

Altered Expression and Subcellular Localization of Diacylglycerol-Sensitive Protein Kinase C Isoforms in Diabetic Rat Glomerular Cells

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Protein kinase C (PKC) is implicated in the pathogenesis of diabetic nephropathy. This study was designed to identify the expression of diacylglycerol (DAG)-sensitive PKC- α , - β II, - δ , and - ϵ isoforms in normal and diabetic rat glomerular cells and to determine the effects of high glucose and insulin on PKC isoform cellular compartmentalization and PKC activity. Diabetic rats treated with or without insulin and normal rats were examined 2 and 4 weeks after streptozotocin/vehicle injection. Renal cortical tissue immunogold-labeled with anti-PKC- α , - β II, - δ , or - ϵ antibody was visualized by electron microscopy. From isolated glomeruli, total cell lysate and cytosol and membrane fractions were immunoblotted with the same anti-PKC isoform antibodies. PKC activity in isolated glomeruli was measured by 32 P-phosphorylation of the epidermal growth factor (EGF)-receptor substrate. Immunogold labeling revealed expression of the four PKC isoforms by glomerular visceral epithelial, endothelial, and mesangial cells of both normal and diabetic rats. Immunoblot analysis of the diabetic rat glomeruli at 2 weeks demonstrated a significant increase in membrane-associated PKC- α , - δ , and - ϵ and a significant decrease in membrane PKC- β II content compared with normal, which were similar at 4 weeks. Insulin treatment normalized membrane PKC isoform contents and caused a significant decrease in the cytosol content of PKC- α , - β II, and - δ and total cellular PKC- α compared with normal. Although PKC activity in the cells of diabetic rat glomeruli was increased by 20% compared with normal, the difference did not reach statistical significance. In insulin-treated diabetic rat glomeruli, PKC activity was significantly decreased compared with non-insulin-treated diabetic rat glomeruli. In conclusion, DAG-sensitive PKC- α , - β II, - δ , and - ϵ isoforms are all found in the three major glomerular cell types in rats, and the expression, compartmentalization, and activity are modulated independently by high glucose and insulin. *Diabetes* 47:668-676, 1998

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DAG, diacylglycerol; DC, detergent-compatible; MAPK, mitogen-activated protein kinase; PLP, paraformaldehydelysine-periodate fixative; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; STZ, streptozotocin.

Early diabetic nephropathy is characterized by excessive growth and fractional expansion of the glomerular mesangium (1-3). Although the cellular mechanisms involved in its pathogenesis are multifactorial (4-10), the Diabetes Control and Complications Trial confirmed that sustained hyperglycemia is necessary for the development of progressive nephropathy in susceptible individuals with type 1 diabetes (11). In addition, analysis of glomeruli in rodent models of diabetes and cultured mesangial cells in high glucose demonstrated that high glucose per se leads to altered cellular function (12-14). Because glucose transport by mesangial cells is insulin-independent (15,16), intracellular glucose increases in direct proportion to extracellular levels. One potential mechanism linking high intracellular glucose with increased accumulation of extracellular matrix proteins is through the diacylglycerol (DAG)-sensitive protein kinase C (PKC) pathway (4,12). In glomerular cells of diabetic animals or mesangial cells exposed to high glucose, de novo synthesis of DAG is postulated to activate PKC, causing cytosol-to-membrane translocation of selective PKC isoforms (12,17-23). Activated PKC triggers the mitogen-activated protein kinase (MAPK) cascade (24-27). Recently, Haneda and associates (21,28) reported activation of MAPK in glomeruli from diabetic rats or in mesangial cells cultured in high glucose. Increased expression of *c-fos* and *c-jun* found in mesangial cells grown in high glucose (29) and glomeruli from diabetic rats (30) may be due in part to MAPK stimulation (31,32). These protooncogenes modulate gene transcription by forming a heterodimer, AP-1, that activates the transforming growth factor- β gene promoter (33). An acute and sustained increase in transforming growth factor- β in diabetic glomeruli (7,34) and in mesangial cells cultured in high glucose (35) stimulates increased gene expression and enhances synthesis of extracellular matrix proteins, including type I and IV collagens, fibronectin, and laminin (36,37). Phorbol ester activation of mesangial cell PKC also causes increased fibronectin mRNA expression (38).

PKC consists of a family of at least 12 isoforms classified into three categories—classical, novel, and atypical—according to their calcium and phospholipid dependence (39). Cultured glomerular mesangial and epithelial cells have been reported to constitutively express several PKC isoforms (20,21,23,40). However, the in vivo phenotypic expression of PKC isoforms in the specific glomerular cell types is not known. The characterization and subcellular localization of

DAG-sensitive PKC α , - β I, - β II, - δ , and - ϵ isoforms in diabetic versus normal rat isolated glomeruli are reported (22,23), but the effect of insulin is unknown. We hypothesized that high glucose and insulin independently alter expression and subcellular localization of DAG-sensitive PKC isoforms in glomerular cells. Therefore, this study was conducted in two parts. First, the DAG-sensitive PKC isoform expression in individual glomerular cell types of normal and streptozotocin (STZ)-diabetic rats at 2 weeks was observed using immunogold labeling. Second, the independent effects of high glucose and insulin on glomerular cell PKC isoform expression, cellular compartmentalization, and total PKC activity were explored in STZ-diabetic rats without insulin treatment and in normal rats compared with STZ-diabetic rats receiving intensive insulin treatment to normalize blood glucose for 2 weeks. Glomerular PKC isoform expression was analyzed further after 4 weeks of diabetes without insulin treatment. PKC expression was demonstrated by immunoblotting. Total PKC activity was measured *in situ* by 32 P-phosphorylation of the EGF-receptor substrate.

RESEARCH DESIGN AND METHODS

Materials. Monoclonal antibodies for PKC- α , - δ , and - ϵ were purchased from Transduction Laboratories (Lexington, KY). Anti-PKC- β II, affinity purified polyclonal antibody, was obtained from GIBCO BRL (Mississauga, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG, 12 nm Colloidal Gold AffiniPure Goat anti-Mouse IgG, EM Grade, and 12 nm Colloidal Gold AffiniPure Goat anti-Rabbit IgG, EM Grade, were obtained from Jackson ImmunoResearch (West Grove, PA).

Electrophoresis reagents and a detergent-compatible (DC) protein assay kit were obtained from Bio-Rad Laboratories (Melville, NY). A chemiluminescence kit was purchased from Canadian Life Technologies (Burlington, Canada). Immobilin polyvinylidene difluoride membranes were from Millipore (Bedford, MA). X-Omat film was from Eastman Kodak (Rochester, NY). [γ - 32 P]ATP was from Amersham (Arlington Heights, IL). All other chemicals were purchased from either Sigma (St. Louis, MO) or ICN Pharmaceuticals (Montreal, Canada).

Diabetic rat model. Male Sprague-Dawley rats (Harlan S/D, Indianapolis, IN) weighing ~270 g were rendered diabetic by a single intravenous injection of STZ (Sigma: 60 mg/kg in citric buffer, pH 4.5). Age-matched normal rats received an equivalent volume of citric buffer, pH 4.5, and served as a control group. Diabetes was confirmed by blood glucose >20.0 mmol/l 24 h after STZ administration. Once diabetes was established, a group of diabetic rats were treated with the subcutaneous sustained insulin release device, Limplant (Linshin Canada, Canada [41]), which administered 1.0–2.0 U of insulin/day to normalize blood glucose.

Rats were killed 2 weeks after STZ/vehicle injection, and the kidney samples were prepared for the following immunogold labeling and immunoblotting analysis and *in situ* PKC assay. Kidney samples from rats 4 weeks after STZ/vehicle injection were also used for immunoblotting.

Immunogold labeling. Diabetic rats 2 weeks after the onset of hyperglycemia and age-matched normal rats were anesthetized with 50 mg/kg of intraperitoneal sodium pentobarbital, and the kidneys were rapidly removed. The cortex was sliced off, immersed in 0.1 mol/l phosphate (PO $_4$) buffer, and cut into 1-mm square blocks. Tissue blocks were washed for 20 min in PO $_4$ buffer and then fixed in paraformaldehyde-yserine-periodate fixative (PLP, 3% paraformaldehyde) for 2 h at room temperature.

Some blocks were fixed in a milder PLP solution containing only 0.2% paraformaldehyde for PKC- δ or 0.5% paraformaldehyde for PKC- ϵ to preserve the antigenicity of these PKC isoforms. The tissue was then washed twice in PO $_4$ buffer for 10 min, followed by a 30-min wash in 0.05 mol/l glycine in PO $_4$ buffer. The samples were dehydrated through an ethanol series: 2 \times 15 min in 50%, 2 \times 15 min in 80%, and 2 \times 30 min in 95%, and then incubated in a 2:1 solution of LR White overnight. The following morning, samples were placed in fresh LR White for 1 h, embedded in gelatin capsules, and polymerized at 50°C for 48 h.

Thin sections (80 nm) were cut, picked up on formvar-coated, 200-mesh nickel grids, and allowed to dry. For immunolabeling, grids were floated on drops of phosphate-buffered saline (PBS), supplemented with 0.05% (vol/vol) Tween-80 and 0.05% (vol/vol) polyethylene glycol (20,000 MW), pH 8.0 (PBS-A), for 3 min, and then incubated in blocking solution (1% [wt/vol] non-fat skim milk powder in PBS-A). After 5 min, excess skim-milk solution was blotted off with filter paper, and the grids were immediately placed on 40- μ l drops of monoclonal PKC- α , - δ , and - ϵ antibodies at concentrations of 12.5–50 μ g/ml for PKC- α , 25 μ g/ml for PKC- δ ,

and 33–50 μ g/ml for PKC- ϵ , or with 25 μ g/ml of polyclonal PKC- β II diluted in PBS-A or PBS-A alone for 2 h. This was followed by five washes in PBS-A. Grids were then incubated in the blocking solution for 5 min, immediately followed by incubation with secondary antibody conjugated to 12 nm colloidal gold particles, diluted 1:20 in PBS-A, for 30 min. Grids were washed three times in PBS-A and twice in distilled water (3 min per wash), positively stained with 5% uranyl acetate for 8 min, and allowed to dry on filter paper. Sections were examined in the Hitachi H-7000 transmission electron microscope and photographed with Kodak 4489 plate film.

Immunoblotting. For immunoblotting analysis, glomeruli were isolated from diabetic rats without (2 and 4 weeks' duration) or with insulin (2 weeks' duration) and from age-matched normal rats by differential sieving, as described previously (34). Immediately after the kidneys were removed, the cortical tissue was placed in ice-cold PBS equilibrated with 95% oxygen and 5% carbon dioxide, pH 7.4, and cut into small pieces. All subsequent procedures were carried out at 4°C. The tissue was passed sequentially through 250- and 104- μ m sieves, and glomeruli were collected on a 75- μ m sieve. The glomeruli were resuspended in PBS, and fewer than five tubular fragments per 100 glomeruli were observed.

Isolated glomeruli were homogenized in ice-cold buffer containing 50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l Na $_3$ VO $_4$, 25 μ g/ml leupeptin, and 2 μ g/ml aprotinin, 1% (vol/vol) Triton X-100, and 1% (wt/wt) SDS, which solubilized the cytosol, membrane, and cytoskeleton contents. The glomeruli were then disrupted by passage through a 26-gauge needle and centrifuged at 15,000g for 20 min at 4°C. The supernatant was used as a total glomerular cell lysate. To obtain glomerular cell cytosol and membrane fractions, isolated glomeruli were homogenized in ice-cold buffer containing 50 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l NaHCO $_3$, 5 mmol/l MgCl $_2$, 1 mmol/l PMSF, 1 mmol/l dithiothreitol, 10 mmol/l benzamide, 1 mmol/l Na $_3$ VO $_4$, 1 mmol/l NaF, 25 μ g/ml leupeptin, and 2 μ g/ml aprotinin (buffer A). Then the glomeruli were disrupted by passage through a 26-gauge needle and centrifuged at 100,000g for 60 min at 4°C (TL-100; Beckman Instruments Canada, Mississauga, Canada). The supernatant containing the cytosolic PKC was diluted in Laemmli buffer, boiled for 2 min, aliquoted, and stored at -70°C. The pellet was washed, and then resuspended in buffer A plus 1% (vol/vol) Triton X-100 and centrifuged at 100,000g for 60 min at 4°C. The supernatant containing membrane-associated PKC was treated the same as the cytosol extracts. Protein was measured with the Bio-Rad DC protein assay (the modified Lowry assay [42]) using bovine serum albumin as the protein standard.

The total cell lysate and cytosol and membrane fractions of glomerular cells containing 15–30 μ g of protein were separated by electrophoresis through a 10% polyacrylamide gel at 100 V for 2–2.5 h using a Bio-Rad transblot apparatus. The separated proteins were transferred to an immobilin polyvinylidene difluoride membrane for 12 h in the cold. The membranes were incubated for 2 h in blocking buffer (5% non-fat dry milk powder, 3% bovine serum albumin, 10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, and 0.05% [vol/vol] Tween-20) at room temperature. The membranes were then probed with monoclonal antibodies to PKC- α , - δ , and - ϵ at a concentration of 0.05 μ g/ml for PKC- α and 0.5 μ g/ml for PKC- δ and - ϵ , and with polyclonal antibody to PKC- β II at 0.1 μ g/ml. The blots were exposed to secondary antibody (either horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit; 1:5,000 dilution) for 30 min at room temperature. Immunodetection was performed with a chemiluminescence kit per manufacturer's instructions, and the membranes were exposed to an X-Omat film for 5 s to 10 min. Bands were analyzed by densitometry with a White/UV Transilluminator, Gel Documentation System, and UV Image Score 5000 (UVPm Diamed Lab Supplies, Mississauga, Canada), and the sample band intensity was then analyzed with the National Institutes of Health's Image software version 1.60 (Bethesda, MD). The normal, diabetic, and diabetic plus insulin samples were always prepared together in a single experiment and run on the same gels. The percent of normal was calculated by comparing data only from the same gels.

Immunoblotting of the 4 DAG-sensitive PKC isoforms using the above protocol was validated by stimulating the freshly isolated glomeruli with 100 nmol/l phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min at room temperature before extracting protein.

Measurement of PKC activity by *in situ* EGF-receptor phosphorylation. PKC activity in isolated glomeruli was measured by *in situ* phosphorylation of the PKC-specific substrate derived from the EGF receptor (VRKRTLRL). This substrate was synthesized, in accordance with the published amino acid sequence (43), with an automated solid-phase peptide synthesizer, and its purity was estimated to be 99% by high-performance liquid chromatography. Digitonin was used to permeabilize glomerular cells to allow the substrate to rapidly enter the cells with [32 P]ATP. This method was originally developed by Heasley and Johnson (43) and used to study total PKC activity in isolated glomeruli by Koya et al. (44). Isolated glomeruli from individual rats were washed with PBS and resuspended with buffer containing 137 mmol/l NaCl, 5.4 mmol/l KCl, 10 mmol/l MgCl $_2$, 0.3 mmol/l

TABLE 1
Characteristics of experimental rat groups

Group	2 weeks			4 weeks	
	Normal	Diabetic	Insulin	Normal	Diabetic
<i>n</i>	16	18	17	3	3
Body weight (g)					
Initial	268 ± 9	269 ± 5	264 ± 9	270 ± 12	271 ± 7
Final	344 ± 13	241 ± 24*	337 ± 12	378 ± 20	221 ± 13
Percent change	29 ± 4	-10 ± 8*	28 ± 5	40 ± 5	-18 ± 6*
Blood glucose (mmol/l)	6.2 ± 0.7	31.2 ± 3.9*	6.2 ± 4.8	5.8 ± 0.4	27.0 ± 7.1
Kidney weight (g)	2.51 ± 0.11	2.85 ± 0.32*	2.50 ± 0.20	2.66 ± 0.25	2.75 ± 0.39
Kidney weight (% of body weight)	0.73 ± 0.04	1.21 ± 0.06*	0.74 ± 0.05	0.70 ± 0.03	1.25 ± 0.25

Data are means ± SD or *n*. **P* < 0.05 vs. the other group(s).

sodium phosphate, 0.4 mmol/l potassium phosphate, 25 mmol/l β-glycerophosphate, 5.6 mmol/l glucose, 5 mmol/l EGTA, 2.5 mmol/l CaCl₂, and 20 mmol/l HEPES (pH 7.2). Some aliquots of glomeruli were incubated with 100 nmol/l PMA for 30 min. The reaction was initiated by adding the buffer supplemented with 100 μg/ml digitonin (final 50 μg/ml), 200 μmol/l ATP (final 100 μmol/l), [³²P]ATP (specific activity 1,000 cpm/pmol), and 600 μmol/l PKC-specific peptide substrate (final 300 μmol/l). After 3 min incubation, the reaction was terminated by ice-cold trichloroacetic acid (final 5% [wt/vol]) containing 10 mmol/l ATP (final 2 mmol/l). Aliquots (50 μl) of the acidified reaction mixture were spotted onto 2.1-cm phosphocellulose paper circles (Whatman P81) and washed in three changes of 75 mmol/l phosphoric acid and once with 75 mmol/l Na₂HPO₄, pH 7.5. After the filter papers were dried, the radioactivity was measured. PKC activity was expressed as picomoles of γ-³²P incorporated into the PKC-specific peptide substrate per minute per milligram of glomerular protein. Nonspecific binding of γ-³²P to the filter paper was determined by performing the assay in the absence of the substrate. The linearity of the kinase reaction over a 3-min period at room temperature was verified in preliminary experiments. Glomerular cell protein was solubilized in buffer containing 1% SDS and 0.2 N NaOH and quantified by using the Bio-Rad DC protein assay.

Statistical analysis. Animal characteristic data were expressed as means ± SD, and immunoblot densitometry measurements and in situ PKC activity were reported as mean ± SE. Data between two rat groups were compared using either Student's *t* test or Wilcoxon's rank-sum test. Comparison among three rat groups was analyzed using the one-way analysis of variance (ANOVA) followed by Tukey's studentized range test or the nonparametric Kruskal-Wallis *H* test, followed by Dunn's test as appropriate. A *P* value < 0.05 was considered statistically significant. All the statistical analyses were carried using the Statistical Analysis System (SAS) version 6.12 for Windows 95 (Cary, NC).

RESULTS

Characteristics of the animals. During the 2 or 4 weeks following STZ administration, the diabetic rats not treated with insulin demonstrated sustained hyperglycemia and impaired growth (Table 1). These diabetic rats had enlarged kidneys, particularly evident when the kidney weight was normalized to body weight. Insulin treatment using the Limplant device successfully normalized blood glucose throughout the time course and prevented growth retardation and renal hypertrophy (Table 1).

Immunogold labeling. As shown in Figs. 1 and 2, DAG-sensitive PKC-α (Fig. 1A and B), -βII (Fig. 1C and D), -δ (Fig. 1E and F), and -ε (Fig. 2A and B) isoforms visualized by secondary antibody conjugated to gold particles at a magnitude of 12,000 times were consistently detected in the visceral epithelial, endothelial, and mesangial cells in randomly selected glomeruli from three normal and three diabetic (2 weeks) rats. Gold particles for each PKC isoform were not located in the extracellular matrix, including the glomerular basement membrane and mesangial matrix (Figs. 1 and 2).

The extent of immunogold labeling in the cell types is visualized in Fig. 3A, where PKC-βII represents the typical pattern observed for the other isoforms except for PKC-δ, which demonstrated approximately twice the amount of labeling in mesangial cells compared with the other two cell types in both the normal and diabetic rat glomeruli. To further determine the specificity of anti-PKC-βII, polyclonal antibody to PKC-βII was incubated with the epitope-specific peptide and gold particles were not detected. This method was too insensitive to quantitate the absolute amount of PKC isoform in each cell type. A qualitative estimate of the relative number of gold particles in each cell type demonstrated no differences among the glomerular cell types of diabetic compared with normal. Figure 3B illustrates the absence of labeling of the secondary antibody conjugated to gold particles when incubated without primary antibody.

Immunoblotting. Figure 4 illustrates whole immunoblots for the four PKC isoforms in the total cell lysate and cytosol and membrane fractions from normal rat glomeruli. The positive rat brain control is shown in each blot, and the molecular size of the individual PKC isoform is indicated. Monoclonal antibody to PKC-α, -δ, and -ε detected a single band of 80, 78, and 90 kDa, respectively, in all of the total cell lysate and cytosol and membrane fractions of glomerular cells from normal rats.

Polyclonal antibody to PKC-βII isoform detected two immunoreactive bands of 80 and 65 kDa in the cytosol fraction from each rat group. When PKC-βII antibody was blocked with the isoform-specific peptide, both bands disappeared (data not shown). In the rat brain positive control, only the 80-kDa band was detected. These data suggest that the 65-kDa band was likely a breakdown product of PKC-βII that generally mimicked the 80-kDa band intensity. Only the 80-kDa band was used for densitometry analysis of the cytosol fraction. In the membrane fraction and total cell lysate, a solitary 80-kDa band was detected.

In Fig. 5, representative immunoblots are shown in the upper panels for each glomerular PKC isoform after 2 weeks of diabetes without or with insulin. As illustrated in Fig. 5A, in the insulin-treated diabetic rats, the PKC-α content in total cell lysate (59 ± 12%, mean ± SE) and cytosol fraction (60 ± 11%) was significantly lower than observed in normal. In the diabetic rat glomeruli, membrane-associated PKC-α content increased to 150 ± 14% (*P* < 0.05) of the normal rat glomerular content. In the insulin-treated diabetic rat glomeruli,

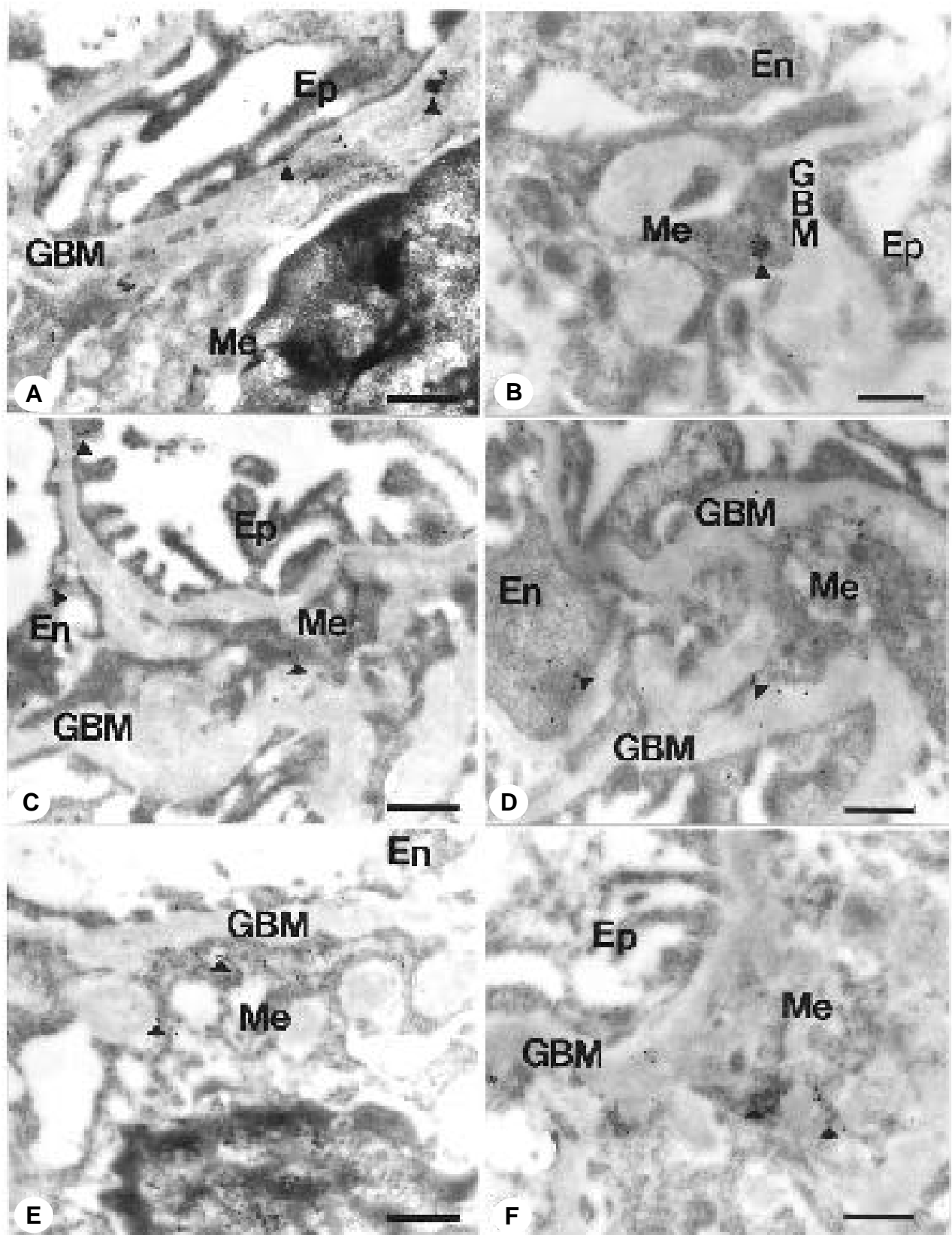


FIG. 1. Representative transmission electron micrographs of thin sections of normal (*A, C, E*) and diabetic (*B, D, F*) rat kidney glomeruli, immunolabeled with primary antibodies to PKC isoforms and secondary antibodies conjugated to 12 nm gold particles. Ep, epithelial cell; En, endothelial cell; Me, mesangial cell; GBM, glomerular basement membrane; arrowheads, gold particles; bars, 0.5 μ m. *A* and *B*: Immunogold labeling of PKC- α in tissue fixed in PLP with 3% paraformaldehyde, using monoclonal anti-PKC- α antibody. *C* and *D*: Immunogold labeling of PKC- β II in tissue fixed in PLP with 3% paraformaldehyde, using polyclonal anti-PKC- β II. *E* and *F*: Immunogold labeling of PKC- δ in tissue fixed in PLP with 0.2% paraformaldehyde, using monoclonal anti-PKC- δ antibody.

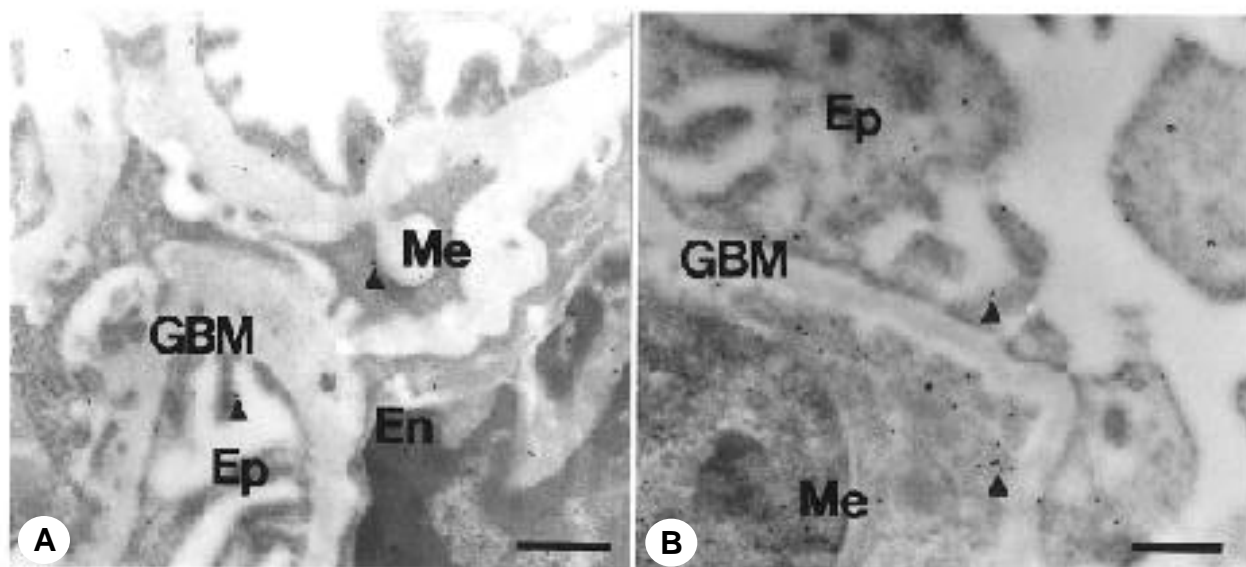


FIG. 2. Representative transmission electron micrographs of thin sections of normal (A) and diabetic (B) rat kidney glomeruli, immunolabeled with primary antibody to PKC- ϵ and secondary antibodies conjugated to 12-nm gold particles. Ep, epithelial cell; En, endothelial cell; Me, mesangial cell; GBM, glomerular basement membrane; arrowheads, gold particles; bars, 0.5 μ m.

membrane PKC- α content ($78 \pm 10\%$) did not differ from normal. In the diabetic rat glomeruli, PKC- β II content decreased in the total cell lysate ($48 \pm 9\%$, $P < 0.05$) and the membrane fraction ($73 \pm 6\%$, $P < 0.05$), and increased in the cytosol fraction ($177 \pm 22\%$, $P < 0.05$), compared with normal (Fig. 5B). Insulin treatment normalized PKC- β II both in the membrane fraction and in the total cell lysate and markedly reduced the cytosol fraction content ($43 \pm 5\%$, $P < 0.05$) compared with normal. As shown in Fig. 5C, in the diabetic rat

glomeruli, total cell lysate and cytosol content remained unchanged, whereas membrane-associated PKC- δ increased ($152 \pm 16\%$, $P < 0.05$) compared with normal. There were no significant differences in PKC- δ content in any of the total cell lysate or cytosol or membrane fraction. Figure 5D shows the immunoblot data for PKC- ϵ . In the diabetic rat glomeruli, an increase in membrane-associated PKC- ϵ ($182 \pm 33\%$, $P < 0.05$ vs. normal) was detected, with no change in total cell or cytosol content. Insulin treatment normalized the mem-

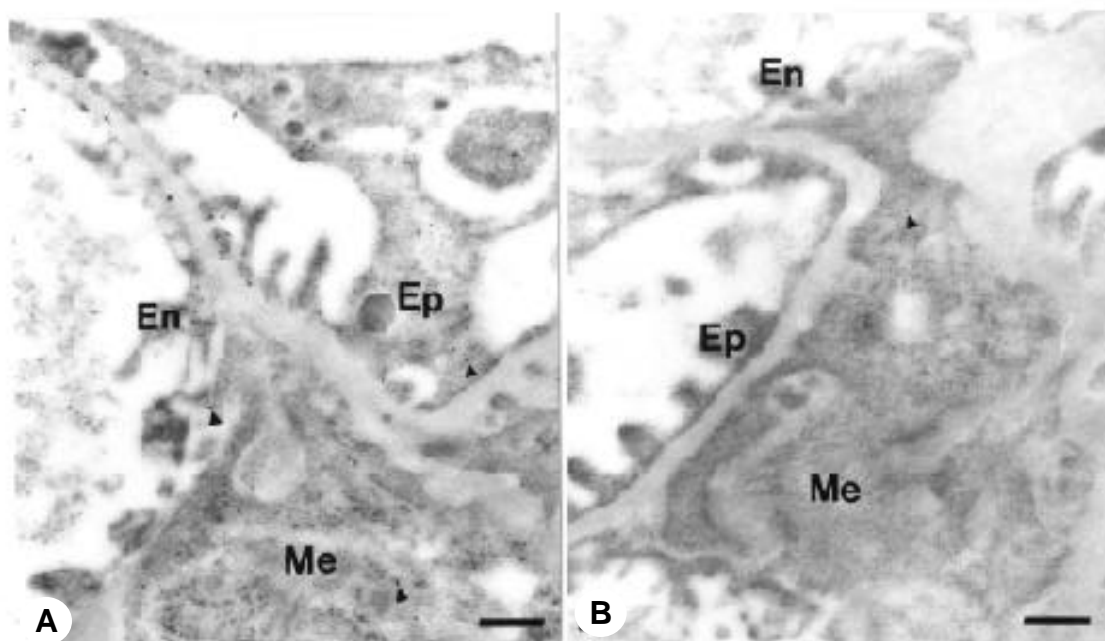


FIG. 3. Representative transmission electron micrographs of thin sections of normal rat kidney glomeruli. A: Labeling with primary antibody to PKC- β II plus secondary antibody conjugated to 12-nm gold particles. B: Secondary antibody conjugated to gold particles alone. Ep, epithelial cell; En, endothelial cell; Me, mesangial cell; GBM, glomerular basement membrane; arrowheads, gold particles; bars, 0.5 μ m.

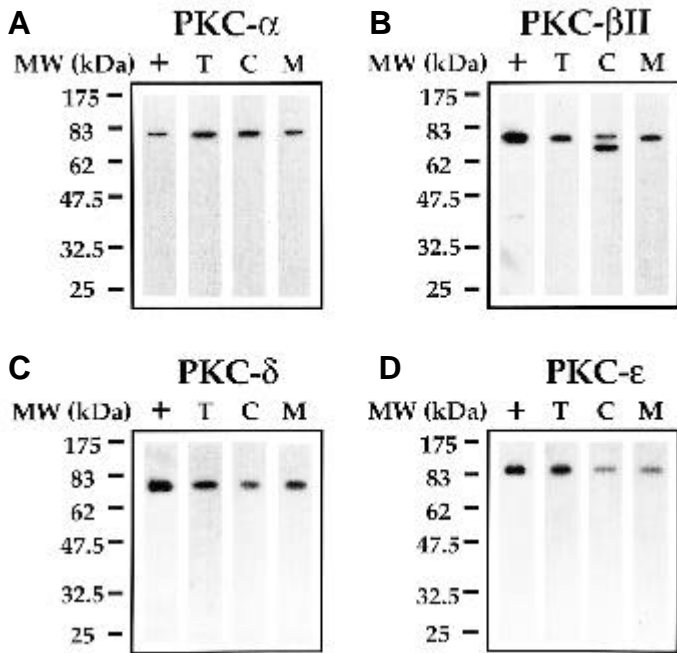


FIG. 4. Whole immunoblots for DAG-sensitive PKC- α (A), - β II (B), - δ (C), and - ϵ (D) isoforms in the total cell lysate (T) and the cytosol (C) and membrane (M) fractions of normal rat glomerular cells with rat brain control (+).

brane-associated PKC- ϵ ($97 \pm 11\%$) and caused a decrease in the cytosol content ($54 \pm 18\%$, $P < 0.05$) compared with normal. The total cell PKC- ϵ content remained unchanged during insulin treatment.

Figure 6 summarizes immunoblot densitometry analysis of the DAG-sensitive PKC isoforms in the cytosol and membrane fractions of glomerular cells from normal and diabetic rats 4 weeks after STZ injection. At this time point, in the cytosol fraction of the diabetic rat glomeruli, not only PKC- β II ($597 \pm 238\%$) but also PKC- δ ($194 \pm 17\%$) content were significantly increased compared with normal. In the diabetic rats with 4 weeks' duration of hyperglycemia, increased membrane-associated PKC- α ($147 \pm 17\%$, $P < 0.05$) and - δ ($181 \pm 21\%$, $P < 0.05$) contents compared with normal were similar to those observed at 2 weeks. PKC- ϵ in the diabetic rat glomerular membrane fraction also appeared increased ($158 \pm 29\%$), but the difference did not reach statistical significance ($P = 0.13$).

Figure 7 illustrates the response to acute PMA stimulation (30 min) of the DAG-sensitive PKC isoforms in the cell glomeruli from normal rats. All the isoforms translocated from the cytosol to the membrane fractions, indicating that during the isolation of the glomeruli, these PKC isoforms remained predominantly inactive.

PKC activity by in situ EGF-receptor phosphorylation. Total PKC activity in digitonin-permeabilized glomerular cells was measured in the basal condition and after 30 min

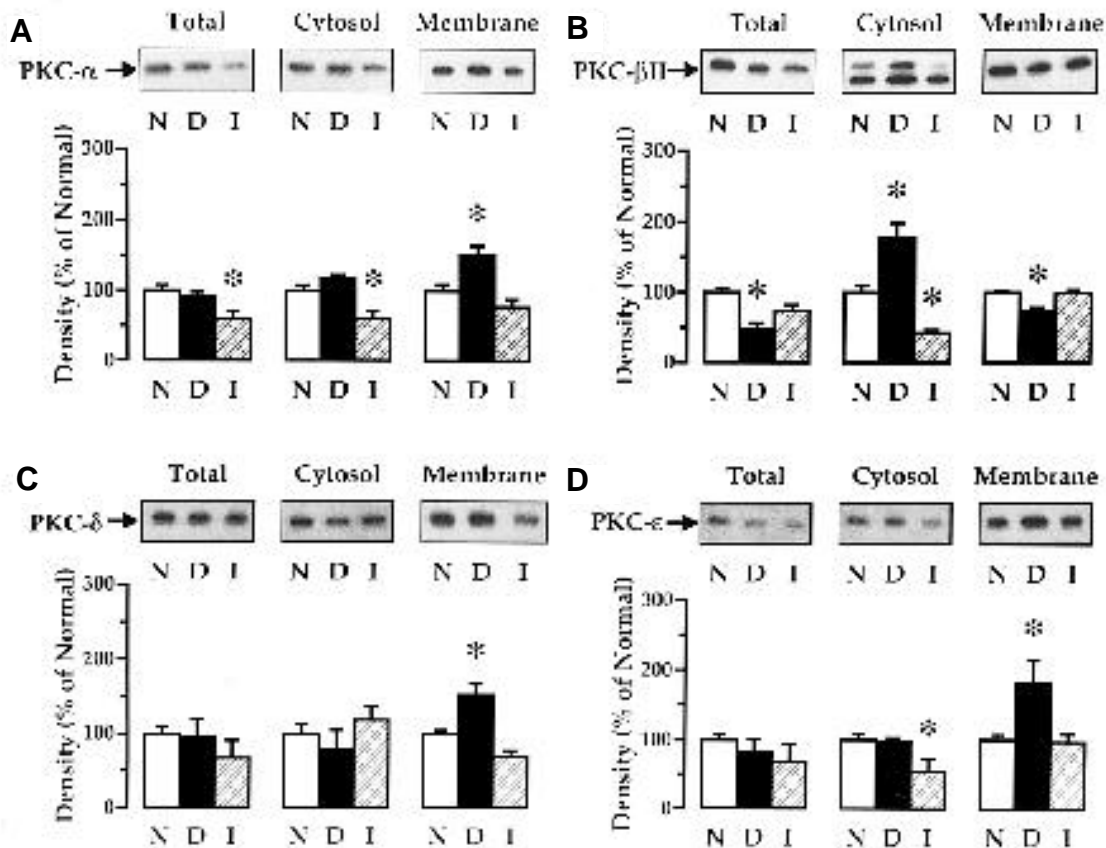


FIG. 5. Immunoblot analysis of DAG-sensitive PKC- α (A), - β II (B), - δ (C), and - ϵ (D) isoforms in the glomerular cells isolated from normal (N), diabetic (D), and insulin-treated diabetic rats (I) 2 weeks after STZ/vehicle injection. The upper panels show representative immunoblots of each PKC isoform in the total lysate and cytosol and membrane fractions of glomerular cells. The graphs illustrate the densitometry measurements (mean \pm SE, $n = 4-7$) expressed as a percent of normal rats (total as 100%) for each PKC isoform. Diabetic (D) and insulin-treated diabetic (I) rat glomeruli PKC isoform contents are compared with normal (N). * $P < 0.05$ vs. normal.

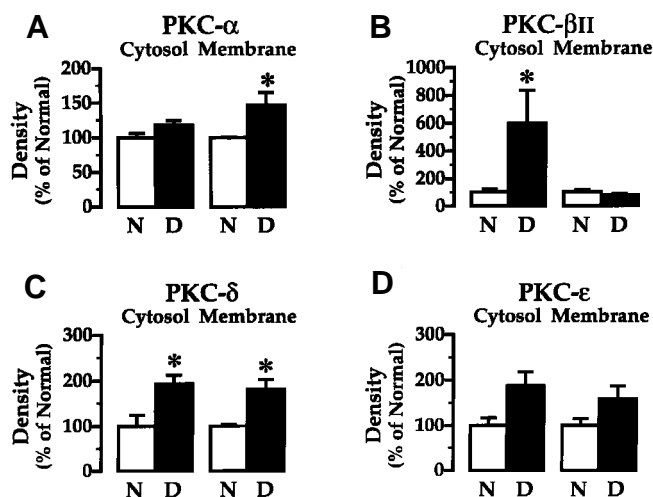


FIG. 6. Densitometry measurements of immunoblot analysis of DAG-sensitive PKC- α (A), - β II (B), - δ (C), and - ϵ (D) isoforms in the glomerular cells isolated from normal (N) and diabetic (D) rats 4 weeks after STZ/vehicle injection. Data are expressed as a percent of normal rats (total as 100%) for each isoform. * $P < 0.05$ vs. normal rats.

stimulation with 100 nmol/l PMA. In diabetic rat glomeruli, the basal PKC activity was 20% higher than normal, but the difference was not statistically significant (70 ± 17 vs. 58 ± 13 pmol \cdot min $^{-1}$ \cdot mg $^{-1}$ protein). In insulin-treated diabetic rat glomeruli, the basal activity was decreased to 27 ± 4 pmol \cdot min $^{-1}$ \cdot mg $^{-1}$ protein ($P < 0.05$ vs. diabetic). PMA-stimulated PKC activity did not differ significantly among the three rat groups (183 ± 22 , 178 ± 28 , and 151 ± 28 pmol \cdot min $^{-1}$ \cdot mg $^{-1}$ protein for normal, diabetic, and insulin-treated diabetic rat glomeruli, respectively).

DISCUSSION

This study reports for the first time, using immunogold labeling, that the DAG-sensitive PKC- α , - β II, - δ , and - ϵ isoforms are all expressed in normal rat glomerular epithelial, endothelial, and mesangial cells. Hüwiler et al. (40), using immunoblotting, reported that both the cytosol and membrane fractions of glomeruli isolated from normal rats constitutively express PKC- α , - β , - δ , - ϵ , and - ζ isoforms. Expression of PKC- α , - δ , - ϵ , and - ζ were also confirmed in cultured rat mesangial (20,21,40) and epithelial cells (40). However, these studies failed to detect PKC- β isoform expression in cultured mesangial or epithelial cells. Thereafter, Ganz et al. (45) identified the presence of PKC- β I but not PKC- β II isoform in cultured mesangial cells using PKC- β I- and - β II-specific antibodies. It is likely that each isoform plays an important role in maintaining normal glomerular cell function. Although immunogold labeling identified the expression of PKC isoforms in each glomerular cell type, this method was not sensitive enough to detect possible changes in PKC isoform content or cellular compartmentalization in diabetes compared with normal.

We hypothesized that high glucose and/or insulin alter(s) glomerular cell PKC isoform expression and cellular compartmentalization. Using immunoblotting, PKC- α , - β II, - δ , and - ϵ were recovered in both the cytosol and membrane fractions of the glomerular cells from normal and diabetic rats. Increased recovery of PKC- α , - δ , and - ϵ isoforms in the

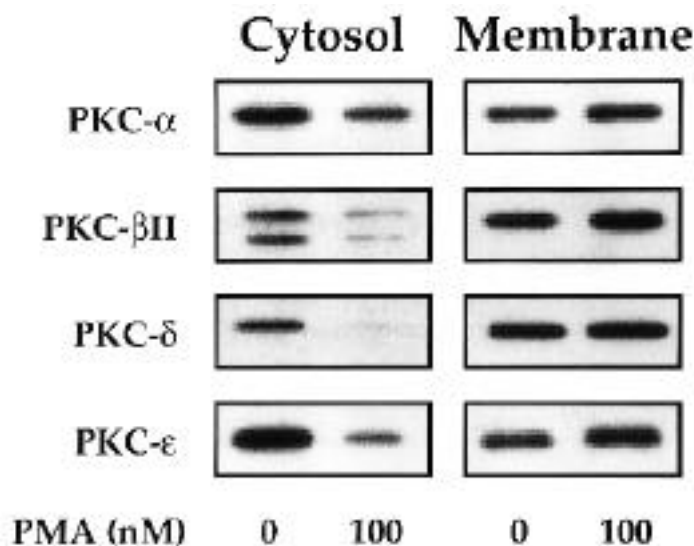


FIG. 7. PMA-stimulated cytosol-to-membrane translocation of DAG-sensitive PKC- α , - β II, - δ , and - ϵ isoforms in the glomerular cells from normal rats. Isolated glomeruli were incubated without or with 100 nmol/l PMA for 30 min and then fractionated into cytosol and membrane. Experiments were carried out on three separate occasions with similar results.

glomerular cell membrane fraction of diabetic rats after 2 weeks of hyperglycemia was observed. By contrast, PKC- β II expression in the membrane fraction of diabetic rat glomeruli was decreased compared with normal. Ishii et al. (22) demonstrated increased membrane localization of PKC- β II and no subcellular redistribution of PKC- α , - δ , or - ϵ in glomerular cells from STZ-diabetic rats with 2 weeks' duration of diabetes (without insulin). Additionally, the same group recently reported that the membrane-associated PKC- α and - β I isoforms in rat glomeruli 12 weeks after the onset of diabetes were not only increased, but also likely activated, as determined by the phosphorylation of the PKC isoform proteins (23). The methods described in our study that differ from others (22,23) include rapid isolation of glomeruli without in vivo perfusion; high yield of PKC isoforms without an extra purification step; detection using monoclonal antibodies specific for PKC- α , - δ , and - ϵ ; and detection of PKC- β II with only the affinity-purified polyclonal antibody from GIBCO BRL and not from other suppliers. These methodological differences likely explain the dissimilarity in findings among different laboratories.

The increased content of glomerular cell membrane-associated PKC- α , - δ , and - ϵ in diabetic rats, in the absence of increased recovery in the total cell lysate observed in our study, suggests that high glucose may activate these DAG-sensitive PKC isoforms in glomerular cells. However, the absence of a simultaneous decrease in cytosolic content and no difference in basal PKC activity between normal and diabetic rat glomeruli indicate that if PKC activation occurs in high glucose, the magnitude is small.

Similar to other cells (46,47), including cultured mesangial cells (20,48), PMA induced rapid cytosol-to-membrane translocation of all the DAG-sensitive PKC isoforms examined and a 3.6-fold increase in PKC activity in the cells of normal

rat glomeruli. Although the basal PKC activity in the cells of diabetic rat glomeruli was higher than normal (70 ± 17 vs. 58 ± 13 pmol \cdot min⁻¹ \cdot mg⁻¹ protein, respectively), this did not reach statistical significance. Koya et al. (44) reported increased basal PKC activity in cells of 2-week diabetic rat glomeruli compared with normal (28 ± 1 vs. 17 ± 1 pmol \cdot min⁻¹ \cdot mg⁻¹ protein, respectively, $P < 0.05$). The differences in methods between our group and Koya et al. include the EGF-receptor substrate, which differs by one amino acid, and our higher concentration of the substrate in the reaction buffer. The higher phosphorylation rates reported in our study indicate that either more types of PKC isoforms or a larger amount of each are contributing to the reaction.

In diabetes, only a small fraction of the PKC isoforms may be translocated, as detected in an enriched membrane fraction, and activated due to increased DAG content (17–19,49,50). PKC- α translocates to the membrane fraction of mesangial cells cultured in high glucose (20,21,23,51). Therefore, increased membrane-associated PKC- α in the cells of diabetic rat glomeruli may be due to hyperglycemia per se. The effect of high glucose on the membrane association of PKC- δ and - ϵ remains controversial (20,21,51). In this study, the biological significance of the decreased membrane-associated PKC- β II and increased cytosol content in diabetic rat glomeruli is unclear. Agonists implicated in the pathogenesis of diabetic nephropathy, such as endothelin-1 (52), angiotensin-II (53), thromboxane (54,55), and platelet-derived growth factor (45), stimulate glomerular-cell DAG-sensitive PKC isoforms, the action of which may be enhanced in high glucose.

Insulin has been reported to stimulate cytosol-to-membrane translocation of PKC- α , - β II, and - ϵ in insulin-sensitive tissue, including adipocytes and myocytes (56–58). Insulin's effect on subcellular distribution of PKC isoforms in insulin-insensitive cells is unknown. In the current study, when blood glucose was controlled by insulin, the increased expression of membrane-associated PKC- α , - δ and - ϵ isoforms was completely normalized, indicating that these alterations are not due to a pharmacological effect of STZ. In the cytosol fraction, PKC- α , - β II, and - ϵ were significantly decreased in glomerular cells of insulin-treated rats, even when compared with normal. Decreased PKC- α in the cytosol fraction of insulin-treated rats was accompanied by no change in the membrane fraction and decreased content of this isoform in the total cell lysate, suggesting that PKC- α was inactivated or downregulated during the 2-week time course by insulin. This speculation is supported by the decreased PKC activity in insulin-treated diabetic rat glomeruli. In the total cell lysate, which was solubilized with SDS and Triton X-100, thereby including not only the cytosol and membrane fractions but also the cytoskeleton fraction, glomerular PKC- β II and - ϵ contents did not differ in the insulin-treated rats from those in the normal rats. These findings may be explained by a redistribution of these isoforms to another cellular compartment, e.g., cytoskeleton, in the presence of insulin.

In conclusion, DAG-sensitive PKC- α , - β II, - δ , and - ϵ isoforms are all expressed in the three major glomerular cell types in both normal and diabetic rats. Diabetes leads to increased membrane localization of PKC- α , - δ , and - ϵ isoforms in the cells of glomeruli. In diabetes, a selective membrane increase of specific PKC isoforms may be associated with their low-

level activation and involvement in the pathogenesis of early diabetic nephropathy. Insulin has an independent effect on the subcellular localization of PKC- α , - β II, and - ϵ isoforms and PKC activity of glomerular cells.

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Author Queries (please see Q in margin and underlined text)

Some of the isoform Greek letters were different in the file than on the hard copy. I made them consistent with the hard copy. Please check them carefully.

Q1: Please spell out DC.>

Q2: As meant that the glomeruli were disrupted? If not, please clarify what was disrupted and centrifuged.>

Q3: Please check spelling of author's name, Hühler in text and Hühler ref. 40.>

Please clarify what the numbers in parentheses mean in refs. 45 and 53. Supplement numbers?>