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High glucose concentrations and protein kinase C isoforms in vascular smooth muscle cells

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High glucose concentrations and protein kinase C isoforms in vascular smooth muscle cells. High extracellular glucose activates protein kinase C (PKC), a family of kinases vital to intracellular signaling. However, which PKC isoforms are involved and where in the cell they operate is unclear. We tested the hypothesis that only those PKC isoforms binding to diacylglycerol (DAG) are activated by high glucose. We also reasoned that the isoforms would translocate to different parts of the cell, where they presumably serve different functions. The PKC isoforms α, β, δ, ϵ, and ζ were studied. Twenty mM glucose caused an increase in total PKC activity at six hours, which was maintained at 24 hours. High glucose decreased the angiotensin II-induced calcium signal. This effect was reversed by preincubating the cells with the PKC inhibitor staurosporine. Glucose induced a translocation of all PKC isoforms except PKC ζ by Western blot. Confocal microscopy showed that PKC α, β, and ϵ were translocated into the nucleus. PKC δ showed strong association with cytoskeletal structures. The effects were sustained at 24 hours for PKC isoform β and to a lesser extent for PKC δ and ϵ, but not for PKC α. Thus, PKC isoforms differ in their propensity to be activated by high glucose. Those isoforms binding to DAG are activated. Both cytoskeletal and nuclear signaling may be involved.

The calcium- and phospholipid-dependent protein kinase signaling system, termed protein kinase C (PKC), is associated with many vascular cell functions that are abnormal in diabetes, including cell contraction [1], permeability [2], basement membrane production [3], signal transduction for hormones and growth factors [4, 5], and proliferation [6]. High extracellular glucose concentrations can activate PKC. Lee MacGregor and Fluharty [7] found increased PKC activity in cultured capillary endothelial cells exposed to high glucose concentrations. PKC activation was also observed in kidneys, hearts, and retinas from diabetic rats [8], as well as in isolated glomeruli [9]. Williams and Schrier showed that exposure of mesangial cells [10] and vascular smooth muscle cells [11, 12] to elevated glucose concentrations induces a long-term increase in PKC activity. They and others also observed that glucose-induced PKC activation interferes with agonist-induced intracellular calcium signaling [10, 13, 14]. High glucose concentrations also result in an increased synthesis of 1,2-diacyl-*sn*-glycerol (DAG) [8, 9, 15, 16]. Shiba et al [17] studied retinal cells and further proposed that the increased DAG synthesis may be responsible for the PKC activation.

PKC does not exist as a single enzyme, but instead consists of several distinct isoforms [4]. To date, 11 different polypeptides, $\alpha,\beta1,\beta2,\gamma,\delta,\epsilon,\zeta,\eta,\theta,\tau$ and λ have been identified, which are classified into different groups according to their structure and function [18]. PKC $\alpha,\beta1,\beta2$ and γ belong to the A group of the PKC family [19]. PKC δ,ϵ and η belong to the B group [19]. Both of these groups depend on DAG for activation and require phospholipids as cofactors [20]. PKC ζ constitutes a different branch of the PKC family, the so-called atypical PKCs. This group lacks cysteine-rich binding sites for DAG and is therefore not activated by DAG or by phorbol ester [21, 22].

PKC isoforms not only have different enzymatic properties, but also exert different functions [4]. Further, the distribution of the isoforms following cell activation is different [23]. Some isoforms are translocated from the cytosolic compartment to cellular membranes and cytoskeletal structures [23, 24], while others are translocated into the nucleus [25, 26] where they may play a role in signaling [27]. The diversity of PKC raises the question whether or not all isoforms are activated by high glucose.

We tested the hypothesis that high glucose activates those isoforms binding to DAG. We also reasoned that the isoforms would translocate to different parts of the cell. We used Western blots to study the activation and subcellular distribution of the isoforms $\alpha,\beta,\delta,\epsilon$, and ζ. We confirmed and extended our confocal microscopy results.

Methods

Materials

Phorbol ester TPA (phorbol -12- myristate 13-acetate), histone type III-S, DEAE-cellulose and all other materials, if not stated otherwise, were purchased from Sigma (Deisenhofen, Germany). γ-$^{[32P]}$ATP was obtained from Amersham (Arlington Heights, IL, USA). 1,2-Diolein and phosphatidylserine were purchased from Avanti Polar Lipids (Birmingham, AL, USA). The fluorescent probe fura-2 AM was purchased from Serva (Heidelberg, Germany). Mannitol (20 mM) was used in all control experiments.

Preparation of vascular smooth muscle cells

Rat aortic vascular smooth muscle cells were cultured by procedures modified from Chamley-Campbell, Campbell and

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Ross. Male Wistar-Kyoto rats (12 to 14 weeks) were anesthetized, bled, and their thoracic aortas were excised. After adherent fat and connective tissue were removed, the aortas were cut longitudinally and the endothelial cells were removed by gently scraping with fine forceps. The aortas were then minced into small pieces and were incubated at 37°C for two hours in PBS without calcium, but with 1 mg/ml collagenase (type I, 150 IU/mg, Worthington Biochemical Corp., Freehold, NJ, USA), 0.5 mg/ml elastase (type III, 40 IU/mg, Sigma) and 0.5 mg/ml trypsin inhibitor (Sigma). After two hours, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS, Gibco, Eggenstein, Germany) was added to the suspension to inactivate enzymes. The cells were then centrifuged at 120 g for 10 minutes and the pellet resuspended in DMEM-12 with 10% FCS. The cells were next seeded at a density of 3 to 5 $\times 10^5/cm^2$ and were cultured at 37°C in 95% air plus 5% CO_2. Cells from passages 2 to 4 were used in all experiments. The phenotype of the cultured vascular smooth muscle cells was determined by staining the cells for α-actin and desmin (Boehringer Mannheim, Mannheim, Germany).

Measurement of protein kinase C activity in vascular smooth muscle cells

PKC activity was measured in cultured confluent cells using a modification of the procedures described by Heasley and Johnson [29]. Cell cultures (in 96 well plates) were incubated in control medium (5% glucose), high glucose or mannitol for the described time periods. The solutions were then aspirated and replaced with 40 ml of a solution containing 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_3PO_4, 0.4 mM K_3PO_4, 20 mM HEPES (pH 7.3), 10 mM $MgCl_2$, 50 mg/ml digitonin, 10 mM b-glycerophosphate, 7.5 mM EGTA, 2.5 mM $CaCl_2$, 600 mM KRTLRR peptide (Bachem, Heidelberg, Germany) and 250 mM γ-[^{32}P] ATP (20 mCi/ml). The reaction was stopped after 15 minutes at 30°C with 10 ml of ice-cold 50% (wt/vol) trichloroacetic acid. With the peptide and ATP concentration used, the rates of kinase activity were linear over 20 minutes. Aliquots (45 ml) of the reaction mixture were then spotted on phosphocellulose (Whatman P-81, Kent, UK) and washed six times for 10 minutes with 75 mM phosphoric acid. The filters were then counted in a scintillation counter and the results expressed in cpm/well.

Measurement of calcium concentration in vascular smooth muscle cells

Cytosolic calcium in single cells was measured as previously described [30, 31]. The calcium measurements were performed using a Spex DM 3000 CM spectrofluorometer, which was connected to a Nikon Diaphot epifluorescence microscope and a variable-aperture photometer for isolating individual cells on the microscope stage (Spex Industries Inc., Edison, NJ, USA). The cells were loaded with fura-2 by a 20 minute incubation in PBS containing 5 μM fura-2-AM (added from a 5 mM stock solution in DMSO). Fluorescence of calcium-bound and unbound fura-2 was determined by rapidly alternating (0.05 sec) the exciting radiation between 340 and 380 nm and separating the resulting emission signals at 505 nm electronically. Maximal fluorescence ratio (R_{max}) was determined by adding 40 μM ionomycin (using a 0.01 M stock solution in DMSO). Minimal fluorescence (R_{min}) was obtained by adding 0.25 M EGTA at pH 7.8. The ratio of the two signals was used to calculate the intracellular free calcium concentration as described by Gollasch et al [30]. All experiments were carried out at room temperature.

Western blotting

Western blot was done as described previously [26]. After the experiments, the cultured vascular smooth muscle cells were treated with ice-cold homogenization buffer (20 mM Tris/ HCl, pH 7.5, 250 mM sucrose, 3 mM EGTA, 10 mM mercaptoethanol, 1 mM phenylmethane-sulphonyl fluoride and 50 mM leupeptin), and were then immediately homogenized. The homogenate was then spun in a TLA 100-2 rotor (Beckman) at 100,000 rpm for 10 minutes and the supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer containing 1.0% Triton X-100 and shaken at 4°C for 30 minutes. The homogenate was then diluted with buffer to a final concentration of 0.5% Triton X-100 and centrifuged at 100,000 rpm for another 10 minutes. The supernatant was used as the particulate fraction. Both PKC-containing fractions then underwent chromatography using 10% SDS-Page gels. Ten to 30 μg of protein were loaded into each lane. The fractions were then electroblotted by the semi-dry technique onto PVDF membranes (Immobilon-P, Millipore, Eschborn, Germany). The membranes were successively incubated, first with blocking buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 7.5, 10% non-fat dry milk powder (Merck, Darmstadt, Germany), 0.2% (vol/vol) Tween-20 and 0.02% NaN_3 for 120 minutes at room temperature. The next incubation was conducted in affinity-purified, isoenzyme specific antibody diluted in incubation buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 7.5 and 1% BSA at room temperature. We used highly specific polyclonal antibodies directed against peptide sequences of PKC that reacted specifically with the α, β, γ, δ, ϵ and ζ subspecies of PKC (antibodies were from Gibco, Gettysburg, MD, USA (1:80 to 1:100); the antibody against PKC-α was a monoclonal (1:200) and from UBI (Lake Placid, NY, USA). A final incubation was carried out in TBS with alkaline phosphatase conjugated anti-rabbit or anti-mouse IgG (Oncogene Science, NY, USA). The membranes were thoroughly washed after each incubation with a buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 7.5, and 0.2% (vol/vol) Tween-20. Quantification of the appropriate PKC band was done by densitometry on a Biorad video densitometer 620. The signals were then integrated and the results were expressed in arbitrary units.

Immunocytochemistry

The techniques are as described elsewhere [26]. The cells were fixed with 3% paraformaldehyde and permeabilized with 80% methanol at −20°C. After incubation with 3% skimmed milk in phosphate buffer solution (SM/PBS) for 60 minutes, the preparation was incubated for one hour at room temperature with the PKC antibodies (see above) diluted in PBS with 0.1% BSA (1:80), washed thrice with PBS and then exposed to the secondary antibody (FITC-conjugated anti-rabbit or anti-mouse IgG, at 1:100, 1% BSA/PBS; Pierce Chemicals, Oud-Beijerland, Holland) for 60 minutes. The preparation was mounted with 50% glycerol under a glass coverslip on a Nikon-Diaphot (Tokyo, Japan) microscope. A Biorad MRC 600 confocal imaging system (Bio-Rad Laboratories, Freiburg, Germany) with an argon laser was used. At least 10 to 18 cells from each of at least seven experiments were examined under each experimental condition. The

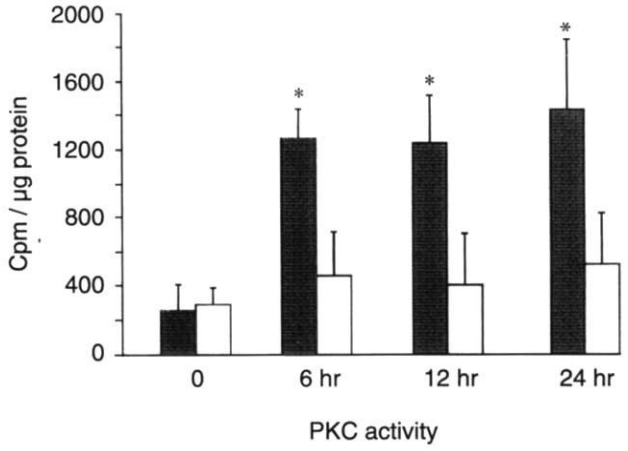

Fig. 1. *The effect of high glucose on total PKC activity.* Glucose 20 mM (■) increased PKC compared to 20 mM mannitol and 5 mM glucose (□). With 20 mM glucose, the increase in PKC activity after 6 hr was further increased at 12 hr, and remained at that level at 24 hr. $^*P < 0.05$ as compared to control values.

results were reproduced by two separate investigators and multiple experiments were done. The observers were unaware of the experimental design and antibodies used.

Quantification in nuclear, cytoplasmic and periplasmic membrane regions was done with histogram/area functions in the MRC-Comos software. The subcellular regions were outlined manually and the calculated mean fluorescent intensity was obtained for the delineated regions. Data are presented as the ratio of the mean fluorescent intensity in the respective regions to the mean fluorescent intensity of the whole cell area.

Statistical analysis

Statistical analysis was carried out on a Macintosh II computer (Apple Inc. Cupertino, CA, USA) using a commercially available program (Statview, Cricket Software Inc., Philadelphia, PA, USA). The results (mean ± SEM) represent duplicate measurements made on seven to ten separate occasions. The non-parametric Wilcoxon test was used. A P value ≤ 0.05 was deemed significant. The terms increase and decrease are applied only when the results were statistically significant.

Results

Elevated glucose concentration and PKC activity

Figure 1 shows the effect of high glucose on total PKC activity. Extracellular 20 nM glucose increased PKC activity compared to 20 mM mannitol or 5 mM glucose. With 20 mM glucose, the increase in PKC activity after six hours was further increased at 12 hours, and remained at that level at 24 hours. We also incubated with high glucose up to 72 hours and found an increased PKC activity. Furthermore, phorbol ester down-regulated glucose-induced PKC activity and led to a complete disappearance of PKC activity after 24 hours. All experiments were carried out in the presence of 1% FCS after exposing the cells to medium without FCS for 20 hours.

Elevated glucose and $[Ca^{++}]_i$

We measured angiotensin II- and vasopressin-induced changes in $[Ca^{++}]_i$ after exposure of cultured cells to 20 mM glucose for 12 hours. Control cells were exposed to 5 mM glucose. To exclude osmotic effects, we carried out additional experiments with mannitol (20 mM). Angiotensin II induced a rapid increase in $[Ca^{++}]_i$,

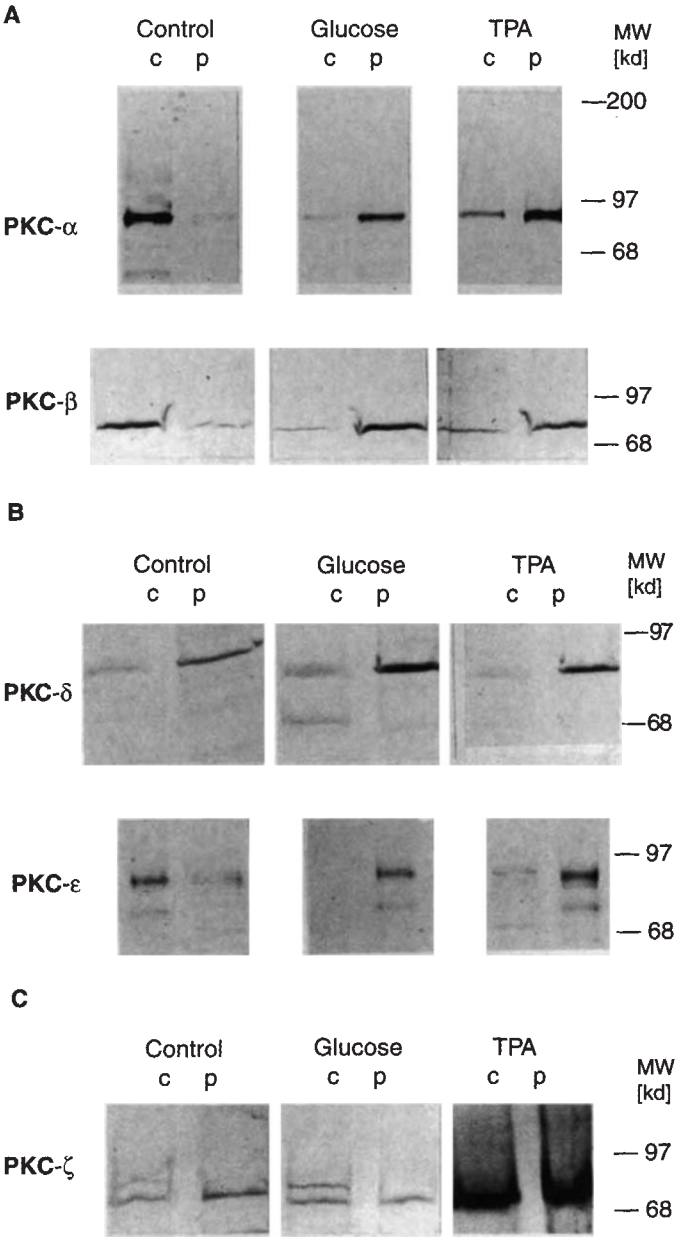

Fig. 2. *(Western blotting) PKC α (lane A) showed a single band at 87 kD.* Glucose 20 mM for 6 hr led to a shift in PKC α immunoreactivity from the cytosol to the particulate fraction. PKC β (lane **A**) showed a single band at 84 kD, with an increase in PKC β in the particulate fraction after 20 mM glucose. PKC δ (lane **B**) showed a single band at 84 kD. The immunoreactivity was higher in the particulate fraction compared to the cytosolic fraction in resting cells. Glucose 20 mM increased the PKC δ immunoreactivity in the particulate fraction while the cytosolic fraction was not altered. PKC ε (lane B) in control cells was almost completely located in the cytosolic fraction with two immunoreactive bands at 91 and 80 kD. Glucose 20 mM for 6 hr led to a complete shift of PKC ε from the cytosolic to the particulate fraction. PKC ζ (lane **C**) showed a double band at 70 and 76 kD in the cytosolic fraction. Glucose 20 mM had no effect on the distribution of PKC ζ immunoreactivity. Treatment with the phorbol ester TPA (10^{-7} M) for 10 min was used as a positive control.

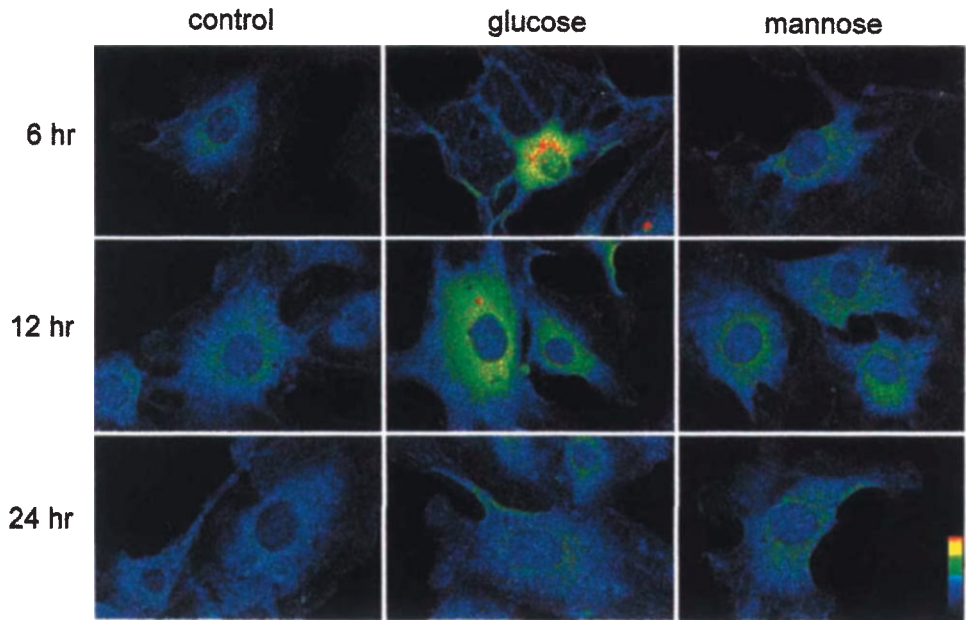

Fig. 3A. *Confocal microscopy of PKC α and β.* PKC isoforms are shown on the left; the graphical quantification of the data is given on the right. Symbols are: (▨) membrane; (▨) cytosolic; (■) nucleus. (upper panels, PKC α) Glucose 20 mM for 6 hr led to an increase in immunoreactivity in the cytosol and the nucleus. After 12 hr, PKC α was still increased in the cytosol. However, the nuclear staining had almost disappeared. After 24 hr, the PKC α in the cytosol was significantly decreased, although an enhanced staining of the perimembraneous region was detected. Mannitol also led to a significant, albeit much smaller, increase in cytosolic immunoreactivity for PKC α.

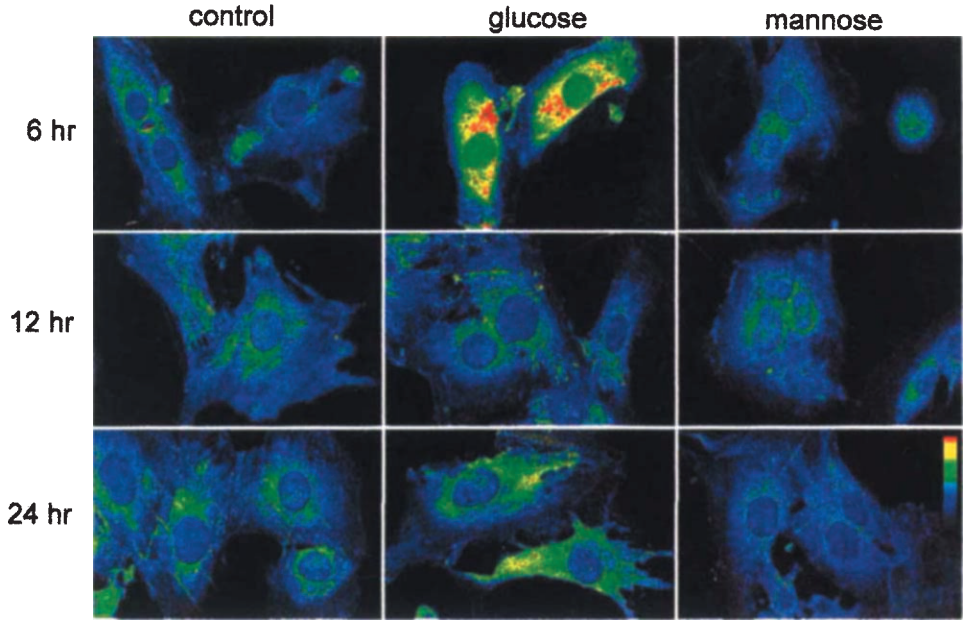

Fig. 3B. (lower panels, PKC β). Glucose 20 mM for 6 hr induced a rapid increase in PKC β immunoreactivity in the cytosolic region. In addition, immunoreactivity for PKC β was increased in the nucleus, although to a much lesser degree. Focal spots of PKC β appeared at the plasma membrane of the cells. After 12 hr of 20 mM glucose, the effect on PKC β immunoreactivity in the cytosolic compartment was greatly reduced. At 24 hr, PKC β was increased in the cytosol, compared to 12 hr. Reproduction of this figure in color was made possible by a grant from Bayer Company, Loverkusen, Germany.

which returned to almost basal levels within 20 seconds. Exposure of the cells to 20 mM glucose decreased the angiotensin II-induced calcium signal (478 ± 165 nM vs. 608 ± 142 nM, $P < 0.05$). A similar effect was observed on vasopressin-induced changes in $[Ca^{++}]_i$. Mannitol had no influence on the agonist-induced calcium signal.

We next investigated the hypothesis that the glucose-induced decrease in calcium signaling was mediated by protein kinase C. Using staurosporine (10^{-8} M), we found that the decreased calcium signal induced by angiotensin II (10^{-7} M) after 12 hour high glucose concentration was restored towards normal (data not shown). Incubation with staurosporine increased the angiotensin II-induced calcium peak in glucose-treated cells from 478 ± 165 nM to 680 ± 133 nM ($P < 0.05$), while mannitol-treated cells were not affected.

Protein kinase C isoforms by Western blot

We then examined the effects of high glucose on the translocation of PKC isoforms from the cytosolic to the particulate fraction and compared these effects with mannitol. The cells were probed with specific antibodies against PKC forms α, β (this antibody recognizes both β-1 and β-2 isoforms), γ, δ, ϵ and ζ. As

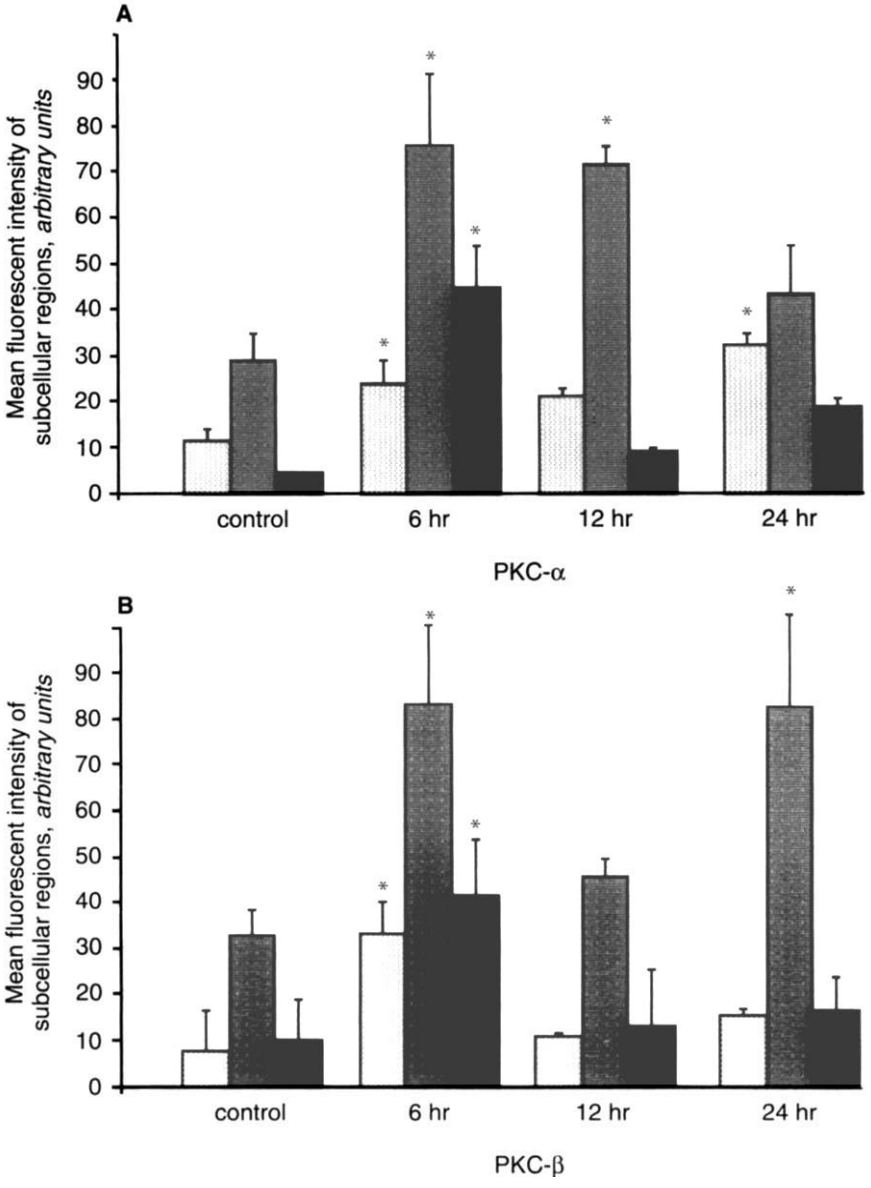

Fig. 3. *Continued.*

shown in Figure 2, we were able to demonstrate the presence of PKC α and β (group A PKC), δ and ϵ (group B PKC), and ζ (atypical PKC). No immunoreactivity for PKC γ was detected. Specificity was demonstrated by using specific oligopeptides which prevent binding of the antibodies to the isoforms (data not shown). In row A of Figure 3 is shown the effect of high glucose on group A PKC isoforms, namely PKC α and β. The antibody against PKC α showed a single immunoreactive band at 87 kD. In vascular smooth muscle cells incubated in 5 mM glucose, almost all immunoreactivity of PKC α was located in the cytosolic fraction. Glucose 20 mM for six hours led to a shift of PKC α immunoreactivity from the cytosolic to the particulate fraction. The effect resembled those of the phorbol ester TPA (100 nM) for 20 minutes. The second of the group A PKC isoforms examined was PKC β. The antibody showed a single immunoreactive band at 84 kD. In resting cells cultured in 5 mM glucose, the immunoreactivity for PKC β was located mostly in the cytosolic fraction and to a lesser extent in the particulate fraction. Exposure to glucose 20 mM for six hours increased PKC β immunoreactivity in the particulate fraction, while PKC immunoreactivity in the cytosolic compartment decreased. Row B of Figure 2 shows the effect of high glucose concentration on PKC isoforms δ and ϵ. The antibody against PKC δ showed a single immunoreactive band at 84 kD, higher in the particulate fraction compared to the cytosolic fraction in the resting cells. Glucose 20 mM increased the PKC δ immunoreactivity in the particulate fraction while the cytosolic fraction was not altered. The lack of translocation suggests a minor effect of glucose on activation of PKC δ. Immunoreactivity of PKC ϵ in control cells was almost completely located in the cytosolic fraction. The antibody against PKC ϵ showed two bands at 91 and 80 kD. Twenty mm glucose for six hours led to a complete shift of PKC ϵ from the cytosolic to the particulate fraction. Both bands were translocated. Row C, Figure 2 shows the effects of high glucose on the atypical PKC isoform ζ. Western

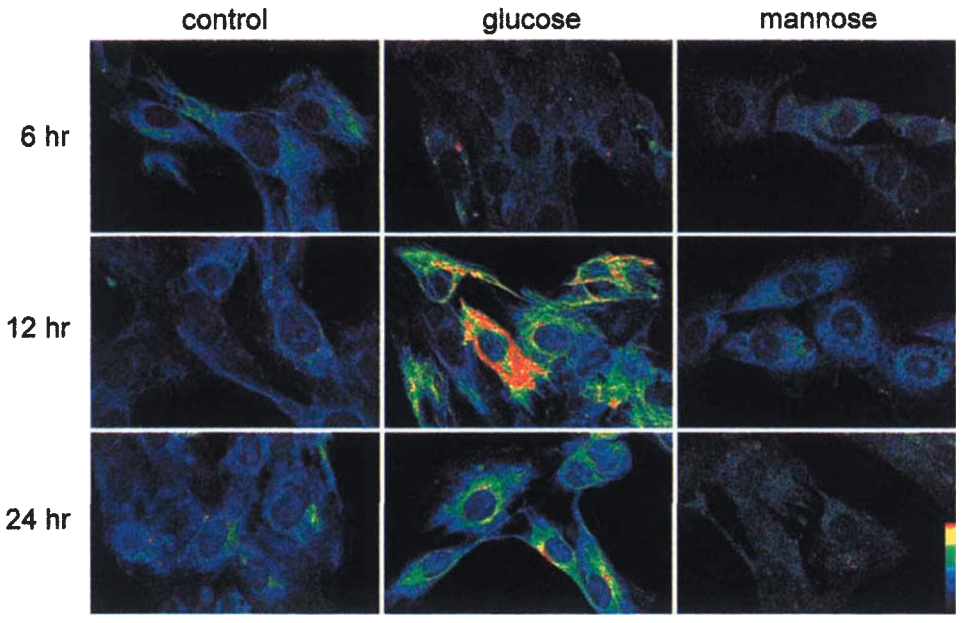

Fig. 4A. *Confocal microscopy of PKC δ and ϵ.* The isoforms are shown on the left, while the graphical quantification is given on the right. Symbols are: (▩) membrane; (■) cytosolic; (■) nucleus. (upper panel, PKC δ) Immunoreactivity for PKC δ in control cells (left) was sparse. A fibrillar distribution with focally enhanced regions in the cytosol was seen. 20 mM glucose for 6 hr (middle) caused immunoreactivity for PKC δ to appear in concentrated "hot spots" throughout the cytosol. However, no significant increase was observed. After 12 hr of 20 mM glucose, PKC δ was increased in the cytosol, but not in the nucleus.

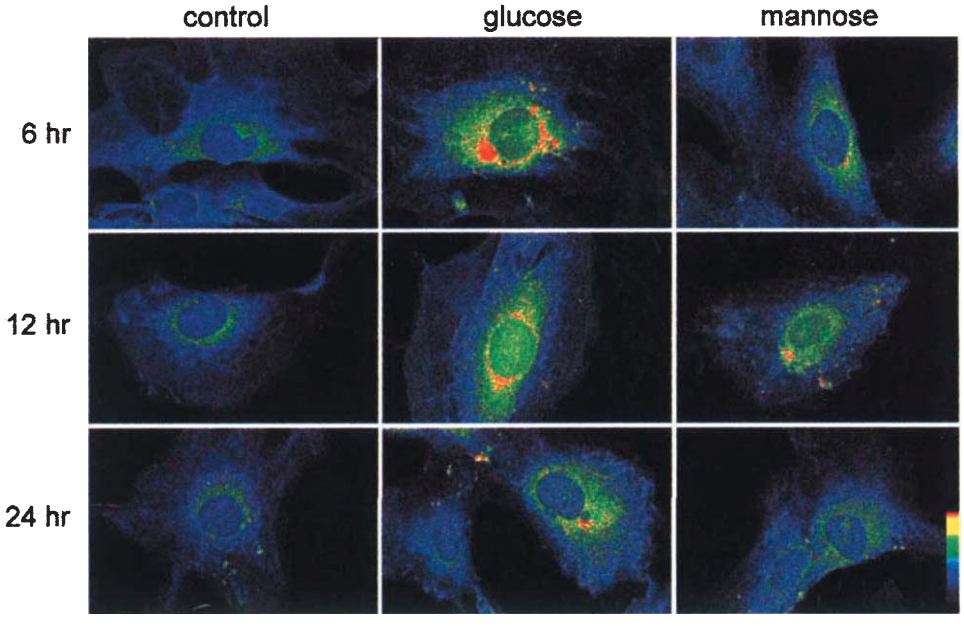

Fig. 4B. (lower panel, PKC ϵ) No immunoreactivity for PKC ϵ was detectable in the nucleus. After 6 hr of 20 mM glucose, PKC ϵ was increased in the cytosol, especially in the perinuclear region. The nucleus also showed an increase in PKC ϵ. After 12 hr of 20 mM glucose, the perinuclear staining was still increased compared to control cells. At 24 hr of 20 mM glucose, the nuclear staining approached control, while the perinuclear immunoreactivity still showed an increase. Mannitol slightly increased in PKC ϵ immunoreactivity in the perinuclear and nuclear staining at 12 hr. Reproduction of this figure in color was made possible by a grant from Bayer Company, Loverkusen, Germany.

blot showed a double band at 70 and 76 kD in the cytosolic fraction. In the particulate fraction only the lower band was present. In contrast to the effect of 20 mM glucose on the other PKC isoforms, exposure of the cells to high glucose had no effect on the distribution of PKC ζ immunoreactivity. TPA also had no effect on PKC ζ.

Confocal microscopy

Figures 3, 4 and 5 show the effects of 20 mM glucose on the intracellular distribution of group A PKC isoforms α and β (Fig. 3), Group B PKC isoforms δ and ϵ (Fig. 4) and the atypical PKC isoform ζ (Fig. 5) using fluorescence (FITC)-labeled antibodies. The left panel always shows the microscopic images of individual cells in control 5 mM glucose medium, the center panel is the 20 mM high glucose medium, and the right panel is the 20 mM mannitol medium. From top to bottom are shown the 6, 12, and 24 hour exposures. The computer generated color images blue, green, yellow and red represent increasing amounts of PKC isozyme immunoreactivity. As in the Western blot experiments, the specificity of the antibodies for the individual isoforms were demonstrated by using specific oligopeptides which prevent binding of the antibodies to the specific PKC isoforms (data not shown).

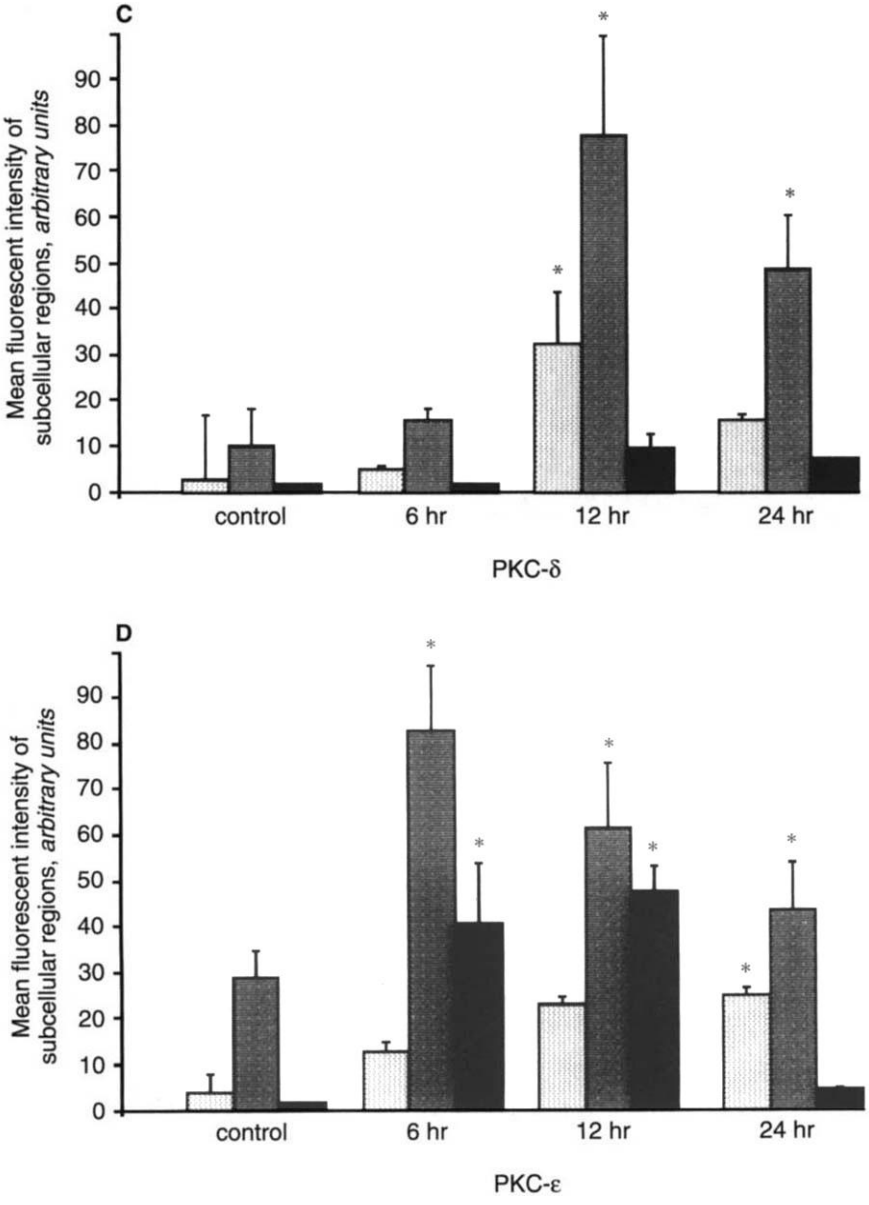

Fig. 4. *Continued.*

In resting control vascular smooth muscle cells, PKC α showed a coarse, granular distribution in the cytosol, especially in the perinuclear region (Fig. 3, upper panel). Glucose 20 mM for six hours led to an increase in immunoreactivity in the cytosol and the nucleus. We also observed an increased staining along the cell membranes. After 12 hours, PKC α immunoreactivity was still increased in the cytosol and at the cell membrane. However, the nuclear staining had almost disappeared. After 24 hours, the PKC α immunoreactivity in the cytosol was significantly decreased; nevertheless, an enhanced staining of the perimembranous region was detected. Mannitol also led to a significant, albeit much smaller, increase in cytosolic and perimembraneous immunoreactivity for PKC α. In contrast to glucose, no nuclear staining was observed. Thus, it seems that the translocation of PKC α observed in the Western blot experiments was due to a shift of PKC α to nuclear and cell membranes. It is also possible that cytosolic PKC immunoreactivity after glucose treatment is associated with cytoskeletal proteins, thereby appearing in the particulate fraction.

The distribution of PKC β immunoreactivity in control cells showed patchy areas throughout the cytosol with an enhancement in the perinuclear region (Fig. 3, lower panel). Glucose 20 mM for six hours induced a rapid increase in PKC β immunoreactivity in the cytosolic region. In addition, immunoreactivity for PKC β was increased in the nucleus, although to a much lesser degree. Focal spots of PKC β appeared at the plasma membrane of the cells. After 12 hours of 20 mM glucose, the effect on PKC β immunoreactivity in the cytosolic compartment was greatly reduced. Focal spots of immunoreactivity remained visible at the plasma membrane. For instance, after 24 hours of 20 mM glucose, PKC β immunoreactivity was increased throughout the cytosol. In contrast to 20 mM glucose for six hours, no nuclear staining was observed; PKC β was increased throughout the whole cell body

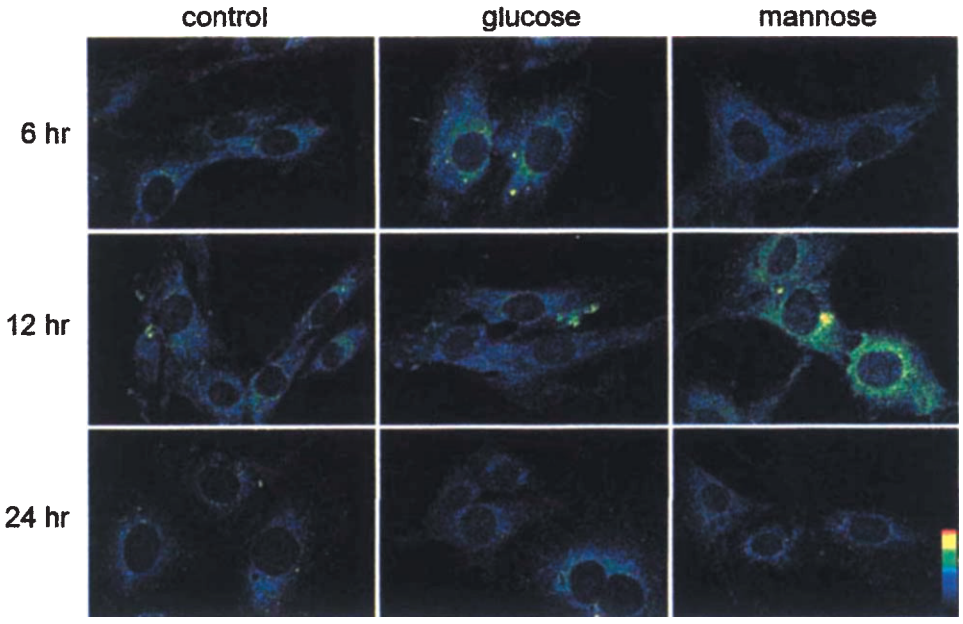

Fig. 5. *Confocal microscopy of PKC ζ.* The isoform is shown on the left; the graphical quantification is given on the right. Symbols are: (▨) membrane; (▩) cytosolic; (■) nucleus. PKC ζ in resting cells was distributed in a speckled fashion throughout the cytosol. Exposure to 20 mM glucose did not influence the expression nor the subcellular distribution of the isoform. The effect of mannitol was not significant at 12 hr. Reproduction of this figure in color was made possible by a grant from Bayer Company, Loverkusen, Germany.

with no accumulation in the perinuclear region. Mannitol had no effect on the immunoreactivity of PKC β compared to control cells.

Immunoreactivity for PKC δ in control cells was sparse (Fig. 4, upper panel). A fibrillar distribution with focally enhanced regions in the cytosol was seen. No immunoreactivity was detected in the nucleus or in the perimembranous region. After six hours of 20 mM glucose, immunoreactivity for PKC δ appeared to be concentrated in "hot spots" throughout the cytosol. However, no significant increase in immunoreactivity was observed at this time. However, after 12 hours of 20 mM glucose, we found a dramatic increase in PKC δ. The immunoreactivity had increased throughout the cytosol and showed a fibrillar pattern. No significant PKC δ increase in the nucleus was observed. A similar fibrillar pattern with less immunoreactivity was observed when the cells were exposed to 20 mM glucose for 24 hours. Exposure of the cells to mannitol had no effect.

PKC ε showed a fine granular pattern in the perinuclear region in control cells (Fig. 4, lower panel). No immunoreactivity was detectable in the nucleus. After six hours of 20 mM glucose, PKC ε immunoreactivity increased significantly in the cytosol, especially in the perinuclear region. The nucleus also showed an increase in PKC ε immunoreactivity. After 12 hours of 20 mM glucose, the perinuclear staining was still increased compared to control cells. The nucleus showed a uniform increase in PKC ε. After 24 hours of 20 mM glucose, the nuclear staining for PKC ε approached control levels, while the perinuclear immunoreactivity still showed a significant increase compared to control cells. In contrast to the previous PKC isoforms investigated, exposure of the cells to high mannitol concentration led to a significant increase in PKC ε immunoreactivity in the perinuclear region and even to an increased nuclear staining at 12 hours.

Figure 5 shows the effect of elevated glucose concentration on the subcellular distribution of PKC ζ. Immunoreactivity of this PKC isoform was only weakly detectable with confocal microscopy. PKC ζ in resting cells was distributed in a speckled fashion throughout the cytosol. Exposure to 20 mM glucose did not influence the expression level or the subcellular distribution of the isoform. Mannitol caused a slight, but nonsignificant increase in immunoreactivity in the cytosol.

Discussion

Consistent with earlier reports, we found that high glucose induced a sustained activation of total PKC activity in vascular smooth muscle cells [11, 12]. Craven and DeRubertis [9] and King and coworkers [8, 17] suggested a possible mechanism whereby glucose activates PKC. They found that the increased flux of glucose through an intracellular pathway culminates in the enhanced synthesis of DAG. They concluded that the increase in DAG contributes to the glucose-induced PKC activation. Ayo et al [32] were able to confirm this notion. Rossi et al [33] showed that *de novo* synthesis of DAG from glucose occurs via dihydroxyacetone phosphate, glycerol 3-phosphate, acylation of phosphatidic acid, and dephosphorylation by phosphatidate phosphatase. Other groups found increased DAG in tissue from diabetic animals [8, 15, 16, 34]. We found that those PKC isoforms binding to DAG are increased while the isoform PKC ζ, which cannot bind to DAG, was not affected. This isoform lacks the conserved domains C1 and C2, which are necessary for DAG binding and for activation by phorbol ester [21]. Our data confirmed this hypothesis and extended it to vascular smooth muscle cells.

To our knowledge, we are the first to investigate the effects of high glucose on separate PKC isoforms in vascular smooth muscle cells. Shiba et al showed that PKC α and β are increased in homogenates from retina of diabetic animals [17]. Confocal microscopy allowed us to monitor the translocation of the isoforms. We detected five PKC isoforms, α, β, δ, ε, and ζ in vascular smooth muscle cells. Expression of PKC α and β has been described by several other groups [reviewed in 35]. PKC γ is exclusively expressed in brain tissue and has not been detected in blood vessels [36]. Khalil et al [37] found PKC ε and δ in ferret aortic smooth muscle cells. However, Andrea and Walsh could not confirm the presence of PKC δ in these cells [35]. We used specific oligopeptides designed for blocking the specific binding of

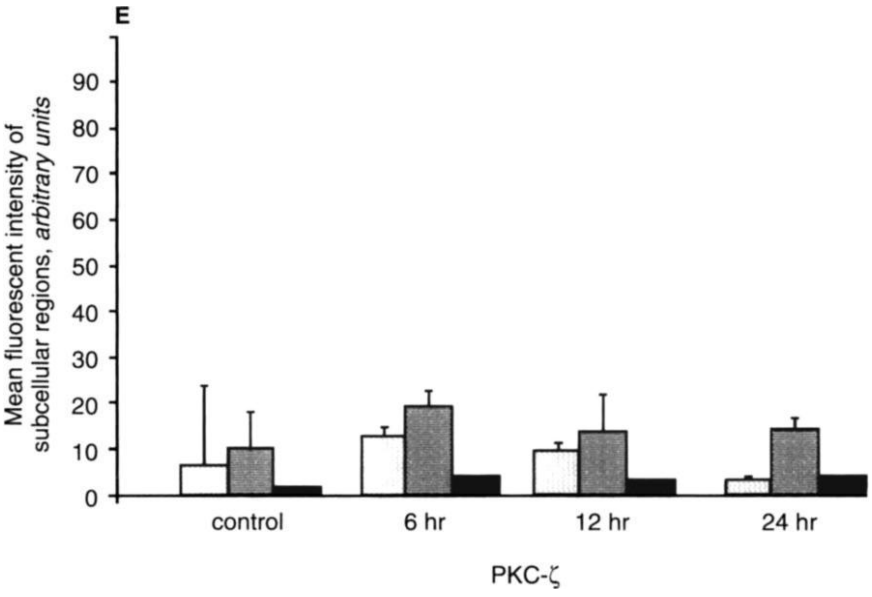

Fig. 5. *Continued.*

our antibodies to the PKC isoforms to rule out cross reactivity in our experiments. Although we found five different isoforms, the level of protein expression varied considerably. As we reported earlier [25], the level of PKC isoform expression seems to depend on the differentiation state of the vascular smooth muscle cells and may not be detectable after multiple passages. This fact may explain the differences in PKC isoform patterns observed by others.

High glucose induced different effects on the subcellular distribution of PKC isoforms α, β, δ, and ϵ, while the PKC isoform ζ was not affected. The isoforms α, β and ϵ were translocated into the nucleus which suggests that they serve some particular function there. We recently observed intranuclear translocation of the isoforms α and β in response to angiotensin II- and PDGF-receptor activation [26]. Others previously demonstrated that PKC isoforms can be translocated into the nucleus after exposure of cultured cells to phorbol ester [25, 38]. PKC activity in isolated nuclei has been measured in several cell types [39–41]. PKC phosphorylates several substrates in the nucleus and various functions have been proposed [27]. We believe that the intranuclear translocation of PKC isoforms in response to high glucose is involved in gene regulation. Our results suggest that PKC α, β or ϵ could be involved in glucose-induced gene activation. Nishio, Aiello and King [42] recently used an mRNA differential display method to characterize glucose-induced alterations of gene expression in vascular smooth muscle cells. They used 22 mM glucose medium and were able to show that a three day exposure led to expression of human elongation factor 2, a gene important to polypeptide synthesis. Whether or not PKC isoforms are involved in mediating the signal for gene expression requires further study; however, the confocal photomicrographs are suggestive.

Glucose-induced PKC activation decreases the angiotensin II-mediated calcium signal in vascular smooth muscle cells [11, 14]. Thus, PKC activation by glucose, and not by osmolality *per se,* is responsible for the decreased intracellular response to vasoactive hormones. PKC mediates negative feedback and controls membrane receptors via phosphorylation [43]. Pfeilschifter [44] suggests that PKC α is responsible for this negative feedback. PKC α may also play a role in the growth response of vascular smooth muscle cells to growth factors [26]. Our knowledge about the functions of the other PKC isoforms is limited. Overexpression of PKC δ results in growth arrest of CHO cells [45] and other cell types [46]. On the other hand, overexpression of PKC ϵ leads to increased growth rates in fibroblasts [46]. Whether the activation of PKC isozymes by glucose alters the intrinsic cellular regulation of growth properties [47], or whether the cellular response to other PKC agonists is primarily altered, is a subject for future study.

We and others have not been able to demonstrate an increase in PKC activity by cell exposure to high mannose concentrations [7–12]. However, in the confocal microscopy experiments, we observed an increase in nuclear staining for PKC ϵ and increased cytosolic immunoreactivity for PKC ζ after 12 hours of osmotic stress with high mannitol concentrations. This finding may indicate that specific PKC isoforms are involved in the regulation of cell volume [48].

We observed a differential temporal effect of high glucose on the various isoforms. PKC α, β and ϵ were translocated into the perinuclear region and into the nucleus within six hours. After 24 hours, the nuclear staining had almost disappeared. However, expression of PKC β, and to a lesser extent PKC ϵ, was increased in the cytosolic region. These results support the work of Inoguchi et al who observed a sustained activation of PKC βII in diabetic animals [8]. Possibly, the late increase in PKC β immunoreactivity reflects increased expression of this isoform. PKC δ on the other hand, showed no response to glucose after six hours, but rather a strong increase in cytosolic immunoreactivity at later time points. Because of the lack of translocation observed in the Western blot experiments, it is unclear whether or not glucose has any effect on the activity of PKC δ. Our results possibly explain the divergent results of high glucose on PKC activation. Several investigators have suggested that high glucose leads to a sustained (days to weeks) increase in PKC activity [8, 10–12, 17]. However, others

could not confirm these observations. Prolonged exposure to glucose resulted in eventual fatigue in PKC activation. Gabbay Siconolfi-Baez and Lebovitz [49] observed lower PKC activity in nerves of diabetic animals. Further, Ishizuka et al [50] reported down-regulation of PKC activity after prolonged (>24 hr) exposure to glucose. Similar down regulation was reported by Cooper, Watson and Dao in the soleus muscle from diabetic rats [51]. According to our results, the different effects on PKC activation observed by these authors may be due to different PKC isozyme expression in the various tissues.

In summary, ours are the first studies to examine the effect of high glucose on the activation and translocation of PKC isoforms in vascular smooth muscle cells. We used Western blots to study translocation and subcellular distribution of the PKC isoforms α, β, δ, ϵ, and ζ. We confirmed and extended our findings with confocal microscopy. Our data support the hypotheses that PKC isoforms differ in their propensity to be activated by high extracellular glucose, that only those isoforms binding to DAG are activated, and that translocation responses differs among the isoforms. We showed that PKC α, β, and ϵ are translocated into the nucleus and that PKC δ is associated with cytoskeleletal structures.

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