 1. Active protein kinase C ϵ -isoform mouse line created by A-to-E point mutation (AE-PKC ϵ -H) mice display increased α -skeletal actin, α -myosin heavy chain (α -MHC), and β -MHC expression. Equivalent amounts of myocardial tissue from control (nontransgenic), low-levels of PKC ϵ transgenic protein (AE-PKC ϵ -L), high levels of PKC ϵ transgenic protein (AE-PKC ϵ -H), and dominant negative PKC ϵ (DN-PKC ϵ) mice were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in methods. *A*: representative Western immunoblots of α -actin expression in control, AE-PKC ϵ -L, and AE-PKC ϵ -H. *B*: representative SDS-PAGE gels demonstrating α -MHC and β -MHC expression in control, AE-PKC ϵ -L, and AE-PKC ϵ -H mice. A total of 6 animals were examined in each group.

PKC ϵ -H mice were also congruous with an increased heart weight-to-body weight ratio (4.5 ± 0.2 in controls vs. 7.4 ± 0.5 in AE-PKC ϵ -H; $P < 0.05$).

Myocardial histological analysis of PKC ϵ transgenic mice. To further characterize the cardiac phenotype of PKC ϵ transgenic mice, we performed histological anal-

yses of myocardial tissue. Briefly, hearts from control and transgenic mice were paraffin embedded and cross-sectioned, and serial sections were stained with hematoxylin-eosin or trichrome.

We found that there were no significant increases in the extent of fibrosis, myocardial disarray, or myocyte hypertrophy in AE-PKC ϵ -L transgenic hearts relative to controls (Fig. 2). More importantly, heart weight-to-body weight ratios remained similar to controls at 20 wk of age, indicating no cardiac hypertrophy in AE-PKC ϵ -L mice at this age (data not shown). Furthermore, atrial thrombosis or calcification and gross cardiac hypertrophy were not discernable. Histological examination of DN-PKC ϵ myocardial tissue revealed normal cardiac cell morphology (data not shown).

In AE-PKC ϵ -H mice, the most prominent pathohistological feature was a widely distributed perimyocardial fibrosis. There were no large patches of replacement fibrosis. There was, however, a marked myocyte disarray and hypertrophy, and many of the hypertrophied cardiomyocytes had enlarged nuclei (Fig. 2). In addition, the occurrence of multinucleate cardiomyocytes having four or more nuclei was enhanced in the transgenic relative to controls (data not shown). Gross observation of hearts from the AE-PKC ϵ -H transgenic mice showed clear cardiac hypertrophy relative to controls. AE-PKC ϵ -H mice also showed organization of large atrial thrombi associated with apparent calcification.

Effect of PKC ϵ activity on cardiac contractile function. Hemodynamic indices for control (nontransgenic), AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice were determined. Among all of the hemodynamic parameters examined (Table 1), there were no significant differences between the control mice versus both the AE-PKC ϵ -L and DN-PKC ϵ mice (Table 1). In contrast, AE-PKC ϵ -H mice exhibited a depressed $-dp/dt$ in addition to attenuation of basal LV dp/dt , indicating an impaired rate of ventricular relaxation (Table 1). Moreover, these mice showed depressed LV peak systolic

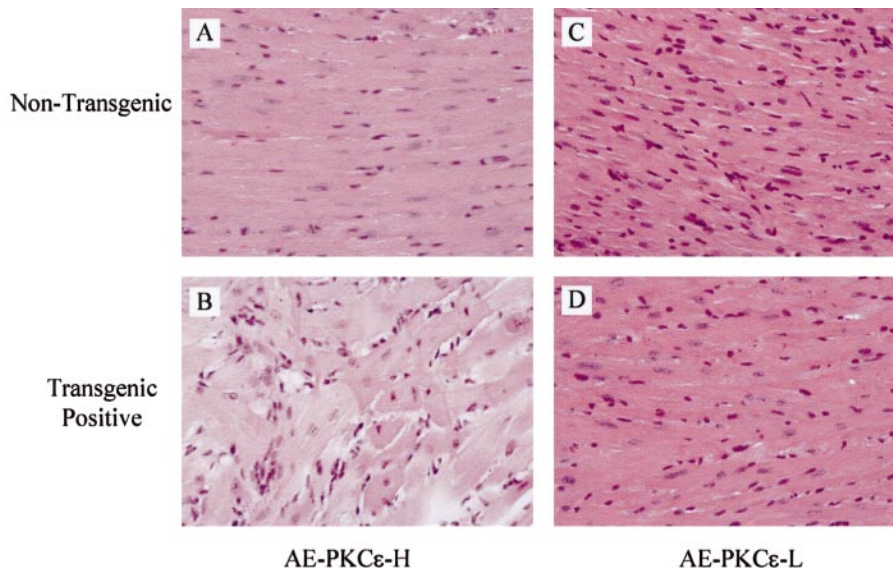


Fig. 2. Histological analyses of myocardial tissue from PKC ϵ transgenic mice. Serial sections of control (nontransgenic), AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice paraffin-embedded myocardium were subjected to histopathological observation after hematoxylin-eosin or trichrome staining. *A* and *C*: nontransgenic myocardium with normal histology. *B*: AE-PKC ϵ -H mice display a widely distributed perimyocardial fibrosis with myofiber disarray and hypertrophy. *D*: AE-PKC ϵ -L mice exhibit a mild histology with no significant levels of myofiber disarray, myocyte hypertrophy, or myocardial fibrosis. DN-PKC ϵ mice possessed normal histology (data not shown).

Table 1. Hemodynamic indices in PKC ϵ transgenic mice

	Nontransgenic (n = 12)	AE-PKC ϵ -H (n = 7)	AE-PKC ϵ -L (n = 7)	DN-PKC ϵ (n = 7)
Heart rate, beats/min	400.53 \pm 0.19	400.54 \pm 0.32	400.38 \pm 0.06	400.44 \pm 0.11
LV peak systolic pressure, mmHg	97.73 \pm 2.52	78.21 \pm 4.95*	101.28 \pm 2.15	92.42 \pm 1.85
LV end-diastolic pressure, mmHg	5.73 \pm 1.41	11.58 \pm 1.72*	4.18 \pm 0.54	4.21 \pm 0.59
LV diastolic pressure, mmHg	2.73 \pm 0.91	7.98 \pm 1.29*	1.75 \pm 0.51	1.94 \pm 0.37
dP/dt, mmHg/s	6,074 \pm 248	3,784 \pm 269*	6,188 \pm 299	5,771 \pm 291
-dP/dt, mmHg/s	4,716 \pm 224	2,846 \pm 248*	5,230 \pm 272	4,486 \pm 116

Values are means \pm SE; n, no. of mice. AE-PKC ϵ -H, constitutively active protein kinase C ϵ -isoform that is created by a high protein A-to-E point mutation; AE-PKC ϵ -L, low-level protein AE-PKC ϵ ; DN, dominant negative; LV, left ventricle; -dP/dt, negative dP/dt. Control (nontransgenic), AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice were subjected to in vivo cardiac function analyses as described in MATERIALS AND METHODS. Of the mice examined, only the AE-PKC ϵ -H mice displayed impaired cardiac function relative to controls, exhibiting decreased LV peak systolic pressure, dP/dt, -dP/dt with increased LV end-diastolic pressure and LV diastolic pressure. * P < 0.05 vs. control.

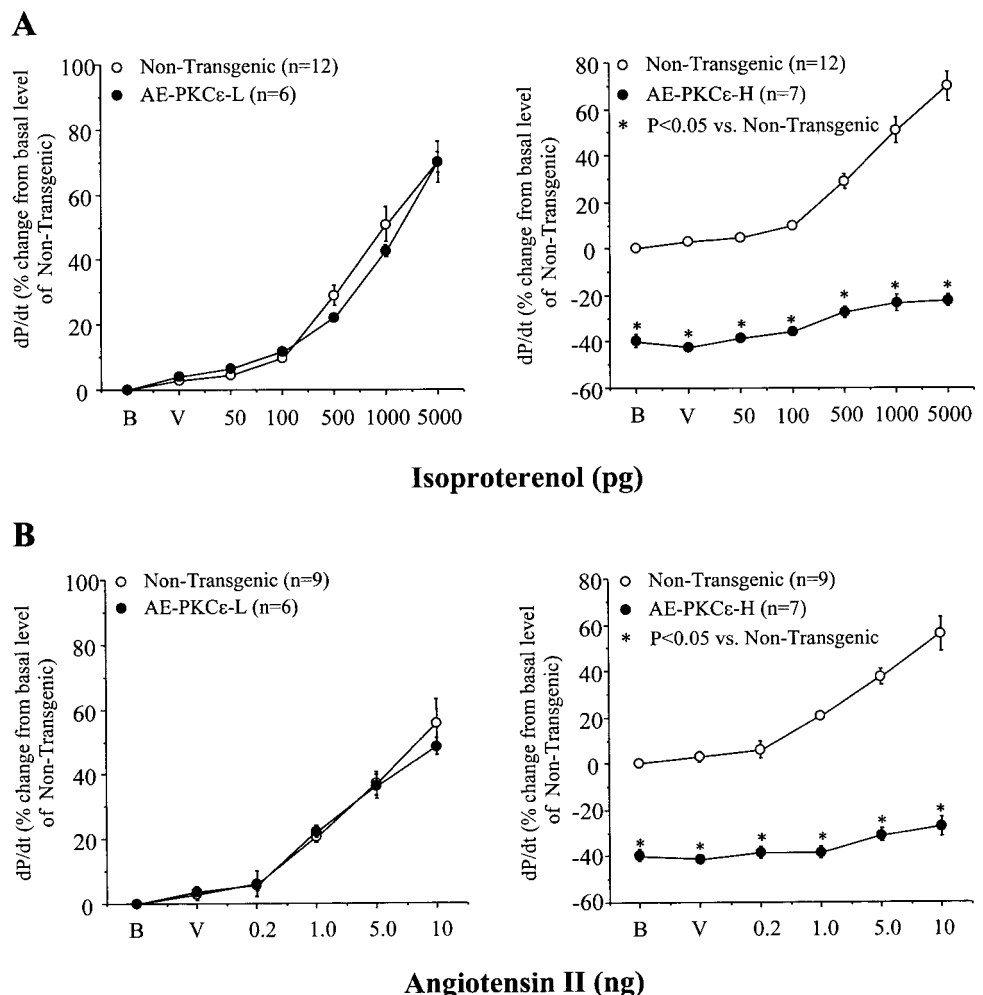
pressure, elevated LV end-diastolic pressure, and elevated LV diastolic pressure (Table 1).

Furthermore, using LV dP/dt as an index of cardiac contractility, we assessed cardiac function under basal, isoproterenol-challenged, or angiotensin-challenged conditions in control, AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice. Under basal conditions, we detected no discernable differences in contractility among control and AE-PKC ϵ -L mice (Fig. 3 and Table 1). Similarly, there were no differences in contractility among control

and DN-PKC ϵ mice (data not shown). However, in AE-PKC ϵ -H mice, basal cardiac contractility was significantly depressed (Fig. 3 and Table 1).

When AE-PKC ϵ -L and DN-PKC ϵ mice were challenged with increasing doses of isoproterenol, there was a marked and dosage-dependent elevation in dP/dt that was indistinguishable from that observed in controls (Fig. 3A, data not shown for DN-PKC ϵ). Conversely, isoproterenol-induced increases in cardiac contractility were significantly depressed in AE-PKC ϵ -H

Fig. 3. Assessment of cardiac function in PKC ϵ transgenic mice. Relative changes in left ventricle (LV) rate of developed pressure (dP/dt) in response to isoproterenol or angiotensin II challenge were used to evaluate cardiac contractility in control (nontransgenic), AE-PKC ϵ -L, and AE-PKC ϵ -H mice. A: progressive doses of isoproterenol induce similar increases in LV dP/dt among control and AE-PKC ϵ -L mice. This response was significantly attenuated in AE-PKC ϵ -H mice. It should also be noted that baseline (B) dP/dt is depressed in AE-PKC ϵ mice relative to controls. V, vehicle. B: progressive doses of angiotensin II increase LV dP/dt among controls and AE-PKC ϵ -L mice. The response to angiotensin II as well as baseline LV dP/dt is depressed in AE-PKC ϵ -H mice. Data are means \pm SE.



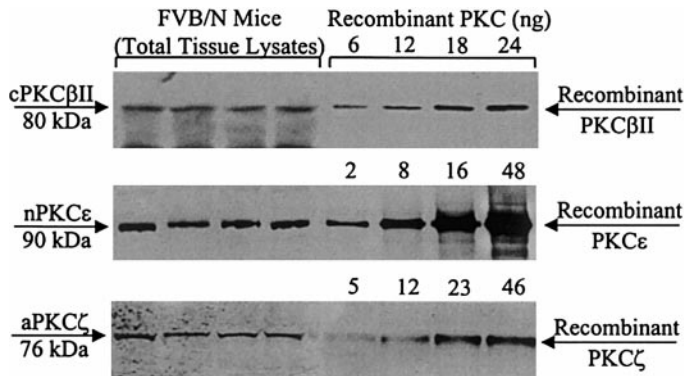


Fig. 4. Absolute protein content of PKC isoforms in the FVB/N mouse heart. Equivalent amounts of cardiac protein from control (nontransgenic) mice were subjected to SDS-PAGE and Western immunoblot analyses along with increasing amounts of recombinant PKC isoforms (α , β I, β II, γ , ϵ , δ , θ , or ζ). Representative immunoblots for the determination of PKC β II, PKC ϵ , and PKC ζ protein content are shown. Five control FVB/N mice were used in each experiment.

mice (Fig. 3A). Treatment with increasing doses of angiotensin II produced similar changes in contractile response in control, AE-PKC ϵ -L, and DN-PKC ϵ , whereas the angiotensin II response was significantly attenuated in AE-PKC ϵ -H mice (Fig. 3B, data not shown for DN-PKC ϵ).

Taken together with the above data regarding the expression of α -actin, α / β -MHC, as well as the histological assessment and hemodynamic values, we conclude that the AE-PKC ϵ -L and DN-PKC ϵ lines represent an unaltered cardiac phenotype. However, the AE-PKC ϵ -H line, which contains high levels of activated PKC ϵ , is characterized as having a severe cardiac hypertrophy with extensive histopathology and

cardiac failure. In fact, AE-PKC ϵ -H mice frequently died suddenly and did not survive past 13 wk of age.

Quantitative assessment of the PKC expression profile in the mouse heart. Several studies (3, 6, 25, 30) demonstrate that activation of individual PKC isoforms is important in mediating cardiac function in the mouse heart. However, virtually no information is available regarding the stoichiometry of the PKC isoform expression profile in the mouse myocardium. To assess the stoichiometric relationships among the PKC isoforms in the mouse heart, we performed quantitative Western immunoblotting to determine the absolute protein content of PKC isoforms in hearts of control mice (Fig. 4). As shown in Table 2, the majority of PKC present in the FVB/N mouse heart belongs to the cPKCs (α , β I, β II, and γ) (\sim 813 pg PKC/ μ g of total protein) with PKC α accounting for 59% of total cPKC. As for the nPKCs (ϵ , δ , and θ) (\sim 165 pg PKC/ μ g of total protein), PKC θ was the most abundant (41% of total nPKC) (Table 2). An ample amount of PKC ϵ (24% of total nPKC) was also identified (Fig. 4), whereas the expression of PKC η was not detected. The mouse heart also expressed an abundant amount of PKC ζ (Fig. 4 and Table 2). Whereas the expression of PKC ι / λ and PKC μ isoforms was detected (data not shown), the absolute protein content of these isoforms was not determined because the corresponding recombinant proteins are not available.

Enhanced particulate association of PKC β II in mice with cardiac hypertrophy and failure. Several lines of evidence indicate an important role for PKC β II in the development of cardiac hypertrophy and failure (4, 17–19, 41, 44). Thus we examined the subcellular localization of this PKC isoform as well as other PKC

Table 2. Content of PKC isoforms in the FVB/N mouse heart

PKC Isoform, MM	Protein Content, pg PKC/ μ g Tissue Protein	Positive Control
cPKCs, kDa		
α (82)	501 \pm 57	Recombinant PKC α , Jurkat cell lysate
β I (80)	1.9 \pm 0.3	Recombinant PKC β I, Jurkat cell lysate
β II (80)	31.4 \pm 1.9	Recombinant PKC β II, Jurkat cell lysate
γ (80)	321 \pm 27	Recombinant PKC γ , Jurkat cell lysate
nPKCs, kDa		
δ (78)	57.8 \pm 3.8	Recombinant PKC δ , Jurkat cell lysate
ϵ (90)	40.3 \pm 4.5	Recombinant PKC ϵ , Jurkat cell lysate
η (77)	Absent	Jurkat cell lysate
θ (79)	67.1 \pm 6.2	Recombinant PKC θ , Jurkat cell lysate
aPKCs, kDa		
ζ (72)	96.1 \pm 18.5	Recombinant PKC ζ , Jurkat cell lysate
ι / λ (74)	Present	Jurkat cell lysate
PKC μ , kDa (115)	Present	Jurkat cell lysate

Values are means \pm SE. MM, molecular mass; Present, protein content was not determined due to lack of available recombinant proteins, but expression was detected with isoform-specific antibodies; Absent, expression was not detected in the FVB/N myocardium. The absolute protein content for cPKCs (α , β I, β II, γ), nPKCs (ϵ , δ , θ), and aPKC ζ was quantified using human recombinant PKC proteins and the following PKC isoform-specific antibodies: PKC α [Transduction Laboratories, immunogen 270–427 amino acid (aa), monoclonal], PKC β I (Sigma, immunogen 658–671 aa, monoclonal), PKC β II (Sigma, immunogen 660–673 aa, monoclonal), PKC ϵ (Transduction Laboratories, immunogen 1–175 aa, monoclonal), PKC γ (Transduction Laboratories, immunogen 489–697 aa, monoclonal), PKC δ (Santa Cruz, immunogen 657–676 aa, polyclonal), PKC θ (Santa Cruz, immunogen 656–671 aa, polyclonal), PKC ζ (Transduction Laboratories, immunogen 394–590 aa, monoclonal). Expression of aPKC ι / λ (Transduction Laboratories, immunogens 404–587 aa and 397–558 aa, monoclonal) and PKC μ (Transduction Laboratories, immunogen 314–517 aa, monoclonal) was also detected. PKC η (Santa Cruz, immunogen 669–683, polyclonal and Biogenesis, immunogen 673–680 aa, polyclonal) was not detected.

isoforms (α , β I, β II, δ , γ , θ , ζ , ι/λ , μ , and η) in control (nontransgenic), AE-PKC ϵ -L, AE-PKC ϵ -H, and DN-PKC ϵ mice. As expected for AE-PKC ϵ -L and DN-PKC ϵ mice, in which there is no manifestation of cardiac pathology, we found no significant differences in the subcellular distribution of any of the PKC isoforms examined except for PKC ϵ (27) compared with controls (data not shown). In marked contrast to AE-PKC ϵ -L and DN-PKC ϵ mice, we found that, in addition to PKC ϵ , the PKC β II isoform in AE-PKC ϵ -H mice was significantly translocated to the particulate fraction ($17 \pm 2\%$ of total PKC β II in control mice vs. $49 \pm 5\%$ of total PKC β II in AE-PKC ϵ -H mice; $P < 0.05$) (Fig. 5, A and B). Thus these data indicate that activation of PKC β II may serve as a mechanism whereby PKC ϵ activity modulates cardiac hypertrophy and failure in AE-PKC ϵ -H mice.

Cardiac hypertrophy and failure is congruous with increased PKC β II-RACK1 interactions. Several studies (22, 35, 26) have demonstrated that binding of PKC β II to RACK1 is required for PKC β II activation. In view of our present data demonstrating that PKC β II is selectively translocated in AE-PKC ϵ -H mice, we examined PKC β II-RACK1 interactions in control (nontransgenic), AE-PKC ϵ -L, and AE-PKC ϵ -H mice via coimmunoprecipitation. We found that only a relatively small amount of PKC β II was associated with RACK1 in controls (Fig. 6A) and there was no difference among controls and AE-PKC ϵ -L mice (data not shown). However, the amount of RACK1 coimmunoprecipitated with PKC β II was significantly elevated in AE-PKC ϵ -H mice ($154 \pm 7\%$ of control; $P < 0.5$) (Fig. 6A), illustrating enhanced PKC β II-RACK1 interactions in PKC ϵ -associated cardiac hypertrophy and failure. In control

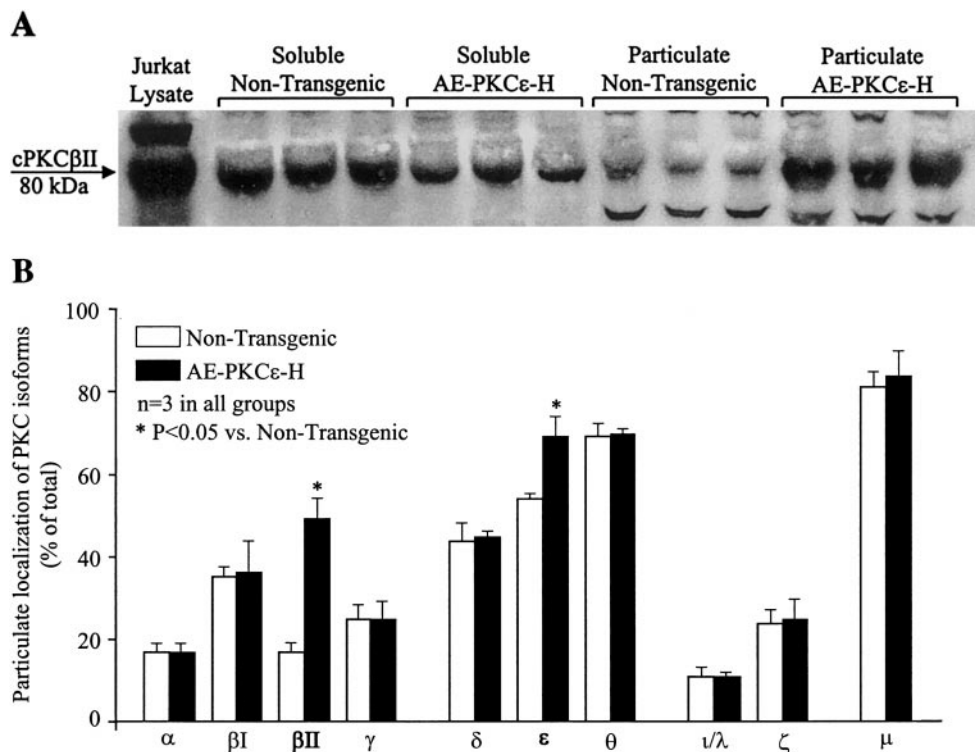
immunoprecipitation experiments where IgG was substituted for PKC β II antibodies, RACK1 was not detected (Fig. 6B, top). Furthermore, there was no interaction of PKC β II with the IgG/bead immunocomplex from nontransgenic or AE-PKC ϵ -H mice (Fig. 6B, middle and bottom).

DISCUSSION

Building on our previous findings (27) regarding increased RACK1 expression and PKC ϵ -RACK1 interactions in AE-PKC ϵ -H transgenic mice, this investigation presents the first evidence demonstrating that, concomitant with the RACK-mediated PKC isoform switching phenomenon (27), there exists enhanced PKC β II translocation and PKC β II-RACK1 interactions in PKC ϵ -associated cardiac hypertrophy and failure. More importantly, in conjunction with previous evidence documenting a role for PKC β II in mediating cardiac contractile dysfunction (38), our data indicate that activation of PKC β II and PKC β II-RACK1 interactions may serve as a key signaling mechanism for the manifestation of a PKC ϵ -dependent hypertrophic phenotype.

Several novel findings were identified in this study. First, we have demonstrated that direct activation of PKC ϵ at high levels is correlated with cardiac hypertrophy and profound cardiac failure, a phenotype that is markedly different from that found with moderate levels of PKC ϵ activation (42). Second, with the use of transgenic DN-PKC ϵ mice, we have demonstrated that attenuation of basal PKC ϵ activity does not affect cardiac size, histology, and function, and that PKC ϵ -associated changes in cardiac phenotype are a consequence

Fig. 5. Cardiac hypertrophy and failure in AE-PKC ϵ -H mice is associated with selective translocation of PKC β II. Cardiac protein samples from control (nontransgenic) and AE-PKC ϵ -H mice were fractionated into soluble and particulate fractions, as described in MATERIALS AND METHODS. Homogenate from each fraction was then subjected to immunoblot analysis for PKC α , β I, β II, γ , δ , ϵ , θ , ζ , ι/λ , μ , and η . A: representative immunoblot depicting the subcellular redistribution of PKC β II to the particulate fraction in AE-PKC ϵ -H is shown. Jurkat cell lysate was used as a positive control for PKC β II expression. B: histogram illustrating that activation of PKC ϵ in AE-PKC ϵ -H mice is associated with a significant translocation of PKC ϵ and PKC β II to the particulate fraction. The distribution of PKC α , β I, γ , δ , θ , ζ , ι/λ , and μ is unaltered in AE-PKC ϵ -H mice, whereas expression of η was not detected. Data are means \pm SE.



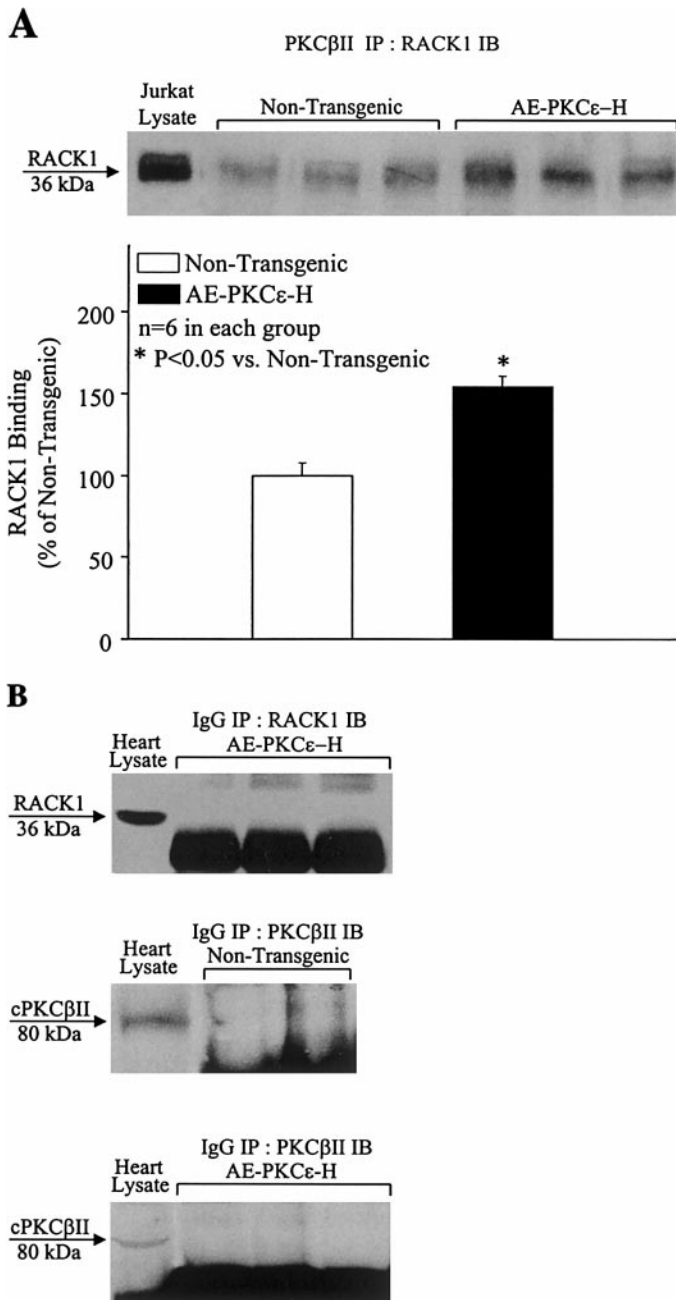


Fig. 6. Cardiac hypertrophy and failure in AE-PKC ϵ -H mice is commensurate with increased PKC β II-RACK1 protein-protein interactions. Cardiac protein samples from control (nontransgenic) and AE-PKC ϵ -H mice were immunoprecipitated (IP) with either anti-PKC β II or IgG (nonimmune controls) before Western immunoblot (IB) analysis with either anti-RACK1 or anti-PKC β II. *A*: representative immunoblot and associated histogram demonstrating increased protein-protein interactions between PKC β II and RACK1 in AE-PKC ϵ -H mice. Jurkat cell lysate was used as a positive control for identification of RACK1. *B*: in nonimmune control experiments where IgG was substituted for anti-PKC β II antibodies, RACK1 was not identified in the IgG/bead-complex of AE-PKC ϵ -H mice (*top*). Similarly, PKC β II was not identified in the IgG/bead-complex of both nontransgenic (*middle*) and AE-PKC ϵ -H (*bottom*) mice. Mouse cardiac tissue lysate was used as a positive control. Data are means \pm SE. RACK, receptor for activated C kinase.

of PKC ϵ activity and not merely increases in PKC ϵ protein expression alone. Third, we assessed quantitatively the PKC isoform expression profile in the mouse myocardium (FVB/N strain). Given the broad usage of this mouse strain in cardiac research, these data should provide invaluable insights into the magnitude of PKC isoform-specific responses to various cardiac stimuli. Finally, the current study is the first to demonstrate a physiological relationship between PKC ϵ -induced expression of RACK1 protein and translocation of PKC β II, as PKC β II-RACK1 interactions were enhanced in mice with a cardiac hypertrophied and failure phenotype. In concert with our previous observations regarding PKC ϵ -RACK1 interactions (27), the present data provide direct evidence that RACK1 serves as a nexus in the genesis of cardiac hypertrophy and failure, directing both PKC ϵ activity (through RACK-mediated PKC isoform switching) and PKC β II activity through a hypertrophic signaling pathway.

The role of PKC isoforms in cardiac hypertrophy and failure. Multiple lines of evidence (4, 7, 12, 17–19, 41, 44) have demonstrated a role for PKC in the development of cardiac hypertrophy and failure. However, elucidation of an isoform-specific role for PKC has proved to be challenging due to the various myocardial expression profiles of PKC isoforms in different species examined and the distinct experimental models of hypertrophy utilized. In the rat heart, for example, aortic banding-induced pressure overload hypertrophy has been shown to preferentially translocate PKC β II and PKC ϵ (12), whereas angiotensin-II-induced hypertrophy in the rat selectively translocates PKC β alone (32). In contrast with pressure overload hypertrophy in the rat heart, aortic banding in the guinea pig induces translocation of PKC α , PKC ϵ , and PKC γ (13). In the human myocardium, limited investigation suggests that the development of cardiac hypertrophy and failure may involve PKC β II (4). Recent studies have employed transgenic mouse models of cardiac hypertrophy. Transgenic expression of constitutively active PKC β II has been shown to induce cardiac hypertrophy and failure (41). Upstream activators of PKC ϵ such as G α q, when overexpressed in the mouse heart, induced myocardial dysfunction, indicating a role for PKC ϵ in the development of cardiac hypertrophy and failure (7).

In the present study, we examined the dose-dependent effects of PKC ϵ activity on cardiac phenotype in the mouse heart. We demonstrated that high levels of PKC ϵ activity induce altered expression of proteins commonly modified in cardiac hypertrophy (α -actin, α -MHC, and β -MHC), and that this altered expression is associated with cardiac failure. Taken together with data by others, in which moderate levels of PKC ϵ activity (100% above the basal value) induce a compensated cardiac hypertrophy, the PKC ϵ transgenic mouse model mimics a progression from compensated to decompensated cardiac hypertrophy and failure: low levels of PKC ϵ activity produce a normal cardiac phenotype that is inherently protected (5, 27), moderate levels of PKC ϵ activity induce a compensated cardiac hypertrophy (11, 42), whereas as high levels of PKC ϵ

activity induce cardiac hypertrophy and failure as reported in the present study.

Expression of myosin isoforms. In the normal mouse heart, MHC exists as three isoforms: V_1 , the homodimer of the α -MHC, V_3 the homodimer of the β -MHC, and V_2 the heterodimer. The myosin composition of the heart is thus dependent on the relative amounts of the α -MHC and β -MHC proteins. Although in hypothyroidism there is a nearly complete shift of isomyosin content in the rodent heart, mouse models of cardiac hypertrophy most often have an incomplete shift that presents as a reciprocal reduction in α -MHC and increase in β -MHC (15, 37, 40). In the AE-PKC ϵ -H mice, we observed a $41 \pm 4\%$ decrease in the level of α -MHC and a concomitant increase of β -MHC expression without a change in the total amount of MHC. The other skeletal MHC isoforms are not detected in the mouse heart (15). These data thus suggest that there was a shift in the myosin isoform abundance corresponding to a reduction of V_1 and an increase in V_2 and V_3 . While the precise mechanism regarding regulation of β -MHC expression is unknown, enhanced activation of PKC β II in the AE-PKC ϵ -H mice may stimulate the promoter of β -MHC (17–19) and thus modulate β -MHC expression (41). Because the α -MHC and β -MHC differ in both Ca^{2+} sensitivity and actin-activated ATPase activity, shifts in myosin isoform abundance may have a significant impact on cardiac function (8, 21).

Quantitative assessment of PKC isoform expression profile in the mouse heart. Numerous studies (11, 27, 38, 41, 42) have utilized the mouse heart to investigate the role of PKC isoforms in mediating various pathophysiological processes, including the development of cardiac hypertrophy and failure. At present, however, only limited information is available regarding the exact protein content of the various PKC isoforms expressed in the mouse heart, and virtually no information exists pertaining to the complete PKC expression profile in the mouse myocardium. To this end, we employed isozyme-specific PKC antibodies to perform quantitative Western immunoblotting (Table 2). We found that the mouse myocardium expressed 10 PKC isoforms [cPKCs (α , β I, β II, γ), nPKCs (ϵ , δ , θ), aPKCs (ν , λ , ζ), and PKC μ]. Quantitative immunoblotting also revealed that, similar to the rabbit myocardium (31), PKC α and PKC γ were the most abundant isoforms in the mouse myocardium. In contrast with the rabbit heart (31), PKC θ was found to be the most abundant novel isoform in the mouse myocardium, whereas as PKC ϵ was expressed at a lower level.

The role of RACKs in cardiac hypertrophy and failure. RACKs represent a group of PKC binding proteins that have been shown to participate in PKC isozyme-mediated development of cardiac pathophysiology (23, 45). However, the role of RACKs in cardiac hypertrophy remains largely unknown. A preliminary study by Reiger and co-workers (32) found that angiotensin II-induced cardiac hypertrophy is associated with increased PKC β -RACK1 colocalization. Alternatively, the expression of peptides (Ψ ϵ RACK peptides) that facilitate the interaction of PKC ϵ with RACK2 in the

mouse heart induced a mild yet nonpathological cardiac hypertrophy, suggesting a role for PKC ϵ -RACK2 interactions in the development of cardiac hypertrophy (23). However, it remains controversial as to whether the mechanism of RACK2-mediated function in hypertrophy involves activation of PKC ϵ . Paradoxically, the level of PKC ϵ activation in mice that express Ψ ϵ RACK is significantly lower (an estimated 20% above basal activity) than the level of PKC ϵ activity observed in our phenotypically normal AE-PKC ϵ -L mice (~ 2.3 -fold increase in phosphorylation activity) (9).

Interestingly, the present study also demonstrated that attenuation of PKC ϵ activity via the use of DN-PKC ϵ mouse protein had no demonstrable effect on cardiac phenotype. The mechanism by which DN-PKC ϵ protein inhibits PKC ϵ activity appears to involve, at least in part, competition between the DN-PKC ϵ protein and the endogenous PKC ϵ protein for RACK2 binding (27). Thus the lack of effect of DN-PKC ϵ on cardiac phenotype suggests a great deal of plasticity with regard to PKC ϵ -RACK2 modulation of cardiac function in the normal myocardium; i.e., although there is an $\sim 50\%$ reduction in PKC ϵ activity, the remaining PKC ϵ activity/PKC ϵ -RACK2 interactions may be sufficient to maintain normal cardiac function. In fact, the DN-PKC ϵ transgenic line is similar to other transgenic models of PKC ϵ inhibition (23) in that partial inhibition of PKC ϵ does not affect cardiac function or development. Alternatively, basal levels of PKC ϵ activity may not be involved in the homeostatic maintenance of cardiac function. Finally, the fact that the DN-PKC ϵ line did not exhibit cardiac contractile dysfunction suggests that the PKC ϵ -associated heart failure phenotype observed in the AE-PKC ϵ -H mouse line is a consequence of PKC ϵ kinase activity, but not overexpression of PKC ϵ protein alone.

PKC ϵ -induced translocation of PKC β II: RACK-mediated PKC isoform-switching. In the present study, we have demonstrated that the expression of the PKC β II isoform is increased in the particulate fraction of hearts from AE-PKC ϵ -H mice, indicating a PKC ϵ -induced preferential activation of the β II isozyme. Our data showed that the total protein expression of PKC β II was not altered in AE-PKC ϵ -H mice, indicating that this is a preferential translocation of the PKC β II isoform. The precise signaling events leading to enhanced PKC β II translocation in AE-PKC ϵ -H mice remain to be completely defined. A plausible mechanism would be RACK1-mediated translocation of PKC β II. To this end, some investigations have shown positive correlation between the level of RACK1 expression and PKC β II translocation (1, 2, 10). In previous studies, we reported that RACK1 is in molar excess of PKC β II, a ratio of $\sim 7:1$. This finding is consistent with the concept that, in addition to interacting with PKC β II, RACK1 may interact with other proteins, thus conferring other biological functions (20, 24, 33, 46), and that the amount of RACK1 available for exclusive PKC β II binding may be less than that implied by the stoichiometric excess of RACK1. Taken together, the fact that RACK1 expression was increased

in AE-PKC ϵ -H mice (27), combined with the present evidence of increased PKC β II-RACK1 interactions in these mice, suggests a significant role of RACK1 in shuttling (34) and henceforth in localizing PKC β II to the particulate fraction where PKC β II function is conferred.

A recently proposed paradigm of PKC isoform-mediated signaling in the myocardium involves the formation of intracellular signaling modules whose multifaceted architecture results in the manifestation of different cardiac phenotypes (30, 43). In previous studies, we have shown that increased RACK1 expression in AE-PKC ϵ -H mice is commensurate with increased PKC ϵ -RACK1 interactions, in addition to the commonly recognized PKC ϵ -RACK2 interactions. These data demonstrate the existence of RACK-mediated PKC isoform-switching (27). In context with the signaling module hypothesis (43), it is plausible that PKC ϵ is recruited into a PKC β II signaling complex by RACK1, and that this recruitment results in the activation of a signaling pathway that utilizes the same constituents of the hypertrophic PKC β II signaling module. In this scenario, PKC ϵ activity proceeds through the PKC β II pathway, a pathway that has been previously shown (4, 17–19, 41, 44) to participate in the genesis of cardiac hypertrophy and failure. On the basis of the present data demonstrating PKC β II translocation and increased PKC β II-RACK1 interactions in AE-PKC ϵ -H mice, we now propose that the signaling mechanism underlying PKC ϵ -induced hypertrophy may involve the recruitment of both PKC β II and PKC ϵ (27) by RACK1, and that their collective and synergistic activities coordinated through the RACK1 molecule may be important determinants of the development of cardiac hypertrophy and failure in AE-PKC ϵ -H mice.

This study was supported by American Heart Association Grant EIG-40167N, National Heart, Lung, and Blood Institute Grants HL-63901 and HL-65431 (all to P. Ping), HL-63034 (to W. K. Jones), HL-43151 and HL-55757 (to R. Bolli), the University of Louisville Research Foundation, and Jewish Hospital Research Foundation.

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