Changes in protein kinase C in early cardiomyopathy and in gracilis muscle in the BB/Wor diabetic rat

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Giles, Thomas D., Jie Ouyang, E. Kenneth Kerut, Michael B. Given, Gayle Eileen Allen, Elizabeth F. McIlwain, and Stan S. Greenberg. Changes in protein kinase C in early cardiomyopathy and in gracilis muscle in the BB/Wor diabetic rat. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H295–H307. 1998.—Hyperglycemia can upregulate protein kinase C (PKC), which may be an important mediator of the progression from normal heart and muscle function to diabetic myopathy in the myocardium and skeletal muscle in type 1 insulin-dependent diabetes mellitus (IDM). We evaluated this possibility during the early stage of IDM in BB/Wor diabetic (D) rats and age-matched BB/Wor diabetes-resistant (DR) rats. Interventricular septal thickness, E wave peak velocity of tricuspid inflow (both minimum and maximum), and left ventricular (LV) weight index were increased, and the rate of change in LV pressure (LV dP/dt) decreased in D rats subjected to M-mode and two-dimensional echocardiography and hemodynamic recording of heart rate, LV pressure (LVP), +LV dP/dt, −LV dP/dt, and LV end-diastolic pressure (LVEDP) in vivo and in vitro 41 days after the onset of hyperglycemia. Whole ventricle basal PKC activity was increased by 44.4 and 18.4% in the particulate and soluble fractions, respectively, from D rats compared with that from DR rats using r-32P phosphorylation of appropriate peptide substrates. When measured by Western blot gel densitometry, particulate PKC-α and PKC-δ content increased by 89 and 24%, respectively, and soluble PKC-β and soluble and particulate PKC-ε were unchanged compared with that of DR rats. Similarly, gracilis muscle PKC activity and PKC-α and PKC-δ were elevated in the gracilis muscle, whereas that of the circulating neutrophil did not differ between the D and DR rats. Thus, in vivo, the early diabetic cardiomyopathy of the D rat is characterized by a restrictive LV with increased septal thickness and is associated with elevated PKC activity and increased amounts of myocardial particulate PKC-α and PKC-δ, which are also seen in the gracilis muscle. We conclude that increased PKC isoforms may play a pivotal role during IDM in the development of diabetic cardiomyopathy and skeletal muscle myopathy.

Protein kinase C (PKC) is a family of enzymes involved in the phosphorylation of enzymatic and regulatory protein substrates (21, 23). Phosphorylation and dephosphorylation of enzymes are important physiological mechanisms utilized for the activation and deactivation of enzymatic activity (15, 21, 23). Twelve isoforms of PKC have been identified and subsequently subdivided into three major categories: 1) Ca2+-dependent and phospholipid- and diacylglycerol (DAG)-activated PKC isoforms (dPKC), 2) Ca2+-independent and phospholipid- and DAG-activated PKC (nPKC), and 3) atypical PKC isoforms (aPKC) that are Ca2+ independent and activated by phosphatidylcholine or phosphatidylethanolamine (15, 21, 23, 36). Activation of PKC may result from mechanical and physical deformation of the myocardial cell membrane (e.g., stretching or distension), the interaction of agonists with their myocardial cell membrane receptors, or elevated intracellular concentrations of the substrates that activate PKC, including DAG, phosphatidylcholine, phosphatidyletha-
nolamine, and Ca\(^{2+}\). Mechanical distention or the interaction of agonists with their myocardial membrane receptors activates phospholipase C, resulting in the hydrolysis of phosphatidylyl 4,5-bisphosphate into inositol (1,4,5)trisphosphate [Ins(1,4,5)P\(_3\)] and DAG. Ins(1,4,5)P\(_3\) releases intracellular Ca\(^{2+}\), which then combines with cytosolic CPKC. This aids in the binding of an inactive form of cPKC to the phosphatidylserine (PS) residues of the cell membrane. The binding of DAG to the membrane-bound inactive form of cPKC results in its activation and subsequent ability to phosphorylate enzymes or receptor proteins. Alternatively, DAG can bind to inactive nPKC. This allows the binding of the DAG-nPKC complex to PS residues in the membrane and results in the subsequent activation of nPKC (15, 21, 23, 36). An alternate pathway for activation of PKC also exists. Activation of phospholipase D hydrolyzes phosphatidylcholine to phosphatidic acid, which is further metabolized to DAG and to phosphatidylethanol. Both DAG and phosphatidylethanol can subsequently activate PKC (15, 21, 36). A similar mechanism exists in skeletal muscle (15, 23, 36).

Phosphorylation of myocardial and skeletal muscle enzymes and proteins by PKC isoenzymes can affect cardiac rhythm and cardiac and skeletal muscle contractility, gene expression, and growth (2, 12, 15–17, 21, 23, 24, 35, 36, 40, 42). Protein kinase C-mediated phosphorylation of troponin T, troponin I, troponin-tropomyosin complex, and troponin-C protein in isolated myocardial and skeletal muscle cells is associated with inhibition of Ca\(^{2+}\)-activated myofibrillar actomyosin MgATPase activity and contractility (21, 23, 24). The PKC-\(\delta\) isoform can increase Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (35), which are also present in the membrane of ventricular myocytes and skeletal muscle (15, 21, 23, 29). Activation of cPKC-\(\delta\) and nPKC-\(\varepsilon\) is associated with myocardial ventricular hypertrophy, including overexpression of \(\beta\)-MHC, vMLC-2, ANF, and ACE (36).

Recent studies in vascular smooth muscle and in cardiac myocytes in culture suggest that elevated plasma levels of glucose, the hallmark of both type 1 and type 2 diabetes mellitus, may be causal to elevated tissue levels of PKC and thereby may play a pivotal role as a mediator of the progression from normal to diseased vasculature and myocardium (1, 8, 16, 25, 26, 39, 42). Hyperglycemia may increase the DAG content of the rat myocardium (16, 25, 26). Total PKC activity also increased in the myocardium of diabetic rats (37, 43). Persistent upregulation of PKC activity may lead to alterations in myocardial contractility and cell growth (2, 13, 15–17, 21, 24, 25, 36, 37, 40, 42), which may compensate for the potential decrease of protein synthesis resulting from impaired sensitivity of the diabetic myocardium to this action of adenosine 3',5'-cyclic monophosphate (cAMP) (32). Moreover, sustained elevations of PKC activity can increase the activity of serum and tissue levels of ACE in the diabetic rat (39).

Increased ACE activity can increase sera and tissue levels of angiotensin II, which can cause myocardial remodeling and hypertrophy (35, 38). We previously reported that treatment of rats with the ACE inhibitor benazepril prevented the development of myocardial dysfunction in streptozocin (ST2)-induced diabetic cardiomyopathy (9). These data all suggest that dysregulation of PKC may play a pivotal role in the development of cardiac and skeletal muscle myopathy in IDM. However, if changes in PKC are causal to the development of diabetic myopathy, we hypothesize that alterations in PKC must be present early in its development at a time when functional abnormalities would be minimal or absent. We tested this hypothesis in BB/Wor diabetic (D) and diabetic-resistant (DR) rats by measurement of the activity and total quantity of PKC and PKC isozymes in ventricular tissue, gracilis muscle, and circulating neutrophils and by measurement of cardiac structure and function in vivo, using hemodynamic measurements and echocardiography, and direct evaluation of myocardial function in vitro, utilizing the isolated working heart preparation.

**METHODS AND MATERIALS**

General experimental protocol. Male BB/Wor D rats (diabetic for 30–41 days) and age-matched DR rats were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases breeding and maintenance facility at the University of Massachusetts. The D rats were maintained on daily subcutaneous injections of 0.6–3.0 U/rat of a long-acting (once a day) protamine, zinc, and insulin suspension (protamine, zinc, and Iletin I, Eli Lilly, Indianapolis, IN) before their feeding period on the basis of their urinary excretion of glucose from the first appearance of glucosuria. DR rats were given equivalent subcutaneous injections of vehicle. Daily injections of insulin were necessary because the D rats, a model for IDM, die within a few days without insulin maintenance. All animals were housed on a 12:12-h light-dark cycle with access to water and standard rat chow ad libitum. All animals were anesthetized intramuscularly with a solution consisting of (in mg/kg) 50 ketamine-4 xylazine before echocardiography and Doppler flowmetry or surgery for collection and measurement of blood for serum glucose concentrations using a standard glucose monitor (Boehringer Ingelheim, Ridgefield, CT). The D and DR rats were subdivided into two cohorts. One cohort was subjected to echocardiography and Doppler flowmetry followed by invasive measurement of cardiovascular dynamics. Their blood was removed and the neutrophils were isolated on a Ficoll-Percoll gradient as described previously (20). The hearts were also removed and the ventricles were rapidly frozen in liquid nitrogen. The second cohort of rats was subjected to echocardiography and Doppler flowmetry and the hearts were then removed and used for in vitro perfusion while the gracilis muscle was removed and frozen in liquid nitrogen. The ventricles, gracilis muscles, and neutrophils were assayed for PKC activity and isozyme content as described below. The experimental protocol (1224) was approved by the Louisiana State University Institutional Animal Care and Use Committee.

Echocardiographic analysis of cardiac performance. M-mode and two-dimensional echocardiograph and Doppler flow recordings were made using a Toshiba model 270 echocardiography instrument with a 7-MHz transducer. The transducer was calibrated with phantoms before use. Two-dimensional echocardiograms were recorded from both parasternal long- and short-axis views, apical four-chamber views and suprasternal views of the aortic valve, and from both ascending and
Henseleit buffer aerated with a 95% O2-5% CO2 mixture, and 50 U/ml were added to the initial perfusate, Krebs-administered to the rats immediately before they were killed, previously in detail (9). Heparin sodium (200 U iv) was perfused on a standard working heart apparatus as described obtained by differentiation of the LVP signals. Values were calculated.

E and A wave velocities and E:A ratio, tricuspid Doppler E and A wave velocities and E:A ratio (both minimum and maximum), and the difference between maximum and minimum E wave Doppler velocities (ΔE Velmax−min) were obtained. Tricuspid ΔE Velmax−min was also calculated.

Determination of LV performance in vivo. The anesthetized rats were placed on a blanket, temperature-controlled surgical table (37°C). A polyethylene catheter (PE-30) connected to a Gould P23Db pressure transducer was advanced into the LV via the right carotid artery. The position of the catheter in the LV was confirmed by the LV pressure (LVP) tracing. After LVP and heart rate (HR) stabilized, HR, LVP, and the positive and negative rate of change of LVP (±LV dP/dt) were continuously recorded and continuously logged on an Apple 650 Quadra computer. The data for 1-min increments were obtained at 300 Hz, and the average values were calculated. +LV dP/dt and −LV dP/dt were obtained by differentiation of the LVP signals.

Determination of cardiac performance in vitro. Hearts were perfused on a standard working heart apparatus as described previously in detail (9). Heparin sodium (200 U iv) was administered to the rats immediately before they were killed, and 50 U/ml were added to the initial perfusate, Krebs-Henseleit buffer aerated with a 95% O2-5% CO2 mixture, to maintain a pH of 7.4. The perfusate was filtered using a Whatman Polycap HD filter (10-μm pore size) to prevent cells and debris from clogging the coronary circulation. All hearts were paced at 250 beats/min and allowed to stabilize before the start of the experiment. Aortic pressure, measured with a Statham P23Db pressure transducer positioned at the level of the aortic valve, was set at 65 mmHg. LVP was measured with a Statham P23Db pressure transducer connected to a 26-gauge needle inserted through the ventricular wall at the "dimple" located at the apex of the heart. The LVP transducer was positioned at a level corresponding to midventricle. Positive and negative LV dP/dt were obtained by differentiating the LVP signal. Left atrial pressure (LAP) was measured via an atrial cannula connected to a Statham P23Db pressure transducer positioned at the level of the left atrium and was varied during the experiment by adjusting the height of the atrial reservoir. Initial LAP was set at 10 cmH2O. After equilibration of the heart, LAP was reduced to 5 cmH2O, increased in 5-cmH2O increments to a maximum of 20 cmH2O, and then reduced to 10 cmH2O at the end of the experiment. The response of the heart to each increase in LAP was allowed to stabilize before the recording of the data. Pressures and derived indexes were recorded on a Grass model 79 polygraph. An Apple Macintosh Quadra 650 computer sampled and digitized the data from the polygraph for storage and subsequent analysis. The cardiac performance parameters measured were maximum developed LV pressure (LVPmax), ±LV dP/dt, −LV dP/dt, and LAP.

Specificity of assay for PKC isozymes. Recent data suggest the possibility that some antibodies specific for PKC isozymes cross-react with other PKC isozymes to give false indexes of the identity and quantity of PKC isozyme present in various tissues (30). To ascertain the existence or absence of constitutive PKC isozymes and the specificity of the antibodies used to test for the presence of the PKC antibody-specific proteins, we analyzed the ventricular homogenate for the presence of PKC isozyme mRNA and protein and subsequently performed PKC antibody isoform neutralization using authentic PKC isozyme peptides as antigens. Transcripts for PKC isozymes were measured by reverse transcriptase-polymerase chain reaction (RT-PCR) in whole homogenates of ventricular myocardium as previously described for alveolar macrophages (10, 11, 20). Briefly, the total RNA of the homogenized ventricle was isolated using Trizol reagent (GIBCO, Gaithersburg, MD). Total cDNA was obtained by reverse transcription of total RNA and was labeled with [32P]dCTP. Total cDNA (10 ng) was amplified by using the specific PKC isozyme primers and [32P]dCTP. Primer sequences for the PKC isozymes are shown in Table 1. The relative amounts of PKC isozyme cDNA were determined by phosphorimager scan and quantitation of the smear and signal bands, normalized to that of β-actin (n = 8) as

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequence</th>
<th>Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td>Forward 5'-TGA ACC CCT AGT GGA ATG AGT-3'</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGC TGC TCT CGT TCT GAA-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-β</td>
<td>Forward 5'-TGT GAT GGA GTA TGT GAA CCG GGG-3'</td>
<td>639</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCG AAG TTT GAG GTG TCG CGC TTG-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-γ</td>
<td>Forward 5'-TGG ATG GGG AAG ATG AGG AGG-3'</td>
<td>347</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAA ATC AGC TTG GTC GAT GCT G-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-δ</td>
<td>Forward 5'-CAC CAT CCT CCA AGA AGA ACG-3'</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTT GCC ATA GGT CCC GTT GGT G-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-ε</td>
<td>Forward 5'-CGA GGA CGA CTT GTT TGA ATC C-3'</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAG TTT CTC AGG GCC ATC GCT C-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-ζ</td>
<td>Forward 5'-CCA TGA AGA TGC CAG AGG GAT C-3'</td>
<td>249</td>
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<tr>
<td></td>
<td>Reverse 5'-TCA TCA ATG GGA GTT AAG AGG G-3'</td>
<td></td>
</tr>
<tr>
<td>PKC-η</td>
<td>Forward 5'-CGA TGG GTT GGA TGG CAT AA A-3'</td>
<td>681</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTA TTC ATG TCA GGG TTG TCT G-3'</td>
<td></td>
</tr>
</tbody>
</table>

PKC, protein kinase C; bp, base pairs.
analyses. PKC isozyme content was determined with Western blot described previously in detail (10, 11, 20). Whole homogenate H298 PKC AND DIABETIC CARDIOMYOPATHY homogenate in homogenization gous D and DR rats were quantitatively pulverized and homogenized in homogenization buffer I (20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM ethylene glycol-bis(β- aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.02% leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Triton X-100) in a cold room. The homogenates were incubated in 2°C and subsequently centrifuged (17,500 g for 30 min at 4°C). The supernatants were then centrifuged at 37,000 g to isolate the mitochondria, the resulting supernatant was centrifuged at 100,000 g at 4°C, and the particulate (membrane) and soluble fractions (cytosol) were stored at −20°C until assayed for protein with the bicinchoninic acid (BCA) method (10, 11). The protein samples (50 or 100 µg) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% (wt/vol) acrylamide separating gel and a 4% (wt/vol) acrylamide stacking gel. The protein was then electrophoretically transferred to nitrocellulose using a Semi-Dry transfer cell (Bio-Rad, Hercules, CA) and a transfer buffer consisting of Tris-HCl (48 mM) and 39 mM glycine (pH 9.2) containing 0.037% (wt/vol) SDS and 20% (vol/vol) methanol.

After nonspecific sites were blocked with blocking solution [phosphate-buffered saline (PBS) without heparin, containing 5% (wt/vol) nonfat milk and 0.05% (vol/vol) Tween-20] for 1 h at room temperature, the nitrocellulose was incubated with the primary antibodies (GIBCO BRL, Grand Island, NY; Transduction Laboratories, Lexington, KY) in buffer II (1/1,000 to 1/2,000 dilution in PBS without heparin, containing 1% (wt/vol) nonfat milk and 0.05% (vol/vol) Tween-20) overnight at 4°C. The nitrocellulose was then washed three times, for 10 min each, with blocking buffer devoid of nonfat milk and then incubated at room temperature for 1 h with horseradish peroxidase-linked secondary antibody (1/5,000 dilution in PBS to remove residual blood and were then frozen in liquid nitrogen. Portions of the frozen ventricles, gracilis muscle, and neutrophils were quantitatively pulverized and then placed in homogenization buffer I (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 0.02% leupeptin, 1 mM PMSF) and homogenized in a cold room with a Polytron set at 7 for 20 s, followed by homogenization for 60 strokes using a Dounce homogenizer. The homogenates were centrifuged as described in Western blot analyses of PKC isozymes for the PKC isozyme assay, and the particulate and soluble fractions were obtained for measurement of PKC activity. The pellets were washed and resuspended in buffer II (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.02% leupeptin, 1 mM PMSF, and 0.1% Triton X-100) again homogenized. The homogenates were solubilized in buffer II without Triton X-100. After a 45-min incubation period at 4°C, the soluble fraction was obtained by ultracentrifugation at 100,000 g for 30 min, and the pellet was retained as the particulate or membrane fraction. Both membrane and soluble fractions were passed through 0.5 ml DEAE columns (Pharmacia, Gaithersburg, MD), washed twice with buffer II (2 ml), and then eluted with 0.4 ml of phosphate buffer containing 200 mM NaCl (37). PKC activity was determined with an Amersham kit for PKC activity (Amersham, Arlington Heights, IL) by measurement of the phosphorylation of the specific substrate octapeptide (RKRTLRLRL) in the presence of Ca²⁺, PS, and DAG using [³²P]ATP (Amersham, Arlington, Waltham, MA). The protein content of the fractions was measured by the BCA method as described previously in detail (10, 11). The enzymatic data were expressed as picomoles per milligram protein per minute after comparison to a standard curve and correction for the nonspecific kinase activities found in the absence of Ca²⁺, PS, and DAG.

Statistical analysis of data. Each experiment contained four to six animals per treatment group. Data were analyzed with analysis of variance (ANOVA) for a randomized complete block or completely random sample design. Differences between and among means were analyzed by Dunnett’s and Duncan’s tests. Biochemical data were analyzed with multiple ANOVA and means were compared with Newman-Keuls test; P < 0.05 was accepted for statistical differences between and among means.
RESULTS

Body weights, blood glucose concentration, and cardiac measurements. Body weights did not differ among the DR and D rats (Table 2). The D rats demonstrated glucosuria between 28 and 49 days after their birth and were diabetic, as evidenced by a glucosuria of +2 to +4, for 6–8 wk before they were killed. At the time of death blood glucose concentrations were increased in D rats compared with those of DR rats (P < 0.05) (Table 2). Although total heart weight and LV mass did not differ among the DR and D rats, there was a small but insignificant increase in LV mass (P = 0.076) and a significant increase in LV mass index (LV wt/body wt; P = 0.013) in the LV obtained from the BB/Wor D rats compared with the LV mass index obtained from the DR rats. When total heart weight was expressed as a percentage of total body weight (heart weight index), no significant difference was observed between DR and D rats (Table 2). Interventricular septal thickness was increased in the hearts obtained from the BB/Wor D rats compared with that of the DR rats (P = 0.03) (Table 3, Fig. 1).

Echocardiographic measurements. Analysis of M-mode echocardiographic recordings revealed an increase in interventricular septal thickness in D rats compared with those obtained from DR rats (P = 0.05), whereas no significant difference in LV internal dimension or LV posterior wall thickness was evident between the two groups of rats (Table 3). Doppler patterns of mitral inflow did not differ between the DR and D rats. However, the E wave peak velocity of tricuspid inflow (both minimum and maximum) was increased in D rats compared with that of the DR rats (P = 0.03) (Table 3, Fig. 1).

Determination of LV performance in vivo and in vitro. LVPmax did not differ between the BB/Wor DR and D rats (Table 4). Moreover, LVEDP was not significantly elevated in the D rats compared with that of the DR rats (Table 4). In contrast, myocardial contractility as reflected by +LV dP/dt (P < 0.05) and the rate of relaxation of the LV as reflected by −LV dP/dt (P = 0.08) were lower in the BB/Wor D rats compared with these parameters in the BB/Wor DR rats (Table 4). However, no significant differences in myocardial function existed in vitro in the paced, isolated hearts obtained from the D and DR rats when maintained at an LAP of 5 or 20 cmH2O (data not shown) and at 10 cmH2O (Table 4).

PKC isozyme mRNA, specificity of antibody, and linearity of PKC isozyme content and enzymatic activity. The constitutive mRNAs present in the ventricle of the D rats as determined by RT-PCR were PKC-δ >> PKC-ε >> PKC-α >> PKC-γ = PKC-ζ >> >> PKC-β (Fig. 2). Similarly, the PKC isozymes detectable in the whole homogenate of ventricle were PKC-δ >> PKC-ε = PKC-α (Fig. 3). When the ventricular homogenate was separated into the soluble (cytosolic) and particulate (membrane) fractions, the PKC isozymes detectable in the membrane fraction of the ventricle were PKC-δ >> PKC-ε = PKC-α, whereas those of the cytosol were PKC-α > PKC-ε > PKC-δ > PKC-ζ > PKC-γ > PKC-β (Fig. 3). PKC-β and PKC-γ were absent in two of five ventricles despite the presence of their mRNA (Fig. 3). Each of the PKC isozyme-specific antibodies elicited a signal when reacted with its authentic isozyme and with the brain extract at a molecular weight that corresponded to the authentic PKC isozyme. PKC-ζ was detectable in the

Table 2. Comparison of body weight, blood glucose, and cardiac weight of BB/Wor DR rats and BB/Wor D rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>338 ± 27</td>
<td>351 ± 32</td>
<td>0.53</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>143 ± 14</td>
<td>347 ± 79</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.17</td>
</tr>
<tr>
<td>Ventricular wt, g</td>
<td>0.776 ± 0.05</td>
<td>0.852 ± 0.09</td>
<td>0.14</td>
</tr>
<tr>
<td>Right ventricle wt, g</td>
<td>0.202 ± 0.024</td>
<td>0.192 ± 0.021</td>
<td>0.507</td>
</tr>
<tr>
<td>Left ventricle wt, g</td>
<td>0.57 ± 0.05</td>
<td>0.66 ± 0.08</td>
<td>0.076</td>
</tr>
<tr>
<td>Interventricular septum wt, g</td>
<td>0.114 ± 0.013</td>
<td>0.129 ± 0.0015</td>
<td>0.03*</td>
</tr>
<tr>
<td>Heart wt/body wt, %</td>
<td>0.285 ± 0.023</td>
<td>0.309 ± 0.031</td>
<td>0.293</td>
</tr>
<tr>
<td>Right ventricle wt/body wt, %</td>
<td>0.060 ± 0.010</td>
<td>0.056 ± 0.007</td>
<td>0.377</td>
</tr>
<tr>
<td>Left ventricle wt/body wt, %</td>
<td>0.169 ± 0.003</td>
<td>0.188 ± 0.013</td>
<td>0.013*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Value obtained from each of 5 BB/Wor diabetic (D) rats was significantly different (P < 0.05) from that obtained from BB/Wor diabetes-resistant (DR) rats.

Table 3. M-mode echocardiographic and Doppler flowmetry analyses of hearts from BB/Wor DR rats and BB/Wor D rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>338 ± 27</td>
<td>351 ± 32</td>
<td>0.53</td>
</tr>
<tr>
<td>M-mode (end diastole)</td>
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<td></td>
</tr>
<tr>
<td>IVSD, cm</td>
<td>0.114 ± 0.013</td>
<td>0.129 ± 0.0015</td>
<td>0.05*</td>
</tr>
<tr>
<td>LVD, cm</td>
<td>0.656 ± 0.137</td>
<td>0.617 ± 0.130</td>
<td>NS</td>
</tr>
<tr>
<td>PWD, cm</td>
<td>0.117 ± 0.008</td>
<td>0.120 ± 0.011</td>
<td>NS</td>
</tr>
<tr>
<td>Mitral</td>
<td></td>
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</tr>
<tr>
<td>DT, s</td>
<td>0.065 ± 0.014</td>
<td>0.045 ± 0.019</td>
<td>NS</td>
</tr>
<tr>
<td>E TVI, cm²</td>
<td>0.013 ± 0.004</td>
<td>0.014 ± 0.003</td>
<td>NS</td>
</tr>
<tr>
<td>A TVI, cm²</td>
<td>0.006 ± 0.002</td>
<td>0.005 ± 0.005</td>
<td>NS</td>
</tr>
<tr>
<td>E:ATVI</td>
<td>0.019 ± 0.006</td>
<td>0.018 ± 0.002</td>
<td>NS</td>
</tr>
<tr>
<td>AV eλmin, m/s</td>
<td>0.245 ± 0.044</td>
<td>0.285 ± 0.068</td>
<td>NS</td>
</tr>
<tr>
<td>AV eλmax, m/s</td>
<td>0.145 ± 0.051</td>
<td>0.150 ± 0.056</td>
<td>NS</td>
</tr>
<tr>
<td>E:A ratio</td>
<td>1.755 ± 0.264</td>
<td>2.327 ± 1.654</td>
<td>NS</td>
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<tr>
<td>Tricuspid</td>
<td></td>
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</tr>
<tr>
<td>DT min, s</td>
<td>0.060 ± 0.031</td>
<td>0.031 ± 0.013</td>
<td>NS</td>
</tr>
<tr>
<td>DT max, s</td>
<td>0.051 ± 0.016</td>
<td>0.030 ± 0.017</td>
<td>NS</td>
</tr>
<tr>
<td>E TVI min, cm²</td>
<td>0.013 ± 0.005</td>
<td>0.012 ± 0.004</td>
<td>NS</td>
</tr>
<tr>
<td>E TVI max, cm²</td>
<td>0.015 ± 0.006</td>
<td>0.014 ± 0.005</td>
<td>NS</td>
</tr>
<tr>
<td>A TVI min, cm²</td>
<td>0.007 ± 0.004</td>
<td>0.004 ± 0.002</td>
<td>NS</td>
</tr>
<tr>
<td>A TVI max, cm²</td>
<td>0.008 ± 0.004</td>
<td>0.005 ± 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>E Vel eλmin, m/s</td>
<td>0.110 ± 0.051</td>
<td>0.215 ± 0.071</td>
<td>0.03*</td>
</tr>
<tr>
<td>E Vel eλmax, m/s</td>
<td>0.146 ± 0.069</td>
<td>0.293 ± 0.104</td>
<td>0.04*</td>
</tr>
<tr>
<td>ΔE Vel eλmax−min, m/s</td>
<td>0.036 ± 0.027</td>
<td>0.078 ± 0.035</td>
<td>0.08</td>
</tr>
<tr>
<td>A Vel eλmin, m/s</td>
<td>0.180 ± 0.062</td>
<td>0.153 ± 0.057</td>
<td>NS</td>
</tr>
<tr>
<td>A Vel eλmax, m/s</td>
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<td>0.158 ± 0.057</td>
<td>NS</td>
</tr>
<tr>
<td>E:A ratio min</td>
<td>0.843 ± 0.926</td>
<td>1.436 ± 0.262</td>
<td>NS</td>
</tr>
<tr>
<td>E:A ratio max</td>
<td>1.880 ± 0.328</td>
<td>1.885 ± 0.379</td>
<td>NS</td>
</tr>
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</table>

Values are means ± SD. Control, BB/Wor DR rats; Diabetic, BB/Wor D rats; IVSD, interventricular septal dimension; LVD, left ventricular dimension; PWD, posterior wall dimension; DT, E wave deceleration time; TVI, time velocity interval; E Vel, E wave velocity; A Vel, A wave velocity; min, minimum value; max, maximum value; ΔE Vel eλmax−min, difference between E wave maximum and minimum velocities. *Value for diabetic rats was significantly different from that for control rats. NS, not significant (i.e., P > 0.05).
cytoplasm of the unstimulated ventricle, PKC-β and PKC-γ were barely detectable in the cytoplasm of the adult rat ventricle, and PKC-α, PKC-ε, and PKC-δ were found in both the cytoplasm and membrane extracts of the adult rat ventricle. Because the antibodies for PKC-β, PKC-γ, and PKC-ζ failed to give any significant signal in the range of 72 to 90 kDa in the particulate fraction that contained significant amounts of PKC-δ and PKC-ε, it is unlikely that any significant cross-reactivity existed between these antibodies for PKC isozymes and PKC-δ and PKC-ε. Moreover, because PKC-δ and PKC-ε did not give any signals at the molecular weight of each other, it is also unlikely that cross-reactivity exists between these isozymes. Finally, preincubation of the PKC isozyme-specific antibodies with their antigen-specific proteins eliminated the detection of a band at the corresponding molecular weight of the antibody-specific isozyme (Fig. 3). Similar results

Fig. 1. Typical echocardiogram showing increased tricuspid E wave and decreased A wave velocities in the BB/Wor diabetic (D) rat (A) and diabetes-resistant (DR) rat (B).
analysis and the enhanced chemiluminescence tech-
d and DR rats in vivo, as identified by Western blot
particulate fraction of the rat ventricles obtained from
the only PKC isozymes found of those tested in the
a heart, with PKC-

b
PKC-
d were the predomi-
, PKC-
a, PKC-
f

were obtained in the skeletal muscle extracts (data not
shown). Thus, under the conditions of the experiment
and with these tissues, the antibodies used appeared to
be isozyme specific.

The gel density units of each of the isoforms of
PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), PKC-\(\gamma\), and PKC-\(\epsilon\) in the
soluble fraction and PKC-\(\alpha\), PKC-\(\delta\), and PKC-\(\epsilon\) in the
particulate fraction of the ventricle were linearly
related to the amount of protein applied to the gels (Fig.
4). PKC-\(\beta\)II and PKC-\(\gamma\) were absent in the particulate
fraction of the ventricle (Fig. 4). Finally, the enzymatic
activity of PKC present in total homogenates and the
particulate and soluble fractions of the ventricles
obtained from DR rats were also dependent on the
amount of protein used in the assay (Fig. 5). Similar
results were obtained with the neutrophil and skeletal
muscle (data not shown).

Ventricular PKC isozyme content and PKC activity.
PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), and PKC-\(\epsilon\) were the predomi-
nant PKC isozymes in the soluble fraction of the rat
heart, with PKC-\(\alpha\) and PKC-\(\delta\) in most abundance (Figs.
6 and 7). In contrast, PKC-\(\alpha\), PKC-\(\delta\), and PKC-\(\epsilon\) were
the only PKC isozymes found of those tested in the
particulate fraction of the rat ventricles obtained from
D and DR rats in vivo, as identified by Western blot
analysis and the enhanced chemiluminescence techni-
quique (Figs. 6 and 7). The PKC-\(\beta\)II and PKC-\(\gamma\) isozymes
were present in low amounts in the soluble fraction of
the ventricles obtained from the D and DR rats but
were absent from the particulate fraction of the ven-
tricles obtained from these groups of rats (Figs. 6 and
7). The content of PKC-\(\alpha\) protein increased by 89.4 and
38.6% in the particulate and soluble fractions, respec-
tively, of the hearts obtained from the BB/Wor D rats
compared with the content of this PKC isozyme in these
fractions of hearts obtained from the BB/Wor DR rats
(Fig. 7). Although the concentration of PKC-\(\delta\) protein
was increased by 24.2% in the particulate fraction of
the hearts obtained from the D rats compared with that
in the ventricles obtained from the DR rats, the amount
of this PKC isozyme in the soluble fractions of the
ventricles obtained from these rats did not differ (Figs.
6 and 7). The concentrations of both PKC-\(\beta\)II and
PKC-\(\epsilon\) did not differ in the soluble and particulate
fractions, respectively, of the ventricles obtained from
the BB/Wor D and DR rats (Figs. 6 and 7). Total PKC
activity in the ventricles obtained from D rats was
(mean ± SD) 985 ± 52 pg·mg protein\(^{-1}\)·min\(^{-1}\) (n = 4),
whereas that of the DR rats was 768 ± 53 pg·mg protein

PKC activity increased by 44.4 and 18.4% in the particu-
late and soluble fractions, respectively, obtained from the
ventricles of the BB/Wor D rats compared with the PKC
activity of the hearts obtained from the BB/Wor DR rats
(Fig. 8).

Gracilis muscle PKC isozyme content and PKC activity.
PKC-\(\alpha\) and small amounts of PKC-\(\beta\)II were the
predominant PKC isozymes in the soluble fraction of the
freshly frozen gracilis muscle, whereas PKC-\(\alpha\) >>
PKC-\(\delta\) >> PKC-\(\epsilon\) in the particulate fraction (Fig. 9).
The gel density of both PKC isozymes increased in the
gracilis muscle obtained from the D rat compared with

<table>
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<th>Parameter</th>
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<th>Diabetic</th>
<th>(P)</th>
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<tr>
<td>Body wt, g</td>
<td>338 ± 27</td>
<td>351 ± 32</td>
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</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>240 ± 115</td>
<td>217 ± 47</td>
<td>0.731</td>
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<tr>
<td>LV(\text{max}), mmHg</td>
<td>125 ± 71</td>
<td>114 ± 30</td>
<td>0.505</td>
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<tr>
<td>+LV dp/dt, mmHg/min</td>
<td>4,272 ± 647</td>
<td>2,912 ± 597</td>
<td>0.021*</td>
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<tr>
<td>−LV dp/dt, mmHg/min</td>
<td>2,790 ± 405</td>
<td>2,210 ± 379</td>
<td>0.080</td>
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<tr>
<td>LVEDP, mmHg</td>
<td>3.6 ± 4.8</td>
<td>12.2 ± 10.9</td>
<td>0.198</td>
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</tbody>
</table>

Values are means ± SD; each value represents responses from 5 rats (in vivo measurements) and 3–4 rats (in vitro measurements) that were measured at an initial left atrial pressure of 10 mmHg. LV\(\text{max}\), maximum developed left ventricular pressure (LVP); LV dp/dt, rate of change of LVP; LVEDP, left ventricular end-diastolic pressure. *Significant difference (\(P < 0.05\)), BB Wor D vs. DR (control) rats.
the DR rat. However, PKC-α increased by 438% (P < 0.05), whereas that of PKC-δ increased by 92% (P < 0.05) (Fig. 10A). PKC activity in the particulate fraction of the gracilis muscle was greater than that of the ventricle and was increased in the gracilis muscle obtained from D rats compared with that obtained from DR rats (Fig. 10A).

Neutrophil PKC isozyme content and PKC activity. The neutrophil content of the fractionated phagocytes was 99.9 ± 0.1% with 98 ± 0.4% viability based on exclusion of Evans blue dye. This was in agreement with previous findings (20). Small amounts of PKC-α and PKC-βII and large amounts of PKC-δ were the predominant PKC isozymes in the soluble fraction of the freshly frozen neutrophils (data not shown), whereas PKC-α and PKC-δ were predominant in the particulate fraction of the freshly isolated neutrophils. (Fig. 10B). The gel density of the PKC isozymes did not increase in the soluble (data not shown) or particulate (Fig. 10B) fractions of the neutrophils obtained from the D rats compared with the DR rats. However, basal neutrophil PKC activity, which was the highest when compared with that of the ventricle and gracilis muscle, increased by 23 ± 24% (P > 0.05) in the particulate fraction of the neutrophils obtained from D rats compared with that obtained from DR rats (Fig. 10B).

DISCUSSION

This study demonstrates with the use of echocardiography, Doppler flowmetry, and hemodynamic measurements that during the early incipient stages of IDM in the BB/Wor D rat interventricular septal thickness, the...
E wave peak velocity of tricuspid inflow, and LV weight index were increased, and myocardial contractility and the rate of relaxation were decreased compared with these parameters in age-matched DR rats. Thus, in vivo, the early diabetic cardiomyopathy of the D rat is characterized by a less compliant LV and a restrictive right ventricle (RV) with increased septal thickness. Although we did not measure skeletal muscle function in the D or DR rats, it is known that skeletal muscle dysfunction occurs during the progression of IDM, as well as enhanced susceptibility to infections (4, 6, 19). The in vivo changes in cardiac function and structure reported herein were minimal and before the onset of fully expressed diabetic cardiomyopathy or impaired skeletal muscle function (33) were accompanied by selective increases in particulate-bound PKC-α and PKC-δ isozymes, with undetectable changes in these or the PKC-βII or PKC-ε isozymes in the soluble fraction. Moreover, they also occurred at a time when neutrophil PKC isozymes did not differ between the D and DR rats. This increase in PKC isozyme content was accompanied by increases of the basal total, soluble, and particulate PKC activity of the ventricles and the particulate fraction of the gracilis muscle, whereas that

<table>
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<tr>
<th>PKC-α</th>
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<td>90kDa</td>
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</table>

Fig. 6. Typical Western blot of PKC-α, PKC-βII, PKC-γ, PKC-δ, and PKC-ε in whole brain homogenate (B) used as a standard tissue containing multiple PKC isozymes and in soluble (cytosol) and particulate fractions (membrane) of heart ventricles obtained from BB/Wor rats with insulin-dependent diabetes mellitus (IDM; D) and BB/Wor rats without IDM (C). Molecular masses of isozymes (in kDa) are at left (for details, see METHODS AND MATERIALS).

![Fig. 6. Typical Western blot of PKC-α, PKC-βII, PKC-γ, PKC-δ, and PKC-ε in whole brain homogenate (B) used as a standard tissue containing multiple PKC isozymes and in soluble (cytosol) and particulate fractions (membrane) of heart ventricles obtained from BB/Wor rats with insulin-dependent diabetes mellitus (IDM; D) and BB/Wor rats without IDM (C). Molecular masses of isozymes (in kDa) are at left (for details, see METHODS AND MATERIALS).](http://ajpheart.physiology.org/)

![Fig. 7. Changes of isozyme content of PKC-α, PKC-β, PKC-γ, PKC-δ, and PKC-ε in soluble (A) and membrane fractions (B) of ventricles of hearts obtained from BB/Wor diabetic (D) and diabetes-resistant (DR) rats. Each mean ± SD represents the responses from 5 animals/group. Data are expressed in gel density units. *Response significantly different from that of DR rats (P < 0.05).](http://ajpheart.physiology.org/)

**Fig. 7.** Changes of isozyme content of PKC-α, PKC-β, PKC-γ, PKC-δ, and PKC-ε in soluble (A) and membrane fractions (B) of ventricles of hearts obtained from BB/Wor diabetic (D) and diabetes-resistant (DR) rats. Each mean ± SD represents the responses from 5 animals/group. Data are expressed in gel density units. *Response significantly different from that of DR rats (P < 0.05).

![Fig. 8. Changes in the enzymatic activity of total PKC activity and PKC activity in cytosolic (soluble) and membrane (particulate) fractions of ventricles obtained from BB/Wor D and diabetes-resistant rats. Means ± SD represent responses from 4 rats/group. Data are expressed in pg phosphorylated substrate·mg protein⁻¹·min⁻¹. *Response significantly different from that of DR rats (P < 0.05).](http://ajpheart.physiology.org/)

**Fig. 8.** Changes in the enzymatic activity of total PKC activity and PKC activity in cytosolic (soluble) and membrane (particulate) fractions of ventricles obtained from BB/Wor D and diabetes-resistant rats. Means ± SD represent responses from 4 rats/group. Data are expressed in pg phosphorylated substrate·mg protein⁻¹·min⁻¹. *Response significantly different from that of DR rats (P < 0.05).

![Fig. 9. Typical Western immunoblot of isozyme content of PKC-α, PKC-β, PKC-γ, PKC-δ, and PKC-ε in the brain (B) and soluble (cytosol) and particulate (membrane) fractions of the gracilis muscle obtained from BB/Wor DR rats (C) and BB/Wor D rats (D). Note presence of PKC-α, PKC-δ, and PKC-ε in particulate fraction and the increase in PKC-α and PKC-δ in the gracilis muscle of the D rat compared with the control DR rat (for details see METHODS AND MATERIALS).](http://ajpheart.physiology.org/)
Interestingly, although both function, and compliance in diabetic humans has been evidence for abnormalities in LV function, diastolic studies in diabetic rats. However, echocardiographic (44). We are unaware of any reported echocardiographic
ance and diastolic dysfunction (4, 13, 14, 22, 26, 31, 38, most consistent of which have been reduced LV compli-
and humans have focused on LV abnormalities, the
abetic cardiomyopathy in the BB/Wor diabetic rat model
muscle particulate (membrane) PKC activity and iso-
that sustained elevations of ventricular and gracilis
zyme content occur before, and may be responsible for,
early transition from normal to diseased myocardium and skeletal muscle during the development of
diabetic myopathies.

Cardiac function and structure. Most studies of dia-
betic cardiomyopathy in the BB/Wor diabetic rat model
and humans have focused on LV abnormalities, the
most consistent of which have been reduced LV compli-
ance and diastolic dysfunction (4, 13, 14, 22, 26, 31, 38, 44). We are unaware of any reported echocardiographic
studies in diabetic rats. However, echocardiographic
 evidence for abnormalities in LV function, diastolic
function, and compliance in diabetic humans has been
demonstrated by several investigators (13, 22, 28, 31).
Interestingly, although both +LV dp/dt and −LV dp/dt
(relaxation) were depressed in the BB/Wor D rat com-
pared with these parameters in the DR rat, the major
functional and structural alteration in the BB/Wor D
rat was detected in the interventricular septum and the
RV when assessed by echocardiography and Doppler
flowmetry. The increased E wave velocity present in the
tricuspid inflow pattern as recorded by Doppler sonog-
raphy may be indicative of a restrictive pattern of
ventricular filling (13, 22, 28, 31). Although an increase
in tricuspid E wave velocity may also reflect an increase
in venous return (preload), it is unlikely that cardiac
output was increased in the BB/Wor diabetic rats
because similar increases of E wave velocity recorded
from the mitral valve were not forthcoming. Evidence
for early RV involvement in experimental diabetic
 cardiomyopathy is also forthcoming from the histopatho-
logical evidence of more severe cardiomyopathy in the
RV compared with the LV in rats with STZ-induced
diabetes (9, 38, 44). This is consistent with the experi-
ence of most investigators, who find that ~12 wk of
hyperglycemia are necessary to produce typical find-
ings of LV dysfunction and overt diabetic cardiomyopa-
thy in BB/Wor D rats (4, 31). Thus the slight increase in
interventricular septal thickness and the trend toward
increased LV mass associated with normal LV systolic
pressure, reduced positive and negative LV dP/dt, and
slightly impaired diastolic function as determined in
vivo by echocardiography and cardiac pressure measure-
ments without in vitro functional changes in the iso-
lated working heart are consistent with evidence that
the BB/Wor D rats were studied at the incipient stages
of diabetic cardiomyopathy in vivo.

PKC and diabetic cardiomyopathy. Elevated plasma
levels of glucose have been shown to increase PKC
activity in two major organ systems that are targets for
diabetes-mediated injury. These include the retinal
capillary endothelial cells in cell culture (33), cultured
aortic smooth muscle and endothelial cells (1, 15, 17,
35, 39, 40), and cardiac myocytes (2, 8, 16, 17, 25, 37).
Diabetes also increases the PKC content and activity in
the hearts and vasculature of experimental animals and
humans (1, 2, 8, 15–17, 25, 36, 37, 41). Many
factors have been suggested to play a role in the
transition from normal to diseased myocardium in
IDM. Among these are alterations in intracellular pH
(40); derangements of intracellular metabolism of Ca2+
transport, and transmembrane permeability to Ca2+
(43, 44); changes in the isozyme patterns of
myosin (13, 43); impaired mitochondrial function (4, 6,
19, 44); altered utilization of glucose and fatty acids (6,
31, 41, 43); decreased activity of cAMP (33); and
increased activity of PKC (8, 16, 25, 26, 37, 42, 43). PKC
may be the most important of these diabetogenic fac-
tors because the obligatory hyperglycemia of IDM can
upregulate PKC (6, 16, 26, 41) and the PKC-δ isozyme
of PKC can modulate intracellular pH and alter intra-
cellular and transmembrane Ca2+ metabolism in part
by increasing Ca2+ influx through L-type Ca2+ channels
(35) present in ventricular myocyte and skeletal muscle
membranes (15, 21, 23, 29). Activation of cPKC-δ and
nPKC-ε is also associated with myocardial ventricular
hypertrophy, including overexpression of β-MHC,
vMLC-2, ANF, and ACE (36, 39), and impairment of
myocardial contractility consequent to stimulation of
β-MHC and vMLC-2 and phosphorylation of troponin
and the troponin-tropomyosin complex (15, 17, 21, 23,
24, 27, 36, 40). Thus many of the changes in myocardial
structure and cardiac and skeletal muscle function in
IDM may result from the combined activation and

![Graph](http://ajpheart.physiology.org/)

Fig. 10. PKC activity (bars at left mmol·mg protein−1·min−1 for 20-min period) and PKC isozyme content (PKC-α, PKC-β, PKC-δ, or PKC-ε; gel density units/mm2) determined by Western immunoblot of freshly isolated and frozen gracilis muscle (A) and neutrophils (B) from BB/Wor diabetic rats and BB/Wor diabetes-resistant rats (control).

![Graph](http://ajpheart.physiology.org/)

Means ± SD represent responses from 5 animals/group. *Response significantly different from that of control rats (P < 0.05).
suppression of the actions of the PKC isozymes on various biochemical reactions of myocardial and skeletal muscle cells and their surrounding supporting cells (18, 25, 27, 41). In support of this speculation are the findings that chronic administration of a selective inhibitor of PKC-β to diabetic rats decreased the vascular and cardiac changes associated with STZ-induced diabetes (1, 17). Our finding of an increase in particulate but not soluble PKC-α and PKC-β protein in the ventricle and gracilis muscle, and increases in total, particulate, and soluble PKC activity in these same tissues when obtained in vivo from the BB/Wor D rat, whereas neutrophil PKC protein and activity did not differ between the D and DR rat, is consistent with the reported increases of DAG and particulate and soluble PKC activity in cardiac ventricular myocytes obtained from STZ-induced diabetic and BB/Wor D rats compared with that of control rats (8, 16, 25, 26, 37, 42). Moreover, it suggests that these changes may be related to progression of the disease rather than to a genetic predisposition for increased PKC in the BB/Wor D rat inasmuch as the neutrophil was refractory to the changes. Also, the finding of overexpression of PKC-δ in the heart of the BB/Wor D rat may have clinical significance because PKC-δ has been shown to modulate cardiac L-type Ca²⁺ channels expressed in Xenopus oocytes (34). Intracellular Ca²⁺ increase and Ca²⁺ sequestration decreased in the myocardium of BB/Wor D rats, STZ-induced diabetic animals, and humans with IDDM and type II diabetes mellitus (2, 44). If the changes in PKC-δ are found to precede the increase of intraventricular and skeletal muscle Ca²⁺, then the changes in the PKC isozyme would be clearly important relative to the transition of normal myocardium and skeletal muscle to diabetes-induced cardiomyopathy and skeletal muscle myopathy.

The basal PKC activity of the whole heart increased in vivo early in the course of IDDM when the changes in cardiac function were first manifest and overt cardiomyopathy was not yet evident. However, the changes in total PKC activity was significantly smaller than expected from the large changes in the content of PKC-α and PKC-δ. PKC-ε has been suggested to be a major PKC isozyme in adult rat hearts (2) but did not increase in this study. We measured five isozymes of PKC. Because PKC represents a structurally homologous group of 12 isozymes that modulate the biochemical function of proteins in a rapid and reversible manner (15, 21, 23, 36), it is possible, although speculative, that the mismatch between total heart PKC activity and the increased PKC-α and PKC-δ proteins reflected an increase of isoform that was not fully active or a decrease in the activity or content of an isoform that was not measured (15, 21, 23, 36). These postulates remain to be resolved. However, it would not be inappropriate to suggest at this time that alterations in PKC isozyme patterns and activity are causal to the later cardiac and skeletal myopathies associated with IDDM, because skeletal muscle function does not change this early in IDDM (4, 6, 19). However, this model clearly supports the conclusion that the PKC isozyme patterns change and PKC activity is elevated in the ventricle and gracilis muscle of the BB/Wor D rat in the early phase of the transition from normal to diseased muscle in IDDM.

Another major finding of this study is that the isozyme profile of freshly isolated ventricles, neutrophils, and skeletal muscle freshly obtained from BB/Wor D rats differed not only from that of the DR rat but also from that of myocytes incubated under cell culture conditions, a finding that has not been previously reported. Previous investigators have reported a preferential increase in PKC-βII isozyme in myocytes in cell culture obtained from the diabetic rat myocardium and vasculature (1, 16, 17, 36). This study not only failed to find particulate PKC-βII isozyme in the ventricle, gracilis muscle, and neutrophil but found the PKC-β isozyme present in very low levels in the soluble fraction and did not increase in the tissues obtained from the BB/Wor D rat when compared with those obtained from the BB/Wor DR rat. Our inability to detect this isozyme in rat tissue was not due to technical reasons inasmuch as we were able to detect significant amounts of PKC-βII isozyme in freshly isolated rat brains (Figs. 5 and 7). The length of the diabetes and the process of cell culture may explain these differences. First, our rats were diabetic for 30–41 days, whereas the rats with STZ-induced diabetes and the BB/Wor D and DR rats used in previous studies were evaluated after 10–12 wk of diabetes (1, 16, 17, 29, 36, 43). Thus the duration of the hyperglycemia characteristic of the diabetic state may have upregulated the PKC-βII isozyme (4, 23, 36, 43). Many cell lines and cells in culture exhibit phenotypic and genotypic transformations that may limit the extrapolation of data obtained to the intact cells from which they were derived in vivo. The peptide growth factors in cell culture medium and the cell culture process itself have been shown to revert many enzymes to their fetal phenotype (3, 5). Thus the predominant PKC isozyme of adult myocytes may be shifted from that of PKC-α, PKC-δ, and PKC-ε in vivo to that of the fetal PKC-β during cell culture (3, 5). Also, the specificity of inhibitors of PKC for an isozyme type must initially be performed in vitro using cultured cells or transformed cell lines (1, 17) and the in vivo selectivity of these inhibitors on an isozyme is also usually inferred from the PKC isozyme(s) inhibited in vitro. The selectivity and specificity of an inhibitor against a PKC isozyme in vivo and in culture may differ. Thus the length of diabetes and the use of cell culture may have influenced the different isozyme patterns observed herein and elsewhere (1, 2, 17, 18, 21, 27, 30, 36). Finally, the difference in PKC isozymes reported herein and elsewhere (1, 2, 17, 18, 21, 27, 30, 36) may result from our measurement of PKC isozymes as they occur in the basal state in contrast to the phorbol ester-stimulated myocardial cells in culture. Each type of measurement is useful. However, the former reflects the PKC isozyme pattern in the intact tissue as it essentially occurs in vivo under the conditions modulating the functionality of the heart. The latter reflects the response of the PKC system to the introduction of a sudden and sustained stimulus to PKC.
Limitations of study. A major limitation of this study is that we measured whole ventricle PKC activity and we only measured five PKC isozymes, whereas previous studies measured the PKC activity and isozyme profile of pure ventricular or atrial myocytes in cell culture (6, 16, 18, 21, 25, 42). We were able to detect increases in several PKC isozymes as well as measure the PKC-βII isozyme in the soluble fraction and PKC-ε isozyme in the particulate fraction of the hearts and gracilis muscle obtained from both BB/Wor D and DR rats. Thus it is unlikely that the small amount of protein derived from the arteries and endothelium within the ventricles or within the gracilis muscle could contribute significantly to the PKC activity or mask the PKC isozyme profile of the larger amount of muscle. In support of this conclusion is the finding that the relative density of the constitutive mRNA for PKC-α, PKC-δ, PKC-ε, and PKC-ζ paralleled the relative density of the PKC isozyme proteins as determined by Western blot analyses.

A second limitation of this study, as stated above, is that the IDM produced changes in the content or the activity of the PKC isozymes that we did not measure. However, we measured the major PKC isozymes known to be present in the myocytes. Additional studies are necessary to determine if IDM changes the content, distribution, or enzymatic activity of the remaining seven PKC isozymes.

A third limitation of this study is that it only measured the changes in PKC isozyme pattern and myocardial function and structure at one time point during the transition from normal to diabetic cardiomyopathic heart. However, few studies have been conducted on the early myocardial changes associated with diabetes mellitus. The echocardiographic and interventricular septal measurements were concordant in individual experiments, suggesting that primary changes in the myocardium of the BB/Wor diabetic rats were predominantly RV and septal in origin and that these changes were consistent with the documented effects of PKC on cell growth (16, 21, 36). However, this study does not reveal the precise meaning of the alteration in PKC isozyme pattern we observed. Although PKC-α is ubiquitous in nature, specific functions have been assigned to some of the more esoteric of the PKC isozymes, such as alterations of L- and T-type Ca2+ channels by PKC-δ and PKC-ε, respectively (16, 21, 35). Speculatively, these PKC isozymes may represent a divergence in cell transduction or lipid signaling and sustained cellular responses contributing to the long-term structural changes of the myocardium in IDM.

Conclusion. At an early stage during the development of diabetic cardiomyopathy (which is characterized by a restrictive RV, small decreases of +LV dp/dt and −LV dp/dt, and an increase in the interventricular septal thickness and mass), total, particulate, and soluble PKC activity of the whole heart is also increased. This is accompanied by elevations in the particulate fraction of PKC-α and PKC-δ isozymes. Similar results were observed in gracilis muscle but not in the circulating neutrophil. Because these isoforms of PKC can produce changes in intracellular pH, Ca2+, contractility, and cell growth characteristic of IDM, the data support the hypothesis that increased PKC activity and changes of the PKC isozyme profile may play an important role in the transition from normal to abnormal myocardium and skeletal muscle in type I diabetes mellitus.

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