Protein Kinase C in Pathological Myocardial Hypertrophy

ANALYSIS BY COMBINED TRANSGENIC EXPRESSION OF TRANSLOCATION MODIFIERS AND Gaq*

Received for publication, June 13, 2000, and in revised form, July 14, 2000
Published, JBC Papers in Press, July 17, 2000
DOI 10.1074/jbc.C000390200

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The ε isoform of protein kinase C (PKC) has a critical cardiotoxic function in normal postnatal developing heart as demonstrated by cardiac-specific transgenic expression of εPKC-selective translocation inhibitor (εV1) and activator (ψεRACK) peptides (Mochly-Rosen, D., Wu, G., Hahn, H., Osinska, H., Liron, T., Lorenz, J. N., Robbins, J., and Dorn, G. W., II (2000) Circ. Res. 86, 1173–1179). To define the role of εPKC signaling in pathological myocardial hypertrophy, εV1 or ψεRACK were co-expressed in mouse hearts with Gaq, a PKC-linked hypertrophy signal transducer. Compared with Gaq overexpression alone, co-expression of ψεRACK with Gaq increased PKC particulate partitioning by 30 ± 2%, whereas co-expression of εV1 with Gaq reduced particulate-associated PKC by 22 ± 1%. Facilitation of εPKC translocation by ψεRACK in Gaq mice improved cardiac contractile function measured as left ventricular fractional shortening (30 ± 3% for Gaq versus 43 ± 2% for ψεRACK/Gaq, p < 0.05). Conversely, inhibition of εPKC by εV1 modified the Gaq nonfailing hypertrophy phenotype to that of a lethal dilated cardiomyopathy. These opposing effects of εPKC translocation activation and inhibition in Gaq hypertrophy indicate that εPKC signaling is a compensatory event in myocardial hypertrophy, rather than a pathological event, and support the possible therapeutic efficacy of selective εPKC translocation enhancement in cardiac insufficiency.

The protein kinase Cs (PKCs) constitute a large family of ubiquitous phospholipid-dependent serine-threonine kinases postulated to have diverse effects in the heart, including mediating cardiac hypertrophy/failure and myocardial protection (1–3). It has been difficult to establish particular roles for individual PKC isoforms due to the absence of isoform-specific agonists and antagonists. Targeted cardiac overexpression of PKC has been helpful in establishing possible effects of myocardial PKC β signaling (4, 5), but the potential for nonspecific or nonphysiological signaling of overexpressed enzymes warrants a cautious interpretation of resulting phenotypes. Based on recent insights into the mechanism for differential PKC isoform subcellular translocation upon activation (6), a strategy was developed whereby the effects of endogenous myocardial PKC isoforms such as εPKC could be elucidated by cardiac-specific expression of peptides that specifically modulate PKC isoform translocation/activation (7, 8). These εPKC-derived peptides, ψεRACK (HDAPIGYD, rat εPKC amino acids 85–92) and εV1 (the first variable region of rat εPKC, amino acids 2–144) have previously been shown to specifically modulate εPKC translocation (7–9). Compared with transgenic overexpression of PKC, a critical advantage of translocation modification is that PKC isoforms are specifically regulated without changing the natural stoichiometric relationships of PKC to its upstream activators and downstream substrates. Cardiac transgenic models expressing either the εPKC translocation inhibitor (εV1) or activator (ψεRACK) were previously created with opposing cardiac phenotypes of dilated cardiomyopathy and physiological hypertrophy, respectively (8), demonstrating that εPKC signaling is both necessary and sufficient for normal myocardial growth in the developing young mouse. This apparently beneficial role for εPKC in normal physiologic cardiac growth is, however, at odds with the widely held notion that pathological cardiac hypertrophy is the consequence of εPKC signaling (1, 10–13). In addressing this apparent contradiction, we hypothesized that the effects of εPKC on a diseased heart might be different than during normal heart development. We further considered that these putative pathologic effects could be identified using εPKC-specific translocation modification in the context of a form of cardiac hypertrophy where εPKC is thought to be a pathological mediator, like the cardiac-specific Gaq-overexpressing mouse, which develops myocardial hypertrophy and contractile depression associated with εPKC translocation (11, 12). εV1 or ψεRACK were therefore co-expressed with Gaq by cross-breeding the respective individual transgenic mice to generate compound transgenic mice.

EXPERIMENTAL PROCEDURES

Transgenic Models—Details of transgenic mice (FVB/N background) overexpressing Gaq, εV1, or ψεRACK under control of the full-length mouse α–myosin heavy chain (MHC) promoter have previously been described (7, 8, 11). Gaq mice express Gaq at ~5 times nontransgenic levels (the line previously described as Gaq-40) and develop nonfailing ventricular hypertrophy with modest contractile depression, which is unresponsive to β-adrenergic agonists (11). ψεRACK transgenic mice develop mild hypertrophy with normal ventricular function and normal response to β-adrenergic agonists (7, 8). The εV1low (lowest expressing) mice used herein have normal cardiac mass and function, whereas higher expressing εV1 lines develop ventricular dilation with wall thinning, diminished systolic function, and heart failure (8). Compound transgenic mice overexpressing εV1 or ψεRACK and Gaq, were obtained by breeding heterozygous εV1low or ψεRACK with heterozygous Gaq-40 mice. Transgenes, alone and in combination, were identified by genomic Southern analysis of tail clip DNA. All experiments were performed with comparison of littermates.

Western Blot Analysis—Samples for measurement of transgene products and PKC expression were prepared as described previously (12). Briefly, mouse ventricles frozen at ~80 °C were homogenized in buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 5 mM diithiofre...
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pot, 10 mm benzamidine, 0.5 mm phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) followed by centrifugation at 100,000 × g for 1 h. The pellet was further extracted with buffer containing 1% Triton X-100 for 30 min on ice. Followed by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. eV1 and Gaq transgene expression were determined by Western blot analysis using anti-FLAG M2 (Sigma) and anti-Gaq,11 (Santa Cruz) monoclonal antibodies, respectively. PKC isoform expression was measured by quantitative immunoblot analysis with anti-ePKC (Santa Cruz) and anti-ePKC antibodies (Transduction Laboratories) using recombinant human ePKC and ePKC (Calbiochem) as quantitative standards (8, 12). Protein loading and transfer efficiency were evaluated by amido black staining of the membrane after immunoblotting.

Assessment of Cardiac Hypertrophy and Function—Morphometric analysis and histological examination of Masson’s trichrome-stained ventricles used standard techniques. Echocardiography was performed to determine cardiac contractile function in a noninvasive manner; left ventricular fractional shortening (the proportion of blood ejected during systole compared with maximal ventricular capacity in diastole) and left ventricular mass were calculated (14). Cardiac gene expression was assayed by RNA dot blot analysis using total RNA (3 systole compared with maximal ventricular capacity in diastole) and ventricular fractional shortening (the proportion of blood ejected during ventricles used standard techniques. Echocardiography was performed for comparison, but were not included in the statistical analysis.

Statistical Analysis—All data are expressed as mean ± S.E. Comparisons between Gaq and dual transgenic mice were evaluated using Student’s t test, and the p value of <0.05 was considered as statistically significant. Values for nontransgenic (NTG) siblings are also presented for comparison, but were not included in the statistical analysis.

RESULTS AND DISCUSSION

Cardiac-targeted transgenic expression of eV1 and resulting inhibition of myocardial ePKC translocation has proven that ePKC activation is necessary for normal physiological myocardial growth in the postnatal period (8). Complementing this finding is the observation that enhancing ePKC activation by expression of ψeRACK stimulates growth of normally functioning myocardium (8). Herein, we utilized in vivo translocation modulation to characterize ePKC effects in Gaq-stimulated cardiac hypertrophy, a form of hypertrophy in which ePKC translocation is increased and in which ePKC has therefore been considered to be a possible pathological mediator (11, 12). Compound transgenic mice were generated and ventricular expression of eV1 and Gaq was compared between parent mice and dual transgenic progeny by Western blot analysis (Fig. 1A). Compound transgenic mice expressed both eV1 and Gaq at the same levels as parent lines, indicating that no cross-talk occurred at the level of transgenic expression. As reported previously, it is not possible to detect ψeRACK expression by Western blot analysis due to the small molecular weight of the peptide (8). However, Northern blot analysis showed no change in ψeRACK and Gaq transgene expression in compound transgenic mice, compared with parent lines (data not shown). Gaq activates PKC via phospholipase C (15). As expected from this signaling pathway, myocardial Gaq overexpression results in increased ePKC partitioning to particulates, i.e. translocation, associated with a decrease in total ePKC content which is considered to be a consequence of chronic ePKC activation (11, 12). We measured the effects of ψeRACK and eV1 on the expression and translocation of ePKC in Gaq overexpressors using quantitative Western blot analysis. ePKC, which is transcriptionally up-regulated but not translocated in Gaq overexpressing hearts (12), was also measured to assay the isoform specificity of eV1 and ψeRACK. Compared with nontransgenic siblings, ψeRACK and eV1 transgenic mice exhibited a 20 ± 3% increase and 15 ± 2% decrease, respectively, of ePKC translocation (measured as the paticulate/cytosol ratio) with no change in ePKC content (data not shown). This is similar to the initial description of these models (7, 8). As shown in Fig. 1B, combined expression of either ψeRACK or eV1 with Gaq did not alter the characterizedly decreased myocardial ePKC content of Gaq overexpressors (12) (NTG 163 ± 8 ng/mg, Gaq 122 ± 9, ψeRACK/Gaq 120 ± 6, n = 4 in each group, p = not significant for ψeRACK/Gaq versus Gaq; NTG 159 ± 9, Gaq 113 ± 12 and eV1/Gaq 115 ± 10, n = 4 in each group, p = not significant for eV1/Gaq versus Gaq). Fig. 1C shows that particulate partitioning of ePKC, which is increased by Gaq overexpression (11, 12), was further augmented 30 ± 2% in ψeRACK/Gaq mice (particulate/cytosol ratio: NTG 1.2 ± 0.1, Gaq 2.1 ± 0.1, ψeRACK/Gaq 2.8 ± 0.2, n = 4 in each group, p < 0.05 for ψeRACK/Gaq versus Gaq) and attenuated by 22 ± 1% in eV1/Gaq mice (particulate/cytosol ratio: NTG 1.2 ± 0.1, Gaq 2.3 ± 0.1 and eV1/Gaq 1.8 ± 0.2, n = 4 in each group, p < 0.05 for eV1/Gaq versus Gaq). Expression and translocation of aPKC, transcriptionally increased in Gaq hearts relative to NTG (12), were not affected in Gaq mice by the ePKC modifying peptides (ePKC content: NTG 852 ± 79 ng/mg, Gaq 1259 ± 93 and ψeRACK/Gaq/11 1198 ± 121; NTG 847 ± 125, Gaq 1368 ± 110 and eV1/Gaq/1359 ± 186; particulate/cytosol ratio: NTG 0.44 ± 0.04, Gaq 0.42 ± 0.03 and ψeRACK/Gaq 0.45 ± 0.03, NTG 0.41 ± 0.04, Gaq 0.40 ± 0.01, and eV1/Gaq 0.40 ± 0.02), confirming the specific effects of these peptides on ePKC (7, 9).

ψeRACK/Gaq mice were healthy when followed for 5 months. Effects of enhanced PKC translocation/activation on Gaq-mediated cardiac hypertrophy and contractile dysfunction were assessed in 12-week-old ψeRACK/Gaq mice. Compared with Gaq transgenic siblings, ventricular and atrial weights in ψeRACK/Gaq mice were reduced (Table I). Left ventricular mass of ψeRACK/Gaq mice calculated from noninvasive echocardiographic analysis was correspondingly decreased (Table II). Echocardiography further revealed that ψeRACK/Gaq
These genes are highly expressed in Gq hearts and had reduced end diastolic and systolic dimensions, i.e., that the cardiac ventricles were smaller before or after contraction, with no change in left ventricular wall thickness (Table II). Thus, the ratio of wall thickness to ventricular radius (h/r) was increased, which suggests concentric ventricular remodeling. Perhaps as a consequence of this more favorable ventricular geometry, eRACK expression enhanced left ventricular contractile function of Gq hearts, measured echocardiographically as the proportion of blood volume ejected per cardiac cycle, or fractional shortening (FS).

A molecular marker of cardiac hypertrophy is increased expression of the embryonic cardiac genes α-skeletal actin, β-MHC, and atrial natriuretic peptide (ANF) in ventricle. These genes are highly expressed in Gq overexpressing hearts, and Northern analysis revealed that α-skeletal actin gene expression was selectively reduced in ventricles of eRACK/Gq mice, whereas ANF and β-MHC gene expression was not changed from their characteristically elevated levels. Since inhibition of ePKC with eV1 in normal hearts was previously shown to selectively increase expression of α-skeletal actin gene (8), the isolated reduction of this mRNA by eRACK in the Gq mice suggests a role for ePKC in transcriptionally regulating actin, but not ANF or β-MHC expression, in the heart. Taken together, the results with eRACK/Gq mice indicate that enhanced ePKC translocation improves ventricular function and normalizes ventricular geometry, but surprisingly, diminishes the extent of cardiac hypertrophy in Gq overexpressors.

Heart failure is not typically observed in Gq overexpressing mice, although the characteristic eccentric hypertrophy is associated with measurable contractile depression. Heart failure also is not a feature of eV1low mice in which hearts are functionally and histologically normal (8). Thus, breeding Gq with eV1low is the equivalent of crossing two nonfailing cardiac models. Strikingly, combined expression of eV1low with Gq caused development of lethal heart failure at 14 ± 2 weeks of age (n = 9). At 12 weeks of age, eV1/Gq mice could not tolerate echocardiographic analysis under even light anesthesia, so a confident assessment of in vivo ventricular function at an age matching that of the eRACK/Gq mice was not obtained. However, gross pathological examination of 12-week-old hearts revealed ventricular dilatation, focal ventricular wall thinning, and massively enlarged atria containing intracavitary blood clots, which are typically observed in chronic low cardiac output states, i.e., heart failure.

**Table I**

| Morphometric parameters in NTG, single and dual 12-week-old transgenic mice |
|-----------------------------------------------|
| Heart/body wt, mg/g                           |
| Dry atria, mg                                 |
| Dry atrium/body wt, mg/g                      |
| Dry ventricles, mg                            |
| Dry ventricle/body wt, mg/g                   |
| Liver/body wt, mg/g                           |
| Lung/body wt, mg/g                            |

**Table II**

| Echocardiographic parameters in NTG, single and dual transgenic mice |
|-----------------------------------------------|
| FS, %                                            |
| ESD, mm                                           |
| EDD, mm                                          |
| PWT, mm                                          |
| FS, %                                            |
| ESD, mm                                           |
| EDD, mm                                          |
| PWT, mm                                          |

* p < 0.05 versus Gq, n = 5–11 per group.

**Fig. 2.** A, 12-week-old transgenic mouse hearts showing concentric remodeling in eRACK/Gq and dilation in eV1/Gq mice. The arrow points to an area of left ventricular wall thinning. B, RNA analysis of gene expression. Quantitative data are indexed to GAPDH (n = 3) and expressed as percent of Gq mice. C, representative Masson’s trichrome stain of left ventricles showing mild interstitial fibrosis (blue staining) in eV1/Gq.
physiological assessment, we performed echocardiographic examination of apparently healthy 6-week-old eV1/Gqq mice to help define the relevant pathophysiology. At 6 weeks, eV1/Gqq hearts exhibited ventricular enlargement and wall thinning with depressed contractile function (fractional shortening) compared with Gqq, which at this age have not yet developed contractile dysfunction (Table II). These findings suggested that eV1<low> inhibits myocardial growth in the young developing Gqq mice, as eV1<high> did in mice with a normal genetic background (8). This notion was confirmed by a 27% reduction in dry ventricular weight of eV1/Gqq mice, compared with Gqq (Table I). In contrast, atrial weight was increased, which, as noted above, probably reflects contractile depression. Finally, the ratio of ventricular wall thickness to ventricular radius (h/r) was significantly diminished, indicating that dilatory remodeling, i.e. ventricular wall thinning with chamber enlargement, was already occurring in 6-week-old eV1/Gqq mice. Thus, inhibition of ePKC translocation, which is already "enhanced" in the Gqq mouse, impairs ventricular function, causes cardiac enlargement with ventricular dilatation, and reduces ventricular mass.

The reciprocal cardiac phenotypes of ψRACK/Gqq and eV1/Gqq mice described above unequivocally refute our hypothesis that ePKC signaling contributes to the pathologies associated with Gqq-mediated hypertrophy. Although ePKC activation occurs in Gqq "superactivation" with ψRACK proved to be therapeutic, whereas even the modest impairment of ePKC translocation caused by eV1<low>, essentially "normalization," greatly exaggerated the underlying Gqq pathology. Most surprising is that the opposing effects on cardiac function were not due to opposite effects on cardiac "hypertrophy" per se, in that ψRACK and eV1 both decreased left ventricular mass of Gqg mice. Rather, it is apparently an ePKC effect on ventricular geometry that is the critical determinant of viability, with benefit accruing from ψRACK-induced concentric remodeling (of the eccentrically hypertrophied Gqq heart (11)) and eV1-induced ventricular dilatation proving fatal. These changes in geometry were surprisingly well defined in that they were not associated with significant changes in ANF or β-MHC gene expression, which are widely considered to be indices of cardiac pathology (16). In the context of our prior study (8), the current results show that the molecular and morphometric effects of ePKC are similar in nontransgenic and Gqq overexpressing hearts, but the effects of ePKC activation and inhibition are amplified in the Gqq model.

An intriguing difference between the current studies of ePKC in the Gqq background and our previous characterization of ePKC translocation modulation in the normal background is the absence of a "hypertrophic" effect of ψRACK plus Gqq. We previously found a modest increase in ventricular mass of 12-week-old ψRACK mice, with concentric remodeling. Compared with Gqq, however, ψRACK/Gqq ventricles were concentrically remodeled, but smaller. This suggests that the intrinsic contractile depression and eccentric remodeling of Gqq mice may itself contribute to the hypertrophic response. ψRACK improved ventricular geometry and function and as a consequence diminished the external stimulus for hypertrophy.

A strength of the current studies is that ePKC activity/translocation was modulated by expressing catalytically inactive peptides rather than by overexpressing ePKC itself, essentially using transgenesis as a means of organ-specific drug delivery. However, the effects of ePKC modulation were measured in the Gqq transgenic mouse, which achieves its phenotype by 5-fold increased expression of Gqq (11). Thus, concerns regarding stoichiometric excess and nonspecific activity of overexpressed signaling proteins (which drove our unique approach of PKC isoform translocation modification) apply to the Gqq model itself. While the rationale for choosing the Gqq model is strong, i.e. changes in ePKC translocation, which suggested it as a pathological mediator (11, 12), the relevance of Gqq overexpression to naturally occurring cardiac disease is uncertain. Additional studies are therefore ongoing to determine the effects of eV1 and ψRACK on cardiac hypertrophy and function in hemodynamically stressed hearts.

In conclusion, the current studies contradict the generally accepted notion that ePKC is a pathophysiological mediator of cardiac disease. Rather, they support the notion that ePKC translocation, either by direct administration of ψRACK peptide, by gene therapy, or through novel pharmacological means, could be used to treat heart failure in the same manner that induction of "physiological" hypertrophy by exercise or growth factors has proven to be therapeutic (17).

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