In Vivo Phosphorylation of Cardiac Troponin I by Protein Kinase Cβ2 Decreases Cardiomyocyte Calcium Responsiveness and Contractility in Transgenic Mouse Hearts

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Abstract

Recently, it has been reported that the protein kinase C (PKC) β isoform plays a critical role in the development of hypertrophy and heart failure. The purpose of the present study was to clarify the mechanism by which activation of PKCβ led to depressed cardiac function. Thus, we used a PKCβ2 overexpressing mouse, an animal model of heart failure, to examine mechanical properties and Ca^{2+} signals of isolated left ventricular cardiomyocytes. The percentage of shortening, rate of shortening, and rate of relengthening of cardiomyocytes were markedly reduced in PKCβ2 overexpression mice compared to wild-type control mice, although the baseline level and amplitude of Ca^{2+} signals were similar. These findings suggested a decreased myofilament responsiveness to Ca^{2+} in transgenic hearts. Therefore, the incorporation of [^{32}P] inorganic phosphate into cardiac myofibrillar proteins was studied in Langendorff-perfused hearts. There was a significant increase in the degree of phosphorylation of troponin I in PKCβ2-overexpressing transgenic mice. The depressed cardiomyocyte function improved after the superfusion of a PKCβ selective inhibitor. These findings indicate that in vivo PKCβ2-mediated phosphorylation of troponin I may decrease myofilament Ca^{2+} responsiveness, and thus causes cardiomyocyte dysfunction. Since chronic and excess activation of PKCβ2 plays a direct and contributory role in the progression of cardiac dysfunction, the PKCβ selective inhibitor may provide a new therapeutic modality in the setting of heart failure. (*J. Clin. Invest.* 1998. 102:72–78.) Key words: hypertrophy • signal transduction • transgenic animal • heart failure • protein kinase

Introduction

Congestive heart failure is a major and growing public health problem with a high mortality rate (1). In response to mechanical and hormonal stimuli, the myocardium adapts to increased work loads through cardiac hypertrophy (2). Initially, the resultant increased work is compensatory with normal wall stress and myocyte function. If the stimulus for pathologic hypertrophy is sufficiently intense and prolonged, decompensated hypertrophy ensues and ultimately leads to congestive heart failure. Although the molecular mechanisms which are responsible for compensated and decompensated hypertrophy have been investigated by experimental and clinical studies, precise mechanisms accounting for the myocyte dysfunction occurring in heart failure remain incompletely characterized (2, 3).

We have recently demonstrated that pathophysiologic elevation of left ventricular diastolic pressure can activate phospholipase C with resultant translocation of protein kinase C (PKC)[1] and accumulation of inositol phosphate in the adult guinea pig heart (4). The activation of this signal transduction pathway has been implicated in the development of in vitro neonatal cardiomyocyte hypertrophy (3). It has also been reported that the level of PKCβ isoform is increased during development of cardiac hypertrophy induced by pressure overload in rats (5). Furthermore, PKCβ expression is elevated in failed human heart, and its contribution to total PKC activity is significantly increased (6). Finally, postnatal cardiac specific overexpression of the PKCβ2 isoform in transgenic mice causes a cardiomyopathy which is characterized by left ventricular hypertrophy, myocardial fibrosis, and decreased in vivo left ventricular performance (7). These morphological and functional changes are reversible by chronic administration of a highly selective inhibitor of the PKCβ isoform. Taken together, these observations suggest that PKCβ plays a critical role in the development of cardiac hypertrophy and heart failure. However, the mechanism by which in vivo activation of PKCβ leads to depressed cardiac function is not yet known.

Therefore, we examined the mechanical properties and calcium transients of isolated left ventricular cardiomyocytes extracted from mice which overexpress the PKCβ2 isoform. We observed decreased mechanical function and calcium responsiveness of these cells which were reversed by a selective PKCβ inhibitor. To elucidate the mechanism by which cardiomyocyte contractility and calcium sensitivity were reduced, phosphorylation of cardiac myofibrillar and membranous proteins, and the levels of calcium cycling and regulatory proteins were quantified. Our results suggest that PKCβ2 induced phosphorylation of the myofilament regulatory protein tropo-

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1. *Abbreviations used in this paper:* PKC, protein kinase C; SERCA2, sarcoplasmic reticulum Ca^{2+} ATPase; T_{50}, time from start to 50% decay of the calcium signal; T_{80}, time from start to 80% decay of the calcium signal.

nin I may play an important role in the diminished cardiomyocyte function observed in the hypertrophied and failing heart.

Methods

Production of PKCβ2 transgenic mice. Transgenic mice were generated as previously described (7). Briefly, the transgene consisted of the adult mouse cardiac myocyte specific α myosin heavy chain promotor ligated to the entire coding sequence for the mouse PKCβ2 isoform. A SacI and MluI fragment of the recombinant plasmid was microinjected into pronuclei of fertilized FVB mice eggs and implanted into pseudopregnant FVB foster mothers. Success of gene transfer was identified by Southern blot analysis of genomic DNA extracted from mouse tail blood with an α myosin heavy chain PKCβ2 transgene-specific probe. The heterozygous transgenic mice from line No. 4 and age-matched nontransgenic littermate control mice (10–12 wk of age) were used for the present study. Notably this is the same line and age as was used in our initial characterization (7). Immunoblot analysis performed with specific antibodies showed that the protein levels of the PKCβ2 isoform in the heart was increased by 20- and 10-fold in the cytosol and membrane fractions, respectively, in the transgenic mice compared to the wild-type mice.

Preparation of isolated left ventricular myocytes. Left ventricular myocytes were isolated from the hearts of mice overexpressing PKCβ2 and from wild-type littermate controls as we have previously described (8–10). Briefly, mice were anesthetized with methoxyflurane, and the heart was rapidly excised and placed in a dish of oxygenated Ca^{2+}-free Joklik's modified buffer pH 7.2 (Gibco-BRL, Gaithersburg, MD). The aorta was cannulated with a 23-gauge needle, flushed briefly with buffer, and mounted onto a perfusion apparatus. The right ventricular outflow tract was excised, and the coronary arteries were perfused at 2.2 ml/min first with Ca^{2+}-free Joklik's buffer for 4 min followed by Joklik's buffer containing 25 μM Ca^{2+}, 75 U/ml collagenase I, 75 U/ml collagenase II (Worthington Biochemical Corp., Freehold, NJ), 1% albumin, and 2% donor calf serum, pH 7.2. The perfusion temperature was maintained at 37°C, and all buffers were continuously bubbled with 95% O_2 and 5% CO_2. After 15–20 min of perfusion, the heart was removed from the perfusion apparatus and transferred to a watch glass containing low Ca^{2+} Joklik's buffer supplemented with 25 μM Ca^{2+} and 2% donor calf serum. The left ventricle was isolated, minced, and gently pipetted into a 20-ml conical tube containing 10 ml of the buffer. The tissue was agitated to release loosened cells into the solution, which were then allowed to settle. Supernatant containing the isolated cells was immediately transferred to a new 50-ml conical tube. Isolation of the cells was repeated four times, and the cell supernatants were pooled. The pooled supernatant was centrifuged at 500 rpm for 1 min, and the cell pellet was resuspended in 20 ml low Ca^{2+} Joklik's buffer. After the cells were allowed to settle for 15 min, they were resuspended in physiological buffer (132 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_26H_2O$, 5 mM glucose, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2) supplemented with 1.8 mM Ca^{2+}.

Measurements of isolated myocyte mechanical properties. The cells were placed in a perfusion chamber on the stage of a microscope (IMT-2; Olympus Optical Co., Ltd., Tokyo, Japan) and constantly superfused with oxygenated physiological buffer at room temperature. Two platinum electrodes placed in the bathing fluid were connected to a Grass S9 stimulator (API-94; Innovative Lab Instruments, Cincinnati, OH), and the myocytes were stimulated with pulses of 2 ms duration at frequencies of 0.25 Hz (15 beats/min) for 40 s. Percent shortening, rate of shortening, and rate of relengthening of cardiomyocytes were quantified (8–10). Myocyte dimensions (length and width) measured from the videotaped images were compared with a calibration micrometer on the microscope stage.

Ca^{2+} measurements. Cytosolic-free Ca^{2+} was measured by ratio imaging of fura-2 fluorescence (8–10). Myocytes were loaded with fura-2 by incubation of a 1-ml suspension of isolated cardiomyocytes for 30 min at 37°C with 7.0 μM fura-2-acetoxymethyl ester in low Ca^{2+} Joklik's buffer. Fura-2-acetoxymethyl ester–loaded cells were allowed to settle, and the resulting pellet was resuspended in physiological buffer as described above. The fura-2–loaded myocytes were placed in a perfusion chamber, and images of the cells were simultaneously acquired through a charge-coupled device (Panasonic GP-CD60; Matsushita Communication Industrial Co., Ltd., Osaka, Japan), viewed on a monitor (PVM-122; Sony Corp., Tokyo, Japan), and illuminated by a PTI delta Scan-1 dual-beam spectrophotofluorometer (Photon Technology International, South Brunswick, NJ) at alternating excitation wavelengths of 340 nm (Ca^{2+} independent) and 380 nm (Ca^{2+} dependent). Emission was monitored at 510 nm. After background subtraction, images at 340 nm were divided by those at 380 nm to determine fluorescence ratios. Signals were analyzed by the Felix software (Photon Technology International).

Five to eight myocytes were analyzed from each of six transgenic and five wild-type littermate mice. The cells used for the mechanical studies were from the same hearts as those used for the Ca^{2+} measurements. Statistical analyses were performed on the basis of the number of hearts studied.

The effects of a PKCβ isoform selective inhibitor LY333531 (Lilly Laboratories, Indianapolis, IN) on myocyte mechanics and calcium transients were tested by a sequential superfusion of 0, 1, and 10 nM/liter concentrations (11). All data were collected for 1 min at 0.25 Hz, and additional 5 min was permitted for equilibration between dosage adjustments.

Heart perfusions. Hearts from anesthetized and heparinized mice were rapidly excised and immediately cannulated for retrograde aortic perfusion with modified Krebs buffer containing (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 0.5 Na_2EDTA, 0.23 KH_2PO_4, and 5.5 glucose. The buffer solution was saturated with 95% O_2 and 5% CO_2 at 37°C. The hearts were initially perfused at a constant flow of 2.5 ml/min for 30 min in a drip-through mode. The perfusion circuit was then switched to a recirculating system containing 2 mCi of [^{32}P] orthophosphate for 30 min. At the end of this ^{32}P-labeling period, the hearts were freeze clamped, powdered in liquid nitrogen, and stored at −80°C as previously described (12, 13). The specific radioactivity of [γ-^{32}P] ATP was determined from the specific activity of [^{32}P] phosphocreatine in each perfused heart (14).

Preparations of membranous and myofibrillar proteins. Microsomal fractions enriched in sarcoplasmic reticulum membranes were prepared from cardiac homogenates of individual hearts (12, 13). The starting material for the myofibrillar protein preparations was the pellet obtained after the first centrifugation of the heart homogenate during membranous preparations. The purification of myofibrils was carried out as previously described (12, 13).

Gel electrophoresis. Polyacrylamide gel electrophoresis of ^{32}Pi-labeled proteins was performed using 15% SDS gels. After electrophoresis, ^{32}Pi-labeled proteins were identified using the PhosphoImager (STORM 860; Molecular Dynamics Inc., Sunnyvale, CA) and autoradiography. Then, the radioactive bands were cut from the fixed gel and counted in scintillation fluid. Phosphate incorporation into these proteins was quantified by dividing the ^{32}P incorporation in each band by the specific activity of [γ-^{32}P] ATP (14), which was determined in each heart, and was expressed as picomoles ^{32}Pi per milligram protein loaded onto the gel lane (12, 13).

Quantitative immunoblotting. Quantitative immunoblotting of cardiac homogenates was used to determine the protein levels of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) 2, phospholamban, troponin I and troponin T (15, 16). The homogenates were electrophoretically separated on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane as previously described. Cardiac tissue from six mice was pooled together and used as an internal control on each gel. The transblots were reacted with monoclonal mouse antibody to SERCA2, phospholamban (Nos. MA3-919 and MA3-922; Affinity Bioreagents Inc., Golden, CO), troponin I and troponin T (Nos. RDI-TRK-4T19-6G9 and RDI-TRK-4T21-C5; Research Diagnostics Inc., Flanders, NJ), and then incubated with an anti–mouse IgG secondary

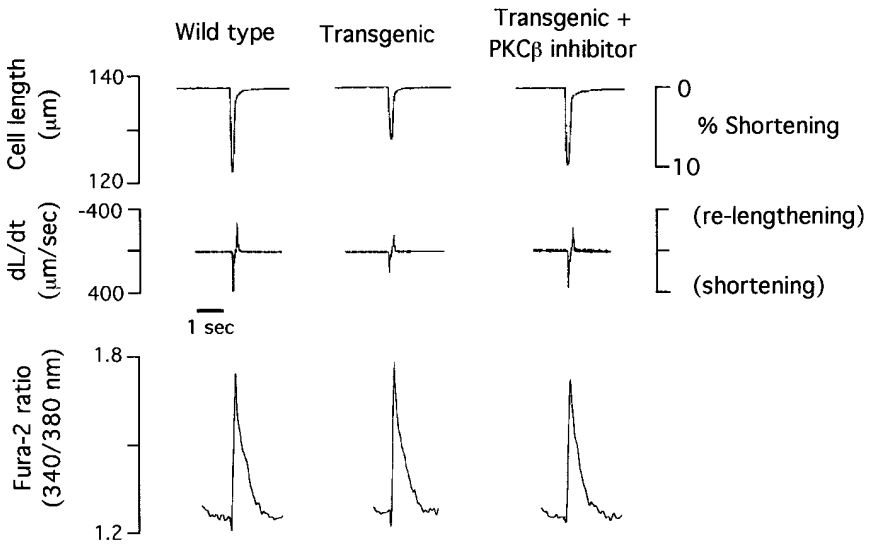

Figure 1. Representative analog recordings of cardiomyocyte mechanics and calcium transients of cells isolated from a wild-type control mouse, a PKCβ2 transgenic mouse, and a transgenic mouse where the cells were superfused with a PKCβ selective inhibitor.

antibody. After they were washed, membranes were developed using a chemiluminescent system, and quantified with ImageQuaNt software (Molecular Dynamics Inc., Sunnyvale, CA).

Statistics. Data are reported in mean±standard error. Statistical analysis was done with unpaired *t* tests to compare myocyte mechanics, calcium signals, ^{32}P incorporation, and protein levels between PKCβ2 transgenic mice and wild-type control mice. If data were not normally distributed or failed equal variance tests after $\log_{10}$ transformations, they were analyzed by nonparametric statistics (Mann-Whitney Rank Sum test). Repeated measurements analysis of variance and posthoc testing were used to compare the changes in myocyte mechanics and calcium signals after the drug superfusion. A P value of < 0.05 was considered significant.

Results

Myocyte mechanical function and calcium transients. Representative analog recordings of isolated cardiomyocyte mechanics for wild-type control and PKCβ2 transgenic mice are shown in Fig. 1. A marked reduction of myocyte contraction was observed in the transgenic mouse. However, the baseline calcium level and the amplitude of calcium transients were similar between wild-type and transgenic mice.

Group data for the morphological and mechanical properties and calcium transients of isolated cardiomyocytes from wild-type and transgenic mice are summarized in Table I. The percentage of myocyte shortening ($P < 0.001$), rate of shortening ($P < 0.001$), and rate of relengthening ($P < 0.001$) were markedly decreased in PKCβ2 transgenic mice compared to wild-type control mice. There was no significant difference in the baseline calcium level or amplitude, the time from start to 80% decay of the calcium signal (T_{80}) or 50% decay of the calcium signal (T_{50}) between wild-type control and transgenic mice. Despite gravimetric cardiac organ and in situ myocyte hypertrophy observed in our initial study (7), myocyte dimensions did not differ in vitro between wild-type and transgenic mice myocytes. This observation is compatible with partial contracture after enzymatic extraction and has been observed by this and other laboratories under these experimental conditions.

Effects of PKCβ inhibition on myocyte mechanics and calcium transients. The effects of a PKCβ isoform selective inhibitor LY333531 on myocyte mechanics and calcium transients were tested. Since LY333531 inhibited PKCβ2 with a half-maximal inhibitory constant (IC_{50}) of 5.9 nM (11), we tested 1- and 10-nM concentrations of this compound. Cardiomyocytes from wild-type and transgenic mice were exposed to incremental doses of the PKCβ inhibitor, and mechanical and Ca^{2+} signaling parameters were measured. As shown in Fig. 1, the decreased myocyte shortening in a transgenic mouse improved after a superfusion of the PKCβ inhibitor without significant change in the calcium transient. Group data for the effects of the PKCβ inhibitor on myocyte mechanics are shown in Fig. 2. The percentage of shortening, rate of shortening, and rate of relengthening were increased by the compound in transgenic mice ($P < 0.01$ at 10 nM), but not in wild-type mice. At a dose

Table I. Mechanical Properties and Calcium Transients of Isolated Left Ventricular Cardiomyocytes from Transgenic and Wild-Type Mice

	Wild Type	Transgenic
Mechanical parameters		
Percentage of shortening (%)	11.8±0.5	5.2±0.4*
Rate of shortening: +dl/dt (μm/s)	358±23	181±16*
Rate of relengthening: −dl/dt (μm/s)	282±24	109±15*
Cell length (μm)	136±6	135±10
Cell width (μm)	52±5	62±11
Calcium kinetics		
Baseline (ratio unit)	1.28±0.07	1.34±0.06
Amplitude (radio unit)	0.56±0.05	0.59±0.07
T_{80} (ms/ratio unit)	1.93±0.16	1.86±0.18
T_{50} (ms/ratio unit)	1.08±0.12	0.95±0.13

Values represent mean±standard error. Five wild-type mice and six transgenic mice were used for the analysis. Baseline, 340/380 ratio at rest; amplitude, height of the calcium peak upon stimulation; T_{80}, 80% decay of Ca^{2+} signal; T_{50}, 50% decay of Ca^{2+} signal. *$P < 0.001$ versus wild-type mice.

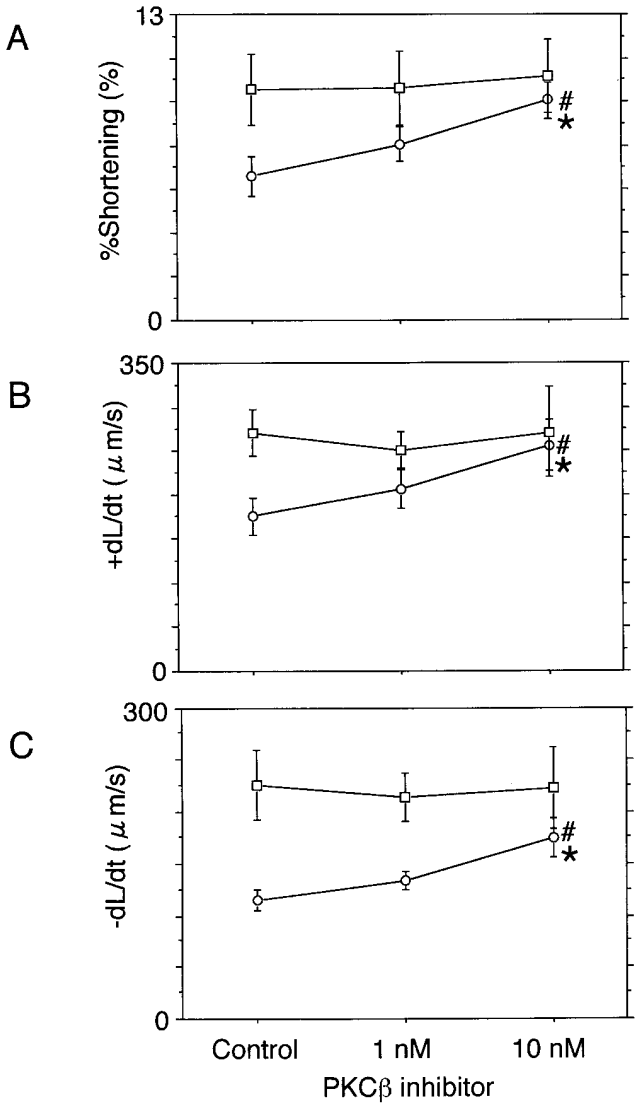

Figure 2. Grouped data for the effects of the PKCβ inhibitor on percentage of shortening (*A*), rate of shortening (*B*), and rate of relengthening (*C*) in isolated cardiomyocytes from wild-type (*open squares*) and PKCβ2 transgenic mice (*open circles*). Values represent the mean±standard error and were obtained using five wild-type and six transgenic mice. *$P < 0.01$ versus control and #$P < 0.05$ versus 1 nM.

of 10 nM, these contractile parameters of transgenic mice approached those of wild-type mice. Effects of the inhibitor on calcium transients are summarized in Table II. No significant changes in baseline, amplitude, T_{80}, and T_{50} were observed after the drug superfusion in both transgenic and wild-type mice.

Protein levels of calcium cycling and regulatory proteins. To determine whether the observed changes in the myocyte function were associated with altered expression of the calcium cycling and regulatory proteins, the relative levels of these proteins were determined by quantitative immunoblotting. No significant differences in phospholamban, SERCA2, troponin I, and troponin T were found between the PKCβ2 transgenic and wild-type mice (Table III).

Phosphorylation of cardiac proteins. The observed decrease in cardiomyocyte function of the PKCβ2 overexpression mice without any changes in the calcium signals suggested that myofilament sensitivity to calcium might be decreased. To elucidate the mechanism by which myofilament sensitivity to calcium was reduced, we examined the degree of phosphorylation of cardiac myofibrillar and membranous proteins. The incorporation of [^{32}P] phosphate into cardiac proteins was studied in Langendorff-perfused hearts. Fig. 3 *A* shows an autoradiogram of a SDS-polyacrylamide gel of myofibrillar proteins. An increase in the degree of phosphorylation of troponin I was observed in the PKCβ2 transgenic mouse compared with the wild-type littermate. These increases were significant ($P < 0.02$) and specific to troponin I, since there were no significant changes in the degree of phosphorylation of other phosphoproteins in the myofibrillar or membranous fraction from the PKCβ2 transgenic hearts compared to wild-type hearts (Table IV). When the increased phosphorylation of troponin I in PKCβ2 transgenic hearts was normalized to that in wild-type hearts (100%), there was a 41% increase observed (Fig. 3 *B*).

Table II. Effects of PKCβ Inhibition on Calcium Transients in Isolated Cardiomyocytes from Wild-Type and Transgenic Mice

	Control	1 nM	10 nM
Baseline (ratio unit)			
Wild type	1.29±0.07	1.31±0.09	1.35±0.07
Transgenic	1.39±0.09	1.48±0.14	1.44±0.12
Amplitude (ratio unit)			
Wild type	0.54±0.05	0.53±0.05	0.56±0.10
Transgenic	0.49±0.07	0.47±0.05	0.47±0.05
T_{80} (ms/ratio unit)			
Wild type	1.86±0.14	2.01±0.28	2.05±0.33
Transgenic	2.04±0.16	2.15±0.27	2.02±0.31
T_{50} (ms/ratio unit)			
Wild type	1.09±0.15	1.13±0.17	1.11±0.22
Transgenic	1.11±0.12	1.10±0.19	1.14±0.24

Values represent mean±standard error. Five wild-type mice and six transgenic mice were used for the analysis. Baseline, 340/380 ratio at rest; amplitude, height of the calcium peak upon stimulation; T_{80}, 80% decay of Ca^{2+} signal; T_{50}, 50% decay of Ca^{2+} signal.

Table III. Protein Levels of Calcium Cycling and Regulatory Proteins in Hearts from PKCβ2 Transgenic and Wild-Type Mice

	Wild-type	Transgenic
SERCA2	21.0±0.6	20.6±0.7
Phospholamban	30.1±0.8	32.8±2.2
Troponin T	3.4±0.1	3.4±0.1
Troponin I	3.5±0.5	3.7±0.4

Values are mean±standard error and are expressed as scan units per milligram protein. Five wild-type and five transgenic mice were used for the analysis. Relative protein levels were determined by quantitative immunoblotting as described in Methods.

Table IV. ^{32}P Incorporation into Cardiac Membranous and Myofibrillar Proteins in Wild-Type and PKCβ2 Overexpression Hearts

	Picomoles ^{32}P/mg protein	
	Wild-type	Transgenic
Myofibrillar proteins		
Troponin T	141±13	121±6
Troponin I	71±3	100±9*
Myosin light chain	85±16	120±16
Membranous proteins		
50-kD protein	122±7	162±24
Phospholamban	60±10	77±7
15-kD protein	71±10	106±20

Values are mean±standard error. Five wild-type and six transgenic mice were used for the analysis. *$P < 0.02$ compared with wild-type mice. Membranous and myofibrillar proteins were isolated and subjected to SDS-PAGE and autoradiography, as described in Methods. The phosphorylated protein bands were identified, cut from the gel, and counted. Phosphate incorporation into the proteins was calculated by dividing ^{32}P incorporation into each band by the specific activity of [γ-^{32}P]ATP in the respective heart.

Discussion

The major findings of the present study were as follows: (*a*) mechanical function of isolated left ventricular cardiomyocytes was depressed in PKCβ2 overexpressing transgenic mice compared to wild-type controls, although the calcium transients were similar; (*b*) these findings suggested that a decrease in calcium sensitivity of the myofilaments resulted in the cardiomyocyte dysfunction; (*c*) the degree of phosphorylation of cardiac troponin I was significantly increased in PKCβ2 transgenic mice and may have contributed to the reduced myofilament sensitivity to calcium; and (*d*) the decreased cardiomyocyte contractility and calcium sensitivity were improved by the PKCβ inhibitor.

Possible mechanisms of decreased contractility in PKCβ2 overexpressing hearts. We initially observed variable fibrosis in the PKCβ2 transgenic heart which may have contributed to altered in vivo left ventricular performance (7). However, the present study clearly demonstrates that the decreased left ventricular function observed in our initial report was, at least in part, a result of diminished intrinsic cardiomyocyte function. The percentage of shortening, rate of shortening, and rate of relengthening were each markedly reduced in isolated left ventricular cardiomyocytes extracted from PKCβ2 overexpressing hearts.

It is well known that altered calcium kinetics modify cardiac contractility (2, 3, 9, 15). However in the present study, calcium transients measured with fura-2 and the levels of calcium cycling proteins were similar between the PKCβ2 transgenic and wild-type mice.

The myosin heavy chain isoform switch from α to β which was observed in the PKCβ2 transgenic mice may have contributed to the decreased contractility (7). However, we demonstrate here that depressed cardiomyocyte function in PKCβ2 transgenic mice was reversed by the PKCβ selective inhibitor and approached that of wild-type littermates. This finding indicates that the excess activation of PKCβ2, but not the isoform switch of myosin heavy chain, is the predominant cause of cardiomyocyte dysfunction.

Although mechanical function of isolated left ventricular cardiomyocytes was depressed in PKCβ2-overexpressing transgenic mice compared to wild-type controls, the baseline calcium levels and amplitude of calcium signals were similar in both groups in the present study. This finding suggested that either a decrease in myofilament ATPase activity or a decrease in calcium responsiveness of myofilaments are possible mechanisms for the cardiomyocyte dysfunction.

Myofilament calcium responsiveness. Studies of myofibrillar mechanical function in human and experimental heart failure have yielded conflicting results: decreased (17, 18) and increased (19) calcium responsiveness have been reported. There are several mechanisms by which myofibrillar calcium sensitivity may be altered, including: (*a*) phosphorylation of

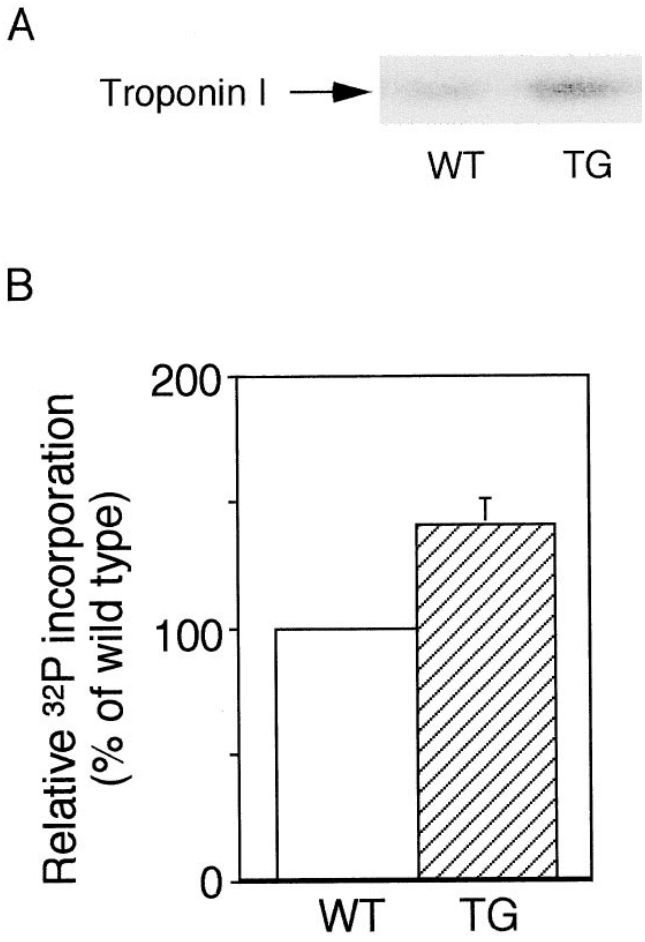

Figure 3. (*A*) Autoradiogram of polyacrylamide gel of a myofibrillar preparation. Myofibrillar proteins were isolated and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography, as described in Methods. (*B*) Comparison of ^{32}P-incorporation into troponin I between PKCβ2 transgenic and wild-type mice. ^{32}P-incorporation into troponin I of PKCβ2 mice was expressed relative to that of wild-type mice (100%). Values represent the mean±standard error. *WT*, Wild-type mice; *TG*, transgenic mice.

myofibrillar proteins (20); (*b*) changes in regulatory contractile protein isoforms (21); and (*c*) regulation of intracellular pH (22).

Noland and Kuo have reported that phosphorylation of troponin I or troponin T by PKC in vitro reduces calcium sensitivity and maximal activity of actomyosin MgATPase, and thus impairs actin–myosin interactions (23). Phosphorylation of serine-23/serine-24 in troponin I is responsible for desensitizing myofilaments, and phosphorylation of serine-43/serine-45 reduces maximal activity of myofibrillar ATPase (24). They also suggested that this might explain the negative inotropic effects of phorbol esters (4). In the present study, we showed that the degree of phosphorylation of troponin I was significantly increased in PKCβ2-overexpressing mice. It is probable that in vivo phosphorylation of cardiac myofibrillar proteins by PKCβ2 decreases myofibrillar calcium sensitivity in these transgenic hearts. To our knowledge, this is the first report demonstrating that troponin I is a substrate for PKCβ in intact beating hearts.

It has also been reported that troponin T isoform shifts occur in diabetic rats and might be a cause for a diminished calcium sensitivity of skinned cardiac muscle and resultant decreased contractility (25). In addition troponin I is partially and selectively degraded in stunned myocardium (26). However in the present study isoform alterations of troponin I or troponin T were not identified by immunoblotting. The protein levels of cardiac troponin I, troponin T, SERCA2, and phospholamban were similar between the PKCβ2 transgenic and wild-type mice.

Acidosis displaces calcium from troponin C with a resultant direct depressant effect on the actin–myosin cross-bridge interaction (22). PKC can potentially modify the regulation of intracellular pH through the activation of the Na^+/H^+ exchanger and secondarily alter myofibrillar calcium sensitivity. A decrease in Na^+/H^+ exchanger activity of cardiac papillary muscle has been reported in streptozotocin-induced diabetic rats (27). A defect in the coupling of PKC signaling with the Na^+/H^+ exchanger has been shown in adult hypertrophied myocardium generated by aortic banding (28). Since PKCβ2 activation was observed in diabetic cardiomyopathy (29), and PKCβ2 overexpression mice had left ventricular hypertrophy (7), we cannot exclude the possibility that regulation of intracellular pH might be impaired in these mice and contribute to diminished cardiomyocyte function.

PKCβ isoforms and heart failure. PKC has been implicated as the intracellular mediator of several neurotransmitters, growth factors, and tumor promoters through multiple signal transduction pathways (30). We have recently demonstrated that in the adult guinea pig heart, left ventricular dilatation produces stretch-mediated activation of phospholipase C which results in inositol phosphate hydrolysis and PKC activation (4). It has also shown that transgenic cardiac-specific Gαq overexpression results in a dilated cardiomyopathy, PKC activation, and overt heart failure. These functional abnormalities are associated with intrinsic cardiomyocyte dysfunction (31). These findings coupled with the similar phenotype observed in the PKCβ2-overexpressing mice strongly implicated overreactivity of the phospholipase C cell signaling pathway in the pathogenesis of cardiac hypertrophy and heart failure (4).

Presently, at least 11 isoforms of PKC have been identified in vivo. Isoform specific activation of PKC has been found in myocardial hypertrophy and failure. The level of PKCβ isoform is increased during development of cardiac hypertrophy induced by pressure overload in rats (5). We examined explanted hearts of patients diagnosed with idiopathic-dilated cardiomyopathy or ischemic cardiomyopathy and found that PKCβ expression was elevated in failed human heart, and its contribution to total PKC activity was significantly increased (6). These lines of evidence suggest that PKCβ may play a critical role in the development of hypertrophy and heart failure. Although we did not examine the protein abundance of PKCε, this isoform appears to be unchanged or reduced in human congestive heart failure (6). PKCα and δ were not significantly altered in this transgenic line (7). Taken together these data provide a mechanism by which PKCβ activation may directly depress myocardial contractility.

PKCβ activation has been also reported in diabetic heart (29). There is higher mortality and morbidity after myocardial infarction in diabetic patients, and the major cause of mortality is ventricular failure (32). Despite the higher incidence of coronary artery disease in diabetic patients, coronary atherosclerosis itself cannot explain the increased frequency of congestive heart failure in such individuals, and the etiology of functional abnormalities has remained uncertain. We demonstrate in the present study that decreased myofilament responsiveness to calcium associated with phosphorylation of troponin I may contribute to these pathologic processes.

In this study the use of a highly selective PKCβ inhibitor reversed the decrease in cardiomyocyte contractility without affecting the calcium transient. This result further supports our proposal that depression of intrinsic cardiac function is in part related to excessive activation of the PKCβ isoform and resultant diminished myofilament sensitivity to calcium. The use of isoform selective PKC inhibitors in a conventional animal model of cardiac failure will further elucidate the role of this signal transduction pathway in the progression from compensated hypertrophy to heart failure.

Conclusion. In conclusion, our findings indicate that increased phosphorylation of cardiac troponin I by PKCβ2 decreases myofilament calcium responsiveness, and thus reduces cardiomyocyte function. Selective PKCβ inhibition improves depressed cardiomyocyte function by increasing myofilament responsiveness to calcium. Since PKCβ isoform activation appears to be involved in the structural and functional changes observed in human heart failure, selective inhibition of the PKCβ isoform may provide a novel therapeutic strategy for the prevention and treatment of this pathologic process.

Acknowledgments

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