Am J Physiol Heart Circ Physiol
280: H946–H955, 2001.

PKCϵ activation induces dichotomous cardiac phenotypes and modulates PKCϵ-RACK interactions and RACK expression

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Received 5 July 2000; accepted in final form 17 October 2000

Pass, Jason M., Yuting Zheng, William B. Wead, Jun Zhang, Richard C. X. Li, Roberto Bolli, and Peipei Ping. PKCϵ activity induces dichotomous cardiac phenotypes and modulates PKCϵ-RACK interactions and RACK expression. *Am J Physiol Heart Circ Physiol* 280: H946–H955, 2001.—Receptors for activated C kinase (RACKs) have been shown to facilitate activation of protein kinase C (PKC). However, it is unknown whether PKC activation modulates RACK protein expression and PKC-RACK interactions. This issue was studied in two PKCϵ transgenic lines exhibiting dichotomous cardiac phenotypes: one exhibits increased resistance to myocardial ischemia (cardioprotected phenotype) induced by a modest increase in PKCϵ activity (228 ± 23% of control), whereas the other exhibits cardiac hypertrophy and failure (hypertrophied phenotype) induced by a marked increase in PKCϵ activity (452 ± 28% of control). Our data demonstrate that activation of PKC modulates the expression of RACK isotypes and PKC-RACK interactions in a PKCϵ activity- and dosage-dependent fashion. We found that, in mice displaying the cardioprotected phenotype, activation of PKCϵ enhanced RACK2 expression (178 ± 13% of control) and particulate PKCϵ-RACK2 protein-protein interactions (178 ± 18% of control). In contrast, in mice displaying the hypertrophied phenotype, there was not only an increase in RACK2 expression (330 ± 33% of control) and particulate PKCϵ-RACK2 interactions (154 ± 14% of control) but also in RACK1 protein expression (174 ± 10% of control). Most notably, PKCϵ-RACK1 interactions were identified in this line. With the use of transgenic mice expressing a dominant negative PKCϵ, we found that the changes in RACK expression as well as the attending cardiac phenotypes were dependent on PKCϵ activity. Our observations demonstrate that RACK expression is dynamically regulated by PKCϵ and suggest that differential patterns of PKCϵ-RACK interactions may be important determinants of PKCϵ-dependent cardiac phenotypes.

protein-protein interactions; cardiac phenotypes; protein kinase C; transgenic mouse; receptors for activated C kinase

THE ϵ-ISOFORM OF PROTEIN KINASE C (PKCϵ), which belongs to the novel subgroup of the PKC superfamily (19, 31, 32), has been identified as an essential signaling element in the development of cardioprotection against ischemia-reperfusion injury (16, 24, 35, 38, 41) and in the genesis of hypertrophic heart failure (1, 4, 17, 21, 34). We (56) have recently developed two transgenic mouse lines that express either low or high levels of constitutively active PKCϵ in a cardiac-specific manner. We have found that mice expressing low levels of constitutively active PKCϵ do not develop hypertrophy and are inherently protected against myocardial ischemia-reperfusion injury (10), whereas mice expressing high levels of constitutively active PKCϵ exhibit cardiac hypertrophy and impaired ventricular function (56). While the phenotypes of these transgenic mice corroborate an important role of PKCϵ in both cardioprotection and hypertrophic heart failure, the underlying molecular mechanisms through which different levels of activated PKCϵ result in distinct cardiac physiological and pathological phenotypes remain unknown.

Receptors for activated C kinase (or RACKs) are a group of PKC binding proteins that have been elegantly characterized and shown to mediate isoform-selective functions of PKC (12, 19, 44). Several studies (11, 20) have demonstrated that PKCϵ interacts selectively with a specific isotype of RACK, RACK2. Information obtained from studies using peptides that either disrupt or enhance PKCϵ binding to RACK2 suggests an important role of RACK2 in cardiac function (14, 16, 20, 24). For example, in models of simulated ischemia, peptides that block PKCϵ binding to RACK2 were found to exacerbate cardiac cell death during hypoxic injury (16, 25). These same inhibitory peptides were also shown to block phorbol 12-myristate 13-acetate (PMA)- and norepinephrine-mediated regulation of cell contraction in rat cardiac myocytes (20). Furthermore, transgenic mice expressing peptides that increase PKCϵ-RACK2 binding are more resistant to ischemic injury (14). PKCϵ has also been reported to interact with RACK1, although this interaction is far less specific than the PKCβII-RACK1 interaction (43).

While the role of RACKs as PKC isoform-selective binding proteins has been well characterized, it remains unknown whether the activation of a specific PKC isozyme modulates the expression of its corre-

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sponding RACK protein(s) and their interactions with PKC. Moreover, it is unknown whether compensatory changes in RACK protein expression and PKC-RACK interactions contribute to the manifestation of PKC-induced physiological and pathological phenotypes. One possibility that has not been explored thus far is that the expression of RACK proteins and their interaction with PKC are dynamically coordinated to achieve PKC-mediated biological functions. Accordingly, we postulated that specific stoichiometric changes in the amount of RACK proteins and their interaction with PKC contribute to the manifestation of specific cardiac phenotypes induced by PKC.

The present study was undertaken as a comprehensive effort to test this hypothesis. With the use of PKCε transgenic mice, we sought to determine whether activation of PKCε modulates the expression of RACK proteins and their interactions with PKCε. Two transgenic lines were studied: one exhibits a cardioprotected phenotype (10), and the other one displays a hypertrophic heart failure phenotype (56). To determine whether the kinase activity of PKCε is necessary for changes in RACK expression to occur, we also examined a third transgenic mouse line that expresses a dominant negative mutant of PKCε. The results demonstrate the existence of compensatory changes in the expression of selective RACK isotypes and distinct congruous changes in their interactions with PKCε in these transgenic mice, supporting a role of these changes in conferring the specific cardiac phenotypes exhibited. These findings support the concept that the expression of a signaling receptor protein (RACK) can be modulated by the activity of its ligand (PKC) to facilitate the biological functions of the ligand. This concept may have vast implications for the role of RACKs in numerous physiological and pathophysiological PKC-dependent processes as well as for the function of other kinases known to interact with receptor proteins.

MATERIALS AND METHODS

Generation and characterization of PKCε transgenic mouse lines. Three transgenic mouse lines expressing cardiac-targeted PKCε mutants were studied. Standard techniques were used for the production and generation of these mice (48). Briefly, a cardiac specific α-myosin heavy chain promoter (48) was used to drive the expression of PKCε cDNA mutants in FVB/N mice. An HA tag was inserted into the 5′ end of all constructs, which allowed differentiation of transgene expression from that of endogenous PKCε. Among the three lines, two express different levels of a constitutively active PKCε (AE-PKCε), which is created by an A to E mutation at the pseudosubstrate domain [amino acid (aa) 159]: one mouse line expresses low levels of the PKCε transgenic protein (AE-PKCε-L) and is inherently protected from cardiac ischemic injury (10), whereas the other line expresses high levels of protein (AE-PKCε-H) and exhibits cardiac hypertrophy and failure (50, 56). The third mouse line expresses the dominant negative PKCε transgene (DN-PKCε), which is created by mutations at the pseudosubstrate domain (aa 159) and at the ATP binding site (K to R, aa 436). This line is free of hypertrophy and does not show any phenotypical differences compared with nontransgenic mice. The phenotypes of these three transgenic mouse lines have been previously characterized (10, 56).

Experimental groups. A total of four groups of mice were studied. *Group I* (control, $n = 6$) consisted of age-matched (10–12 wk) transgenic negative littermates. *Group II* (AE-PKCε-L, $n = 6$) consisted of transgenic mice exhibiting the cardioprotected phenotype. *Group III* (AE-PKCε-H, $n = 6$) consisted of transgenic mice displaying the hypertrophied and heart failure phenotype. *Group IV* (DN-PKCε, $n = 6$) consisted of transgenic mice expressing the dominant negative mutant PKCε.

Determination of PKCε expression in transgenic mice. The expression of PKCε protein was assessed by Western immunoblotting with both antibodies against the HA tag (BABCO) and antibodies against PKCε (Transduction Laboratories). The isoform-selective PKCε phosphorylation activity was determined as previously described (35). Briefly, 50 μg of myocardial sample proteins were immunoprecipitated overnight with PKCε monoclonal antibodies (Transduction Laboratories) and A/G-agarose beads (Santa Cruz Biotechnology). The immunoprecipitation-enriched and -purified tissue PKCε enzymes were then subjected to a phosphorylation assay containing 2.3 μg/ml PMA, 28.8 μg/ml L-α-phosphatidyl-L-serine, and 1 nM of PKCε isozyme-preferred substrate (ERMRPRKRQGSVRRRV).

Expression and purification of RACK proteins. Purified recombinant RACK1 fused to maltose-binding protein and the expression vector for RACK2 were generously provided by Daria Mochly-Rosen of Stanford University (11, 43). Briefly, recombinant RACK2 protein was expressed as (MBP)-FLAG-RACK2 fusion protein using the *Escherichia coli* pMAL-c2 expression vector (New England Biolabs) (11). The MBP-FLAG-RACK2 protein was purified on an amylose affinity column according to the manufacturer's protocol (11). The purities of the MBP-RACK1 and MBP-FLAG-RACK2 proteins were assessed by Coomassie blue staining of 10% SDS-PAGE gels.

Identification of PKCε, RACK2, and RACK1 protein expression. Frozen myocardial tissue samples were powdered in a prechilled stainless steel mortar and pestle. Total cellular protein was obtained by glass-glass homogenization in sample buffer containing 20 mM Tris·HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, and a cocktail of protease inhibitors (50 μg/ml phenylmethysulfononyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). The homogenates were centrifuged at 45,000 *g* for 30 min, and the pellet (particulate fraction) was resuspended in 20 mM Tris·HCl, 1 mM EGTA, 1 mM EDTA, and 12 mM 2-mercaptoethanol, 50 μg/ml phenylmethysulfononyl fluoride, and the above protease inhibitors. Total cellular protein, soluble protein, and particulate protein concentrations were determined (Bio-Rad). Standard Western immunoblotting techniques were used to assess the protein levels of PKCε, RACK2, and RACK1 (38). To assure equal loading of protein, Ponceau stain of nitrocellulose membranes was quantified by densitometric scanning (38).

For quantitative Western immunoblotting, increasing amounts of either recombinant RACK2 or recombinant RACK1 proteins were loaded onto the same SDS-PAGE gel along with 140 μg of total myocardial tissue homogenate from six control hearts (*group I*, nontransgenic). The enhanced chemiluminescence signals generated by the recombinant proteins were used to construct dose-response curves for either RACK2 or RACK1. The dose-response curves were then used to determine the absolute amount of RACK2 or RACK1 protein in tissue samples, which is reported as pico-

grams of RACK2 or RACK1 per microgram of myocardial tissue protein. It is important to note that the antibody recognition site for RACK2 has been identified as the sequence LDD (18) and is identical between the rat recombinant RACK2 used in the generation of the dose-response curve and the mouse RACK2. For RACK1, a basic local alignment search tool (BLAST) sequence comparison revealed 99.7% sequence homology (or 316 of 317 aa) between rat recombinant RACK1 and mouse RACK1. Because the RACK1 antibody immunogen consists of aa 113–317 (Transduction Laboratories), variations in signal intensity as a consequence of differences in antibody binding between rat and "wild-type" mouse RACK1 are highly unlikely.

Immunoprecipitations. Tissue for each immunoprecipitation reaction was prepared as described in *Identification of PKCϵ, RACK2, and RACK1 protein expression* except that the pellet (particulate fraction) was resuspended in 20 mM Tris·HCl, 1 mM EGTA, 1 mM EDTA, 50 μg/ml phenylmethysulfononyl fluoride, and the listed cocktail of protease inhibitors. For each immunoprecipitation reaction, 4 μg of anti-PKCϵ antibodies (GIBCO-BRL) were incubated with 50 μl of protein A/G-agarose beads (Santa Cruz) for 20–40 min at 4°C. In controls, IgG (Sigma) was substituted for anti-PKCϵ antibodies. The protein A/G-agarose-anti-PKCϵ complex was washed three times with phosphate-buffered saline containing 0.1% Triton X-100. The protein A/G-anti-PKCϵ complex was incubated with 800 μg of protein from either soluble or particulate heart fractions overnight at 4°C, washed four times with phosphate-buffered saline containing 0.1% Triton X-100, and then subjected to Western immunoblotting using RACK2 (StressGen Biotechnologies) and RACK1 (Transduction Laboratories) antibodies.

Statistical analysis. All data are presented as means ± SE. Groups were compared using Student's *t*-tests for unpaired data. A *P* value of <0.05 was considered significant.

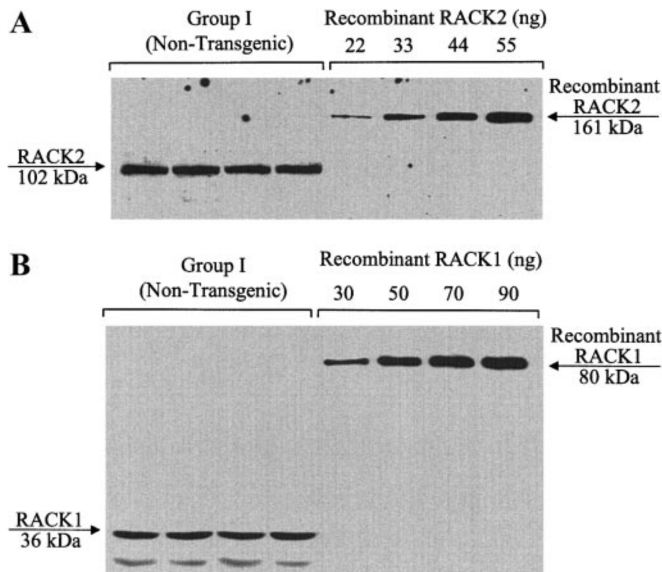

Fig. 1. The control mouse heart expresses abundant amounts of receptors for C kinase (RACK)2 and RACK1. Increasing amounts of recombinant RACK2 or RACK1 proteins were used to determine the absolute amount of RACK2 and RACK1 expressed in control mouse hearts (*group I*, nontransgenic). Representative quantitative Western immunoblots for the determination of RACK2 protein content (*A*) and RACK1 protein content (*B*) are shown. A total of 6 hearts from *group I* were examined for both RACK2 and RACK1.

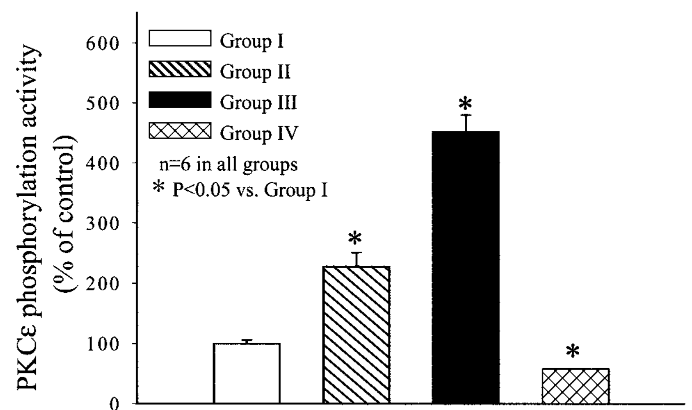

Fig. 2. Protein kinase C (PKC)ϵ activity in PKCϵ transgenic mice. Cardiac tissue from control mice (*group I*, nontransgenic), mice displaying the cardioprotected phenotype [*group II*, mice expressing low levels of a constitutively active PKCϵ created by an A to E mutation at the pseudosubstrate domain (AE-PKCϵ-L)], and mice exhibiting the hypertrophied phenotype [*group III*, mice expressing high levels of a constitutively active PKCϵ created by an A to E mutation at the pseudosubstrate domain (AE-PKCϵ-H)], and dominant negative mice [*group IV*, mice expressing a dominant negative form of PKCϵ created by a K to R mutation in the ATP-binding region in addition to an A to E mutation at the pseudosubstrate domain (DN-PKCϵ)] were examined for PKCϵ-specific phosphorylation activity as described in MATERIALS AND METHODS. A total of 6 hearts from each group were examined. Data are means ± SE.

RESULTS

Expression of PKCs and RACKs in mice. At present, no information is available regarding the stoichiometric ratio of RACK proteins to their respective PKC isoforms. We performed quantitative Western immunoblotting for RACK2 and RACK1 proteins in hearts of control mice (*group I*, nontransgenic). As shown in Fig. 1, *A* and *B*, abundant amounts of RACK2 (132 ± 4 pg/μg of total tissue) and RACK1 (94 ± 3 pg/μg of total tissue) are expressed in the mouse heart. Interestingly, the absolute protein amounts of RACK2 and RACK1 are in large excess of those of their respective PKC isoforms, PKCϵ (40 ± 5 pg/μg of total tissue) and PKCβII (31 ± 2 pg/μg of total tissue), yielding molar ratios of 3:1 for RACK2 to PKCϵ and 7:1 for RACK1 to PKCβII. To our knowledge, this is the first measurement of the stoichiometric molar ratios of PKC isoforms and their corresponding RACKs in the heart.

Activation of PKCϵ induces cardiac phenotypes in a PKCϵ activity-dependent fashion. Transgenic mice with low levels of constitutively active PKCϵ (*group II*, AE-PKCϵ-L) exhibited an increase in both PKCϵ protein expression (993 ± 47% of control, $P < 0.05$) and PKCϵ phosphorylation activity (228 ± 23% of control, $P < 0.05$) (Fig. 2). As expected, the largest increase in constitutively active PKCϵ protein (894 ± 47% of control, $P < 0.05$) was found in the particulate fraction (Fig. 3, *A* and *B*), where 70 ± 3% of total cardiac PKCϵ (AE-PKCϵ plus endogenous PKCϵ) was found to reside. In separate studies, we (10) have demonstrated that these mice exhibit enhanced postischemic functional recovery and ATP levels compared with their trans-

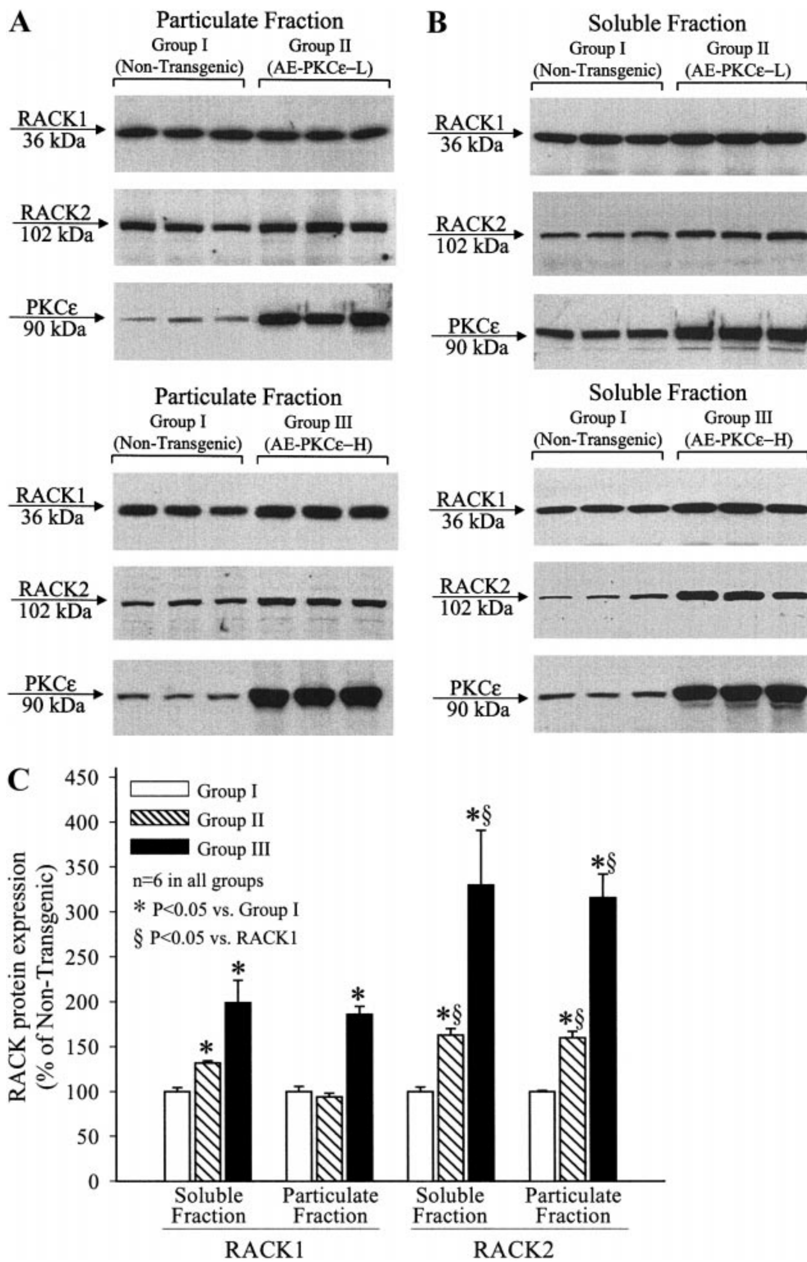

Fig. 3. Compensatory subcellular changes in RACK expression in PKCϵ transgenic mice. Cardiac tissue from control mice (*group I*, nontransgenic), mice displaying the cardioprotected phenotype (*group II*, AE-PKCϵ-L), and mice exhibiting the hypertrophied phenotype (*group III*, AE-PKCϵ-H) were assayed by Western immunoblotting for the expression of PKCϵ, RACK1, and RACK2. *A*: representative immunoblots demonstrating the expression of PKCϵ, RACK1, and RACK2 in the particulate fraction. *B*: representative immunoblots demonstrating the expression of PKCϵ, RACK1, and RACK2 in the soluble fraction. *C*: histogram depicting RACK1 and RACK2 expression in each group. A total of 6 hearts were examined in each group. Data are means ± SE.

genic negative littermates and thus display a cardioprotected phenotype.

As expected, in transgenic mice with high levels of constitutively active PKCϵ (*group III*, AE-PKCϵ-H), the increase in both PKCϵ protein expression (3,735 ± 311% of control, $P < 0.05$) and PKCϵ phosphorylation activity (452 ± 28% of control, $P < 0.05$) (Fig. 2) were significantly greater than in *group II* ($P < 0.05$). Similar to *group II*, the largest increase in constitutively active PKCϵ protein (3,915 ± 478% of control, $P < 0.05$) occurred in the particulate fraction (Figs. 3, *A* and *B*), which was found to contain 69 ± 5% of total cardiac PKCϵ (AE-PKCϵ plus endogenous PKCϵ). In separate studies, we (56) have found that these mice exhibit cardiac myofibrillar disarray, increased expression of α-skeletal muscle actin and atrial natriuretic factor, and impaired contractile function, indicating a hypertrophy and heart failure phenotype.

Phenotypic differences in PKCϵ transgenic mice are congruous with compensatory changes in RACK2 and RACK1 protein expression. We next examined the myocardial expression of PKCϵ, RACK2, and RACK1 in the three transgenic lines. In mice displaying a cardioprotected phenotype (*group II*, AE-PKCϵ-L), in which a low level of PKCϵ activation was present, there was a significant increase in the expression of RACK2 protein (178 ± 13% of control, $P < 0.05$) and only a marginal change in RACK1 protein (120 ± 11% of control). In mice displaying a hypertrophied phenotype (*group III*, AE-PKCϵ-H), a higher level of PKCϵ activation was associated with a further increase in the expression of RACK2 protein (330 ± 33% of control,

$P < 0.05$). In contrast with the cardioprotected phenotype, RACK1 protein expression in the hypertrophied mice was significantly increased (174 ± 10% of control, $P < 0.05$). The increases in PKCϵ, RACK1, and RACK2 expression were also confirmed in isolated cardiac myocytes for *group II* (data not shown).

The above changes in total cellular PKCϵ and RACK expression were also noted when particulate and soluble fractions were analyzed separately (Fig. 3, *A–C*). In mice displaying the cardioprotected phenotype (*group II*), RACK2 protein expression in the particulate fraction was increased to 160 ± 7% of control ($P < 0.05$); however, no significant change in particulate RACK1 expression was identified (Fig. 3*A*). In mice exhibiting the hypertrophied phenotype (*group III*), further increases in the particulate RACK2 protein expression were detected (316 ± 26% of control, $P < 0.05$), and, most importantly, the particulate expression of RACK1 protein was significantly elevated (186 ± 9% of control, $P < 0.05$) (Fig. 3*A*). Surprisingly, increased RACK2 and RACK1 expression was also found in the soluble fraction [163 ± 7 and 135 ± 2% of control ($P < 0.05$), respectively, in *group II* and 433 ± 50 and 199 ± 25% of control ($P < 0.05$), respectively, in *group III*] (Fig. 3*B*).

The subcellular distribution of RACK proteins was also altered in both the cardioprotected (*group II*) and hypertrophied mice (*group III*). In control mice (*group I*), we found that 39 ± 3% of total cellular RACK2 protein and 16 ± 2% of total RACK1 protein was localized in the particulate fraction. In mice displaying a cardioprotected phenotype, particulate-associated RACK2 protein was increased to 57 ± 2% ($P < 0.05$ vs. *group I*). No significant change in the distribution of RACK1 was identified in this group. Conversely, in mice exhibiting the hypertrophied phenotype, there was a significant increase not only in particulate-associated RACK2 (57 ± 3% of total RACK2, $P < 0.05$ vs. *group I*) but also in particulate-associated RACK1 (34 ± 7% of total RACK1, $P < 0.05$ vs. *group I*).

Collectively, these data indicate that *1*) higher levels of PKCϵ activity are associated with greater increases in RACK2 and RACK1 protein expression, *2*) activation of PKCϵ is associated with a redistribution of RACK2 to the particulate fraction in both the cardioprotected and hypertrophied phenotype, and *3*) the PKCϵ-induced hypertrophied phenotype is uniquely associated with increased RACK1 protein expression and redistribution of RACK1 to the particulate fraction.

Compensatory increases in RACK expression require functional activity of PKCϵ. In PKCϵ transgenic mice that display either a cardioprotected (*group II*, AE-PKCϵ-L) or hypertrophied (*group III*, AE-PKCϵ-H) phenotype, increases in PKCϵ protein expression occurred concomitantly with increases in PKCϵ activity. Thus the above experiments do not discern whether the kinase activity of PKCϵ is necessary to modulate RACK expression in these groups. To address this issue, we examined RACK expression in transgenic mice that express a dominant negative mutant of PKCϵ (*group IV*, DN-PKCϵ), in which the expression of PKCϵ protein was markedly increased (3,584 ± 395% of control, $P < 0.05$) but kinase activity was decreased to 58 ± 1% of control (or attenuated by 42 ± 1%, $P < 0.05$) (Fig. 2).

If increased expression of PKCϵ alone is sufficient to induce RACK expression, then RACK2 and RACK1 expression should have been elevated in *group IV*. However, despite the dramatic increase in PKCϵ protein, the expression of RACK2 and RACK1 in *group IV* was unaltered (Fig. 4, *A* and *B*). Similarly, there was no change in the levels of either soluble or particulate RACK2 or RACK1 or in their subcellular distributions (data not shown). These data demonstrate that the coupling of PKCϵ and RACK expression in PKCϵ transgenic mice is dependent on the enzymatic activity of PKCϵ.

Cardiac phenotypes of PKCϵ transgenic mice are congruous with enhanced PKCϵ-RACK interactions. If compensatory increases in RACK expression facilitate PKCϵ-mediated changes in cardiac phenotype, then the observed increases in RACK expression should be associated with increases in PKCϵ-RACK interactions. To determine PKCϵ-RACK interactions, the subcellu-

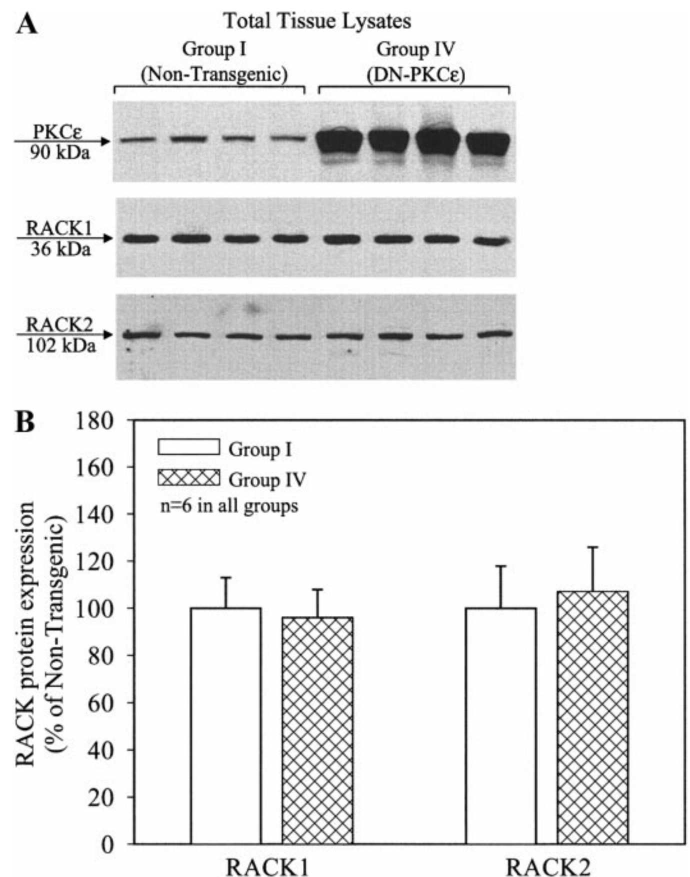

Fig. 4. Compensatory changes in RACK expression require functional activity of PKCϵ. Cardiac protein samples from control mice (*group I*, nontransgenic) and mice expressing the dominant negative (DN) mutant of PKCϵ (*group IV*, DN-PKCϵ) were assayed for the expression of RACK1 and RACK2 protein. *A*: representative immunoblots. *B*: histogram depicting percent changes in RACK1 and RACK2. A total of 6 hearts were examined in each group. Data are means ± SE.

lar myocardial fractions from control mice (*group I*, nontransgenic), mice displaying the cardioprotected phenotype (*group II*, AE-PKCϵ-L), and mice exhibiting the hypertrophied phenotype (*group III*, AE-PKCϵ-H) were subjected to immunoprecipitation with PKCϵ-specific antibodies (11). PKCϵ-RACK2 or PKCϵ-RACK1 interactions were assessed by subjecting immunoprecipitates to Western immunoblotting with either the RACK2 or the RACK1 antibodies, respectively (11).

We found that, in control mice, only a small amount of RACK2 was associated with PKCϵ in the soluble fraction, whereas a significant amount of RACK2 was associated with particulate PKCϵ (Fig. 5*A*). In mice displaying the cardioprotected phenotype (*group II*), a marginal association of PKCϵ with RACK2 was detected in the soluble fraction, but the amount of RACK2 associated with PKCϵ in the particulate fraction was significantly greater than that identified in control mice (178 ± 18% of control, $P < 0.05$) (Fig. 5, *A* and *B*). In both groups, no PKCϵ-RACK1 interactions were detected in either of the subcellular fractions.

In mice exhibiting the hypertrophied phenotype, there was a significant increase not only in particulate PKCϵ-RACK2 interactions (154 ± 14% of control, $P < 0.05$) but also in soluble PKCϵ-RACK2 interactions (165 ± 7% of control, $P < 0.05$) (Fig. 6, *A* and *B*). The functional significance of the interactions in the soluble compartment remains unknown; the present study is the first to document the existence of such interactions. Interestingly, PKCϵ-RACK1 interactions were evident in four mice examined and were found in both the soluble and the particulate fractions in group III (Fig. 6*C*). This interaction is rather unique because physiologically relevant PKCϵ-RACK1 interactions have never been reported.

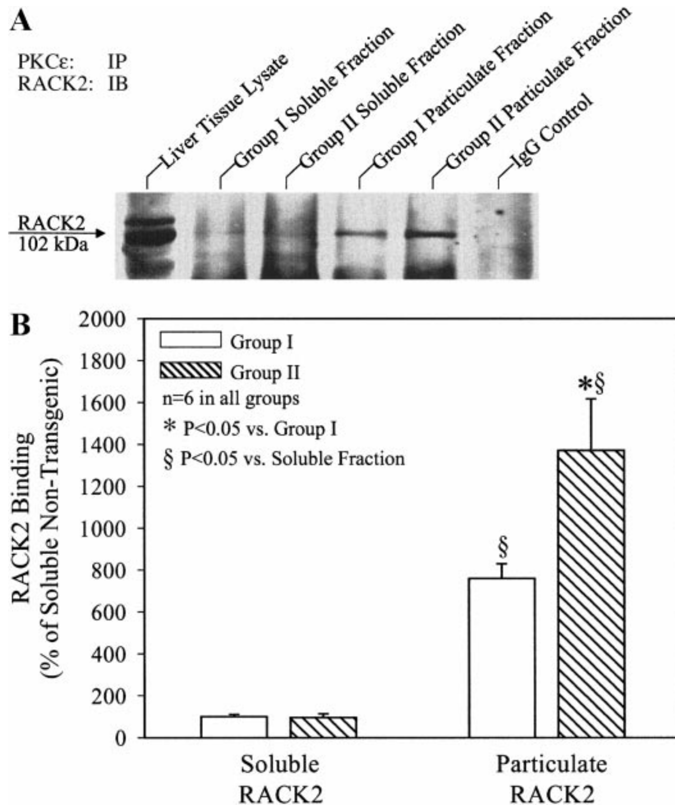

Fig. 5. Transgenic mice with cardioprotected phenotype display increased PKCϵ-RACK2 protein-protein interactions in the particulate fraction. Cardiac protein samples from both the soluble and particulate fractions of control mice (*group I*, nontransgenic) and mice displaying the cardioprotected phenotype (*group II*, AE-PKCϵ-L) were immunoprecipitated (IP) with PKCϵ antibodies before Western immunoblotting (IB) with RACK2. *A*: representative immunoblot. Liver tissue lysates were used as positive control. IgG was substituted for PKCϵ antibodies and used as negative control. *B*: histogram depicting PKCϵ-RACK2 binding. A total of 6 hearts were examined in each group. Data are means ± SE.

Interactions between DN-PKCϵ and RACK2. To explore molecular mechanisms underlying dominant negative inhibition of PKCϵ, we assessed whether the dominant negative mutant of PKCϵ (*group IV*, DN-PKCϵ) was able to interact with the RACK proteins. After immunoprecipitation with anti-HA antibodies and Western immunoblotting with anti-RACK2, we found that DN-PKCϵ interacts with RACK2 in both the soluble and the particulate fractions (Fig. 7). Moreover, in reciprocal immunoprecipitations (immunoprecipitation with anti-RACK2 antibodies and Western immunoblotting using anti-HA antibodies, which detect the DN-PKCϵ protein), DN-PKCϵ was also found to interact with particulate RACK2 (data not shown). These results suggest that dominant negative mutant-mediated inhibition of PKCϵ may involve competition for RACK2 binding sites with endogenously active PKCϵ.

DISCUSSION

The present investigation represents a comprehensive effort to determine whether RACK expression and PKCϵ-RACK interactions are static or dynamically modulated in response to increased PKCϵ activity in vivo. There are several novel findings in this study. First, with the use of transgenic mice that express either low (cardioprotected phenotype) or high (hypertrophied phenotype) levels of cardiac-targeted PKCϵ (AE-PKCϵ), we demonstrated that RACK proteins are dosage dependently coupled to the expression of PKCϵ in a RACK subtype-selective manner. Second, with the use of a dominant negative transgenic mouse line, we found that PKCϵ-mediated increases in RACK protein expression are dependent on the activity of PKCϵ rather than on its protein expression. Third, we found that increased myocardial RACK2 expression is congruous with increased PKCϵ-RACK2 interactions, suggesting that elevated RACK2 expression serves a compensatory role in coordinating the biological function of PKCϵ. Our finding that cardioprotected and hypertrophied mice display different patterns of PKCϵ-RACK2 interactions suggests that the consequence of RACK2-modulated PKCϵ function may be different among these mice, thus contributing to the manifestation of dichotomous cardiac phenotypes. Finally, we report that, in addition to its interaction with RACK2, PKCϵ interacts with RACK1 in mice displaying the hypertrophied phenotype. This is the first demonstration of PKCϵ-RACK1 interactions in vivo. Importantly, PKCϵ-

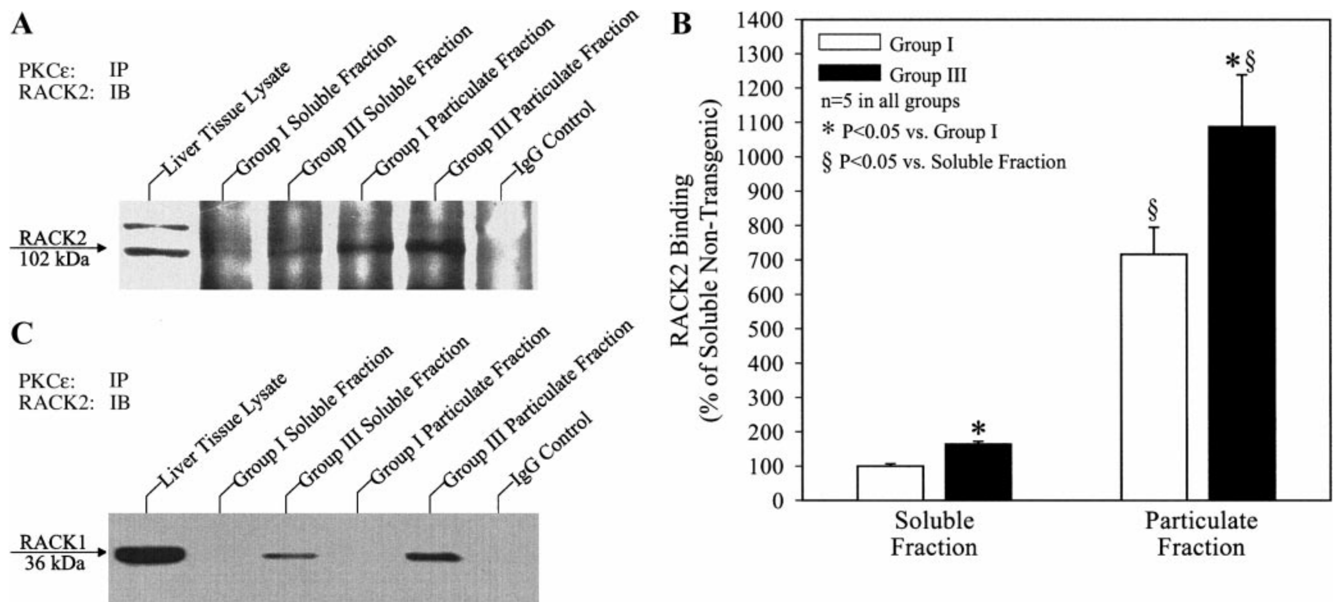

Fig. 6. Transgenic mice with hypertrophic heart failure exhibit enhanced PKCϵ-RACK1 and PKCϵ-RACK2 protein-protein interactions in both the soluble and particulate fractions. Cardiac protein samples from either the soluble or particulate fractions of control mice (*group I*, nontransgenic) and mice exhibiting the hypertrophied phenotype (*group III*, AE-PKCϵ-H) were immunoprecipitated (IP) with PKCϵ antibodies before Western immunoblotting (IB) with either RACK2 or RACK1 antibodies. *A*: representative immunoblot for PKCϵ-RACK2 binding. Liver tissue lysates were used as positive control. IgG was substituted for PKCϵ antibodies and used as negative controls. *B*: histogram depicting PKCϵ-RACK2 binding. *C*: representative immunoblot for PKCϵ-RACK1 binding. Liver tissue lysates were used as positive control. IgG was substituted for PKCϵ antibodies and used as negative control. In control mice (*group I*, nontransgenic), no PKCϵ-RACK1 binding was detected in any of the hearts examined. In mice exhibiting the hypertrophied phenotype (*group IV*, AE-PKCϵ-II), PKCϵ-RACK1 binding was evident. Data are means ± SE.

RACK1 interactions are associated with a functional consequence, i.e., the genesis of cardiac hypertrophy and failure. To the best of our knowledge, this is the first investigation to demonstrate a PKC activation-dependent differential regulation of RACK protein expression in vivo. Our observations are consistent with the hypothesis that differential patterns of RACK expression and PKCϵ-RACK interactions are important determinants of PKCϵ-induced phenotypes in the heart.

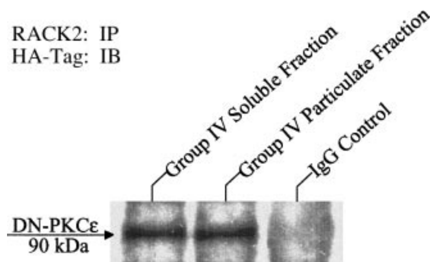

Fig. 7. Dominant negative PKCϵ binds to soluble and particulate RACK2. Cardiac protein samples from either the soluble or particulate fraction of mice expressing the dominant negative mutant of PKCϵ (*group IV*, DN-PKCϵ) were immunoprecipitated (IP) with RACK2 antibodies before Western immunoblotting (IB) with anti-HA-tag antibodies. HA antibodies detect the expression of transgenic PKCϵ but not that of endogenous PKCϵ. These data demonstrate that DN-PKCϵ binds to RACK2 in both the soluble and particulate fractions, indicating that it is able to compete with active endogenous PKCϵ for RACK2 binding.

Role of PKC in cardiac hypertrophy and ischemia. Several lines of evidence (13, 49, 52, 56) support the notion that activation of PKC may be a trigger of cardiac hypertrophy and failure. Transgenic mice expressing cardiac specific PKCβII exhibit enhanced troponin I phosphorylation (49) and develop hypertrophy and impaired ventricular function (52). Activation of PKC has also been found to contribute to $G_{\alpha q}$ overexpression-induced cardiac hypertrophy (13). A previous study (56) in our laboratory has shown that transgenic mice with high levels of constitutively active PKCϵ exhibit enhanced α-skeletal muscle actin and atrial natriuretic factor expression, myofibrillar disarray, an increased ratio of heart weight to body weight, and impaired contractile function.

An important role for PKC has also been identified in ischemic preconditioning (25, 55), a cardioprotective phenomenon whereby brief episodes of ischemia render the heart resistant to subsequent ischemic injury (5, 7, 29). Ischemic preconditioning induces activation of PKC (38), whereas inhibition of PKC abrogates cardioprotection (25, 26, 41). Furthermore, recent investigations (38, 41) have established an isoform-specific role for PKCϵ in preconditioning. Activation of this PKC isoform protects against hypoxia-induced cell death (16, 24, 36), improves postischemic functional recovery (10), and reduces myocardial infarction (40). PKCϵ-mediated cardioprotection appears to involve multiple downstream signaling elements, including the recruit-

ment of Src tyrosine kinases (39), mitogen-activated protein kinases (30, 36, 37), and phosphoinositide-3-kinase (51). However, although the phenotypic consequences of PKCϵ activation are well characterized, the precise molecular mechanisms underlying PKCϵ-dependent phenotypes remain unknown.

RACKs. Since the concept of receptors for activated C kinase (RACKs) was first introduced by Daria Mochly-Rosen (27, 28), several functional roles for RACK1 and RACK2 proteins have been identified (23, 42, 45, 53, 54, 57). RACK2, or β′-coatomer protein β′-COP (18, 47), has been identified as an important element in the regulation of cardiac function (16, 20, 24). Disruption of PKCϵ-RACK2 interactions using peptides derived from the RACK2 binding site on PKCϵ has been shown to inhibit cardiac cell contraction (20) and to exacerbate cell death in hypoxic injury (16, 24). Interestingly, transgenic mice overexpressing peptides that activate PKCϵ (pseudo-RACK2 peptides) are less sensitive to ischemic injury (14). These studies provide important insight into the role of PKCϵ and RACK2 in cardiac function. However, current technology limits quantitative analysis of the subcellular distribution of these RACK peptides, thus making it difficult to achieve a quantitative determination of their expression and of their effect on PKC-RACK interactions (14, 16, 20, 24). In the present investigation, we used constitutively active PKCϵ transgenic mice, in which the active PKCϵ proteins are preferentially localized to the particulate fraction. This approach enabled us to perform quantitative analyses of subcellular RACK protein expression and their interactions with PKCϵ. We found that PKCϵ activation induced RACK2 protein expression and enhanced PKCϵ-RACK2 interactions in the cardioprotected mice. These findings are consistent with the concept that RACK2 proteins and PKCϵ-RACK2 interactions facilitate the PKCϵ-mediated manifestation of cardioprotection against ischemia in vivo, further supporting the hypothesis that RACK proteins and their interactions with activated PKCs bear functional significance in the myocardium.

Regulation of RACK expression. Previous studies regarding regulation of RACK protein expression have been limited to RACK1. Furthermore, whether the activity and/or expression of PKC govern the expression of RACK proteins has never been examined. Some investigators (3, 15) have concluded that the expression of RACK1 protein and the expression/activity of PKC are interdependent, whereas others (2, 9) found that altered RACK1 expression occurs in the absence of a concomitant change in PKC activity and expression. In the rat brain, RACK1 expression was found to parallel PKC activity (3), and RACK1 expression was found to correlate with the expression of PKCβ and PKCα (15). In the human brain, RACK1 expression was not coupled to the expression of PKCβII (2). In rat alveolar macrophages, RACK1 expression is impaired, whereas the total expression of PKC isoforms is preserved (9).

In the aforementioned studies (2, 3, 9, 15), only the expression of RACK1 proteins was examined. Therefore, it remains unknown whether RACK2 expression can be modulated, and, if so, whether regulation of RACK2 proteins is dependent on the activity of PKCϵ, the expression of PKCϵ, or both. Importantly, whether PKC-RACK protein-protein interactions participate in the manifestation of PKCϵ-dependent phenotypes have never been characterized. We report the first evidence that RACK2 protein expression is dynamically regulated by activation of PKCϵ and that distinct adaptive changes in both the expression of RACK proteins and their interactions with PKCϵ are associated with distinct cardiac phenotypes. The level of RACK protein expression in PKCϵ transgenic mice appears to be dictated by the activity of PKCϵ and not by its protein content, because the expression of RACK1 and RACK2 proteins was not changed in mice expressing high levels of a kinase negative mutant of PKCϵ (*group IV*, DN-PKCϵ). Intriguing is the possibility that PKCϵ activity modulates the expression of RACK1 via activation of the transcription activating protein-1 (AP-1). PKCϵ activation has been shown to enhance the DNA-binding activity of the transcription factors nuclear factor-κB and AP-1 (22). Although the transcriptional regulation of RACK proteins is largely undefined, the promoter of RACK1 has recently been found to contain AP-1 binding sites (8).

Signaling mechanisms underlying dichotomous phenotypes. The mechanism leading to the manifestation of different cardiac phenotypes is likely to involve complex signaling events. In a separate study (33), we have found that the mouse myocardium (FVB/N strain) expresses 10 isoforms of PKC (α, βI, βII, γ, δ, ϵ, θ, ι/λ, ζ, and μ). In principal, it is conceivable that overexpression of PKCϵ may result in a given phenotype by altering the expression of other PKC isozymes, particularly in view of the fact that transgenic expression of PKCβII leads to cardiac hypertrophy and failure (49, 52). However, we have not identified significant changes in the expression of any other PKC isoforms in either the cardioprotected (*group II*, AE-PKCϵ-L) or heart failure phenotype (*group III*, AE-PKCϵ-H) (data not shown). Thus the possibility that upregulation of PKCβII may underlie the development of the heart failure phenotype can be excluded. A distinctive feature of mice with hypertrophy is that they exhibit substantial increases in soluble and particulate RACK1 expression compared with cardioprotected mice. More strikingly, increased RACK1 expression is associated with congruous increases in PKCϵ-RACK1 interactions. On the basis of these data, we suggest that PKCϵ activity is directed to an alternative signaling pathway by RACK1 that leads to the manifestation of the hypertrophied phenotype. Another difference between these two phenotypes is the increase in soluble PKCϵ-RACK2 interaction in the former, which was not found in the cardioprotected mice. Presently, nothing is known concerning the functional consequence(s) of soluble PKCϵ-RACK2 interactions. Our data are the first to document the existence of such interactions, a finding that should stimulate further investigation of this phenomenon. It is possible that increases in solu-

ble PKCε-RACK2 interactions may be deleterious, thus contributing to the phenotype of cardiac hypertrophy and failure.

In conclusion, our observations and those of others (14, 15, 16, 24) suggest that the stoichiometric ratio of RACK proteins and their respective PKC isoforms is important for the preservation of PKC biological function. The results of the present study affirm this notion, in that we found that PKCε activity dosage dependently modulates the expression of RACK2 protein and, to a lessor extent, the expression of RACK1 protein concomitantly with distinct PKCε-induced cardiac phenotypes. Our observations provide in vivo examples illustrating a functional consequence of RACK protein expression and its interaction with PKC in both a physiological setting (the cardioprotected phenotype) and pathological condition (the hypertrophied and heart failure phenotype). These findings have broad implications in that they support a unique signaling paradigm whereby the expression of a signaling receptor protein (RACK) can be dynamically modulated by activation of its ligand (PKC) to promote the manifestation of the biological function of the ligand.

This study was supported in part by National Heart, Lung, and Blood Institute Grants HL-58166 and HL-63901 (to P. Ping) and HL-43151 and HL-55757 (to R. Bolli), American Heart Association National Center Grant-In-Aid 9750721N (to P. Ping), and by the Jewish Hospital Research Foundation, Louisville, Kentucky.

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