High glucose level inhibits capacitative Ca^{2+} influx in cultured rat mesangial cells by a protein kinase C-dependent mechanism

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Summary In cultured mesangial cells (MC), capacitative Ca^{2+} influx via store-operated channels (SOC) is potentiated by agents that release Ca^{2+} from intracellular stores, and inhibited by protein kinase C (PKC). Cells grown under high glucose conditions, as a model of the diabetic microenvironment, display reduced Ca^{2+} signalling in response to vasoconstrictors, probably due to downregulation by elevated PKC activity. Since SOC might be relevant to this phenomenon, we assessed Ca^{2+} influx by microfluorometry of fura-2-loaded rat MC cultured for 5 days in normal (5.5 mmol/l, NG) or high glucose (30 mmol/l, HG). The addition of 1–10 mmol/l Ca^{2+} to NG cells equilibrated in Ca^{2+} -free media induced an immediate Ca^{2+} influx with a free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) plateau of 155 ± 50 and 318 ± 114 nmol/l, respectively. Basal influx was reduced to 88 ± 8 and 145 ± 17 nmol/l $[\text{Ca}^{2+}]_i$ (1–10 mmol/l Ca^{2+} , $p < 0.01$) by 30 mmol/l D-glucose. This effect of HG was confirmed by Mn^{2+} quenching of fura-2, indicating reduced entry of divalent cations via the capacitative pathway. Equimolar L-glucose had no effect on Ca^{2+} influx, consistent with a non-osmotic mechanism.

Arginine vasopressin (10 $\mu\text{mol/l}$) elicited weaker release of stored Ca^{2+} and subsequent influx in HG cells (191 ± 33 vs 153 ± 24 nmol/l, 400 ± 76 vs 260 ± 33 nmol/l, 1–10 mmol/l Ca^{2+} , NG/HG, $p < 0.05$). To examine the involvement of PKC in the effect of HG on capacitative Ca^{2+} influx, the enzyme was activated or downregulated by treatment with 0.1 $\mu\text{mol/l}$ phorbol myristate acetate (PMA) for 3 min or 24 h, respectively. PMA acutely inhibited Ca^{2+} influx in NG cells, while PKC downregulation restored it in HG cells. Similarly, the PKC inhibitors staurosporin or H-7 normalized SOC activity in HG cells. In summary, impairment of Ca^{2+} influx via SOC by HG is one mechanism of the reduced MC $[\text{Ca}^{2+}]_i$ responsiveness to vasoconstrictors. This event is mediated by PKC and may contribute to the glomerular haemodynamic changes in the initial stages of diabetes mellitus. [Diabetologia (1997) 40: 521–527]

Keywords Mesangium, diabetes mellitus, protein kinase C, capacitative Ca^{2+} influx, store-operated Ca^{2+} channels.

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Abbreviations: PKC, Protein kinase C; HG, high glucose; AVP, arginine vasopressin; DAG, diacylglycerol; SOC, store-operated Ca^{2+} channel; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol myristate acetate; SSP, staurosporine; VSMC, vascular smooth muscle cells.

Diabetic glomerulopathy is a common long-term complication of insulin-dependent diabetes mellitus [1]. Hyperfiltration, resulting from a fall of afferent arteriolar resistances with increased glomerular capillary pressure, is an early sign of glomerular involvement [1, 2]. Such haemodynamic changes are believed to result from reduced vascular sensitivity to vasoconstrictors [3]. Arteriolar relaxation could be explained by enhanced production of vasodilators, such as prostaglandins [4], or atrial natriuretic peptides [5]. Alternatively, reduced responsiveness of the contractile apparatus of the glomerulus should be considered [3, 6].

Altered receptor number/affinity, or functional uncoupling between receptors and signal transduction mechanisms could be involved [3].

The glomerular mesangium, an intercapillary network of contractile, smooth muscle-like cells surrounded by amorphous extracellular matrix, is the site of typical proliferative lesions in diabetic glomerulopathy [6, 7]. As mesangial cells closely resemble arteriolar smooth muscle with respect to sensitivity to vasoactive agents and the diabetic microenvironment, we and others employed this cell culture model to study the mechanism underlying vascular insensitivity [8–10]. In both cell types, it is now evident that high glucose (HG) inhibits phosphoinositide metabolism and changes of free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) in response to vasoconstrictor stimuli, such as angiotensin II, arginine vasopressin (AVP), or the thromboxane A_2 mimetic, U-46619 [8–11]. Breakdown of inositol-containing phospholipids plays a central role in signal transduction for vasoconstrictors. In fact, receptor-coupled phospholipase C releases equimolar amounts of water-soluble inositol phosphates and diacylglycerol (DAG) from membrane phosphoinositides [12]. Inositol trisphosphate [$\text{Ins}(1, 4, 5)\text{-P}_3$] mobilizes Ca^{2+} from intracellular stores, resulting in an elevation of cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The latter is, in turn, a major regulator of contractile protein interactions. Several groups have attributed impaired signalling to the inhibitory effects of protein kinase C (PKC), a key effector system activated by both vasoconstrictors themselves via DAG, an endogenous activator of PKC, or directly by HG, possibly through the de novo synthesis of DAG from excess glucose [10, 11, 13, 14].

Given our earlier finding of altered $[\text{Ca}^{2+}]_i$ regulation in HG-cultured cells [10], we now examined whether other mechanisms underlie this process, in addition to blunted release of Ca^{2+} from internal stores. We then focused our attention on a recently described pathway for Ca^{2+} entry into cultured cells, that is, 'capacitative', or Ca^{2+} release-activated Ca^{2+} influx, through the so-called store-operated Ca^{2+} channels (SOC). This influx may be a major component of the prolonged $[\text{Ca}^{2+}]_i$ elevation in vasoconstrictor-activated cells [15–17]. Since such a putative channel appears sensitive to PKC in several cell systems [18], we also examined the possibility that HG modulates Ca^{2+} transport via this enzyme. Our results suggest that multiple mechanisms operate to dampen $[\text{Ca}^{2+}]_i$ fluxes in mesangial cells grown in a microenvironment resembling diabetes in vitro.

Materials and methods

Cell culture. Pure lines of mesangial cells were obtained with standard techniques from rat glomerular explants [10, 17, 19]. Four independent lines, characterized as previously described

[20], were used in passages 3–16. RPMI 1640 medium supplemented with 17 % heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Irvine, Scotland, UK), 5 $\mu\text{g}/\text{ml}$ human recombinant insulin (Novo, Copenhagen, Denmark), 10 $\mu\text{g}/\text{ml}$ ceftriaxone (Hoffmann-La Roche, Basel, Switzerland) was used for initial plating and propagation of the cultures. The cells were maintained at 37 °C in a controlled, humidified atmosphere of 95 % O_2 -5 % CO_2 and subcultured at 4–7 day intervals. Prior to each experiment, cells were subcultured in regular complete RPMI medium and 24 h later switched to 5.5 mmol/l or 30 mmol/l glucose Dulbecco's Minimum Essential Medium (DMEM, Paisley, Scotland, UK) supplemented with 17 % FBS in the absence of insulin. Media were replaced every 24 h for 5 days. In control experiments, the non-metabolized isomer L-glucose was added to 5.5 mmol/l D-glucose medium to bring total osmolality to values equivalent to 30 mmol/l.

$[\text{Ca}^{2+}]_i$ measurement. $[\text{Ca}^{2+}]_i$ was measured fluorometrically in cells loaded with the intracellular probe, fura-2 (Molecular Probes, Eugene, Ore., USA) as previously described [17, 19, 20, 21]. Briefly, after withdrawing FBS for the 24 h prior to each experiment, confluent monolayers grown on plastic Aclar coverslips (Allied Engineered Plastics, Pottsville, Penn., USA) were loaded for 40 min at 37 °C with 1 $\mu\text{mol}/\text{l}$ fura-2 in serum-free DMEM, followed by further incubation for 20 min in the same medium without fura-2. Glucose concentrations were maintained at 5.5 or 30 mmol/l throughout the loading procedure. Fluorescence measurements were performed by inserting the coverslips diagonally in a quartz cuvette filled with 2 ml of modified Krebs-Henseleit solution of the appropriate glucose concentration, buffered with 20 mmol/l N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and supplemented with 0.2 % fatty acid-free bovine serum albumin. The monolayers were excited at 340 nm with emission collected at 500 nm in a Perkin-Elmer LS5B spectrofluorometer (Beaconsfield, U.K.) equipped with stirrer and thermostatically controlled cell. Excitation/emission slits were set at 2.5/5 nm, respectively. Calibration of Ca^{2+} -dependent fluorescence was performed by sequential saturation of the dye with 15–40 $\mu\text{mol}/\text{l}$ ionomycin (± 10 mmol/l CaCl_2) to maximum fluorescence (F_{max}), followed by chelation of Ca^{2+} to minimum fluorescence (F_{min}) with 7.5 mmol/l ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) plus 60 mmol/l Tris, pH 10.5. Ratio fluorometry with alternate 340/380 nm excitation at 6-s intervals was employed for validation of each set of experiments. Standard formulae were employed for the calculation of $[\text{Ca}^{2+}]_i$, employing a K_d of fura-2 for Ca^{2+} of 224 nmol/l [19–21]. In the Mn^{2+} -quenching experiments, the fluorescence decay of fura-2 was monitored for at least 3 min after addition of 100 $\mu\text{mol}/\text{l}$ MnCl_2 , and then for an additional 3 min after application of the vasoconstrictors employed to promote Ca^{2+} influx. The figures reproduce the original 340-nm excitation tracings.

Chemicals. Ionomycin, staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine HCl (H-7) were from Calbiochem-Behring (La Jolla, Calif., USA). L-glucose was from Merck (Darmstadt, Germany). All other chemicals were of the purest grade available through Sigma Chemical Co. (St. Louis, Mo., USA).

Statistical analysis

All data are expressed as mean $\pm$ SEM and analysed by one-way analysis of variance.

Fig. 1. Effects of 5.5/30 mmol/l glucose on capacitative Ca^{2+} influx in fura-2-loaded monolayers of cultured rat mesangial cells equilibrated in Ca^{2+} -free media. *Upper panel*, $[\text{Ca}^{2+}]_i$ elevation upon addition of 1–10 mmol/l Ca^{2+} to cells grown for 5 days in 5.5 mmol/l glucose. *Lower panel*, reduced Ca^{2+} influx upon addition of 1–10 mmol/l Ca^{2+} after 5 days in 30 mmol/l glucose. Fluorescence tracings representative of $n = 12$ experiments

Fig. 2 A–D. Effects of 5.5 (A, B)/30 mmol/l glucose (C, D) on capacitative Ca^{2+} influx in fura-2-loaded monolayers of cultured rat mesangial cells. Panels A and C, $[\text{Ca}^{2+}]_i$ elevation upon addition of 0.1 $\mu\text{mol/l}$ arginine vasopressin (AVP) in the presence of 1.25 mmol/l extracellular Ca^{2+} . Note lower amplitude of $[\text{Ca}^{2+}]_i$ spike in cells grown for 5 days in 30 mmol/l glucose. Panels B and D, 1–10 mmol/l Ca^{2+} added after releasing Ca^{2+} from intracellular stores with AVP in Ca^{2+} -free media. Note lack of activation of Ca^{2+} influx in 30 mmol/l glucose. Fluorescence tracings representative of $n = 9$ experiments

Results

As previously observed [10], the growth of cultured rat mesangial cells for 5 days in 30 mmol/l glucose (HG) had no effect on resting $[\text{Ca}^{2+}]_i$. Omitting Ca^{2+} from the bathing medium induced a rapid decrease of $[\text{Ca}^{2+}]_i$ from 82 ± 5 to 55 ± 6 nmol/l in cells maintained in 5.5 mmol/l glucose (NG), and from 78 ± 7 to 52 ± 4 nmol/l in HG cells ($n = 42$). The subsequent addition of extracellular Ca^{2+} to a final

Table 1. Effect of 5-day culture of rat mesangial cells in 5.5/30 mmol/l glucose media on resting and agonist-activated capacitative Ca^{2+} influx

Culture	Glucose concentration		L-Glucose 30 mmol/l
	5.5 mmol/l	30 mmol/l	
Ca^{2+} 1 mmol/l	155 ± 50	88 ± 8^1	135 ± 32
Ca^{2+} + AVP, 0.1 $\mu\text{mol/l}$	191 ± 33	153 ± 24^1	215 ± 52
Ca^{2+} 10 mmol/l	318 ± 114	145 ± 17^1	326 ± 55
Ca^{2+} + AVP, 0.1 $\mu\text{mol/l}$	400 ± 76	260 ± 33^1	454 ± 95

Data are mean $\pm$ SEM nmol/l peak $[\text{Ca}^{2+}]_i$ from $n = 10$ experiments for each agent on independent monolayers equilibrated in Ca^{2+} -free solutions. AVP, Arginine vasopressin. $^1p < 0.01$ vs 5.5 mmol/l glucose

concentration of 1 or 10 mmol/l induced a stepwise, persistent increase of $[\text{Ca}^{2+}]_i$, which reached a maximum within 1 min. The amplitude of this $[\text{Ca}^{2+}]_i$ elevation, presumably due to influx via a plasma membrane Ca^{2+} channel, was significantly larger in cells kept in NG than in the HG monolayers (Fig. 1, Table 1). As the extent of the Ca^{2+} influx occurring upon readdition of Ca^{2+} to cells previously equilibrated in Ca^{2+} -free media is often amplified by prior discharge of intracellular Ca^{2+} stores, the monolayers were treated with AVP, a vasoconstrictor stimulus of phospholipase C, which typically increases $[\text{Ca}^{2+}]_i$ by releasing stored Ca^{2+} . As shown in Figures 2A–B, 0.1 $\mu\text{mol/l}$ AVP triggered a rapid rise of $[\text{Ca}^{2+}]_i$, even in the nominal absence of Ca^{2+} in the bathing media, which was followed by a larger current of Ca^{2+} when the extracellular concentration was restored. Hence, the subsequent Ca^{2+} influx matches the features of a capacitative entry via SOC. In HG cells, both the rapid $[\text{Ca}^{2+}]_i$ spike and the amplitude of the subsequent Ca^{2+} influx were again significantly reduced in comparison with NG cells (Fig. 2C–D). This suggests either that the limited release of Ca^{2+} by AVP results in weaker activation of the Ca^{2+} current, or that HG itself modulates the state of activity of the putative channel. Similar responses were noted with another phospholipase C agonist, angiotensin II, which increased Ca^{2+} influx to 212 ± 44 and 440 ± 38 nmol/l peak $[\text{Ca}^{2+}]_i$ in 5.5 mmol/l glucose, as opposed to 140 ± 10 and 295 ± 41 in 30 mmol/l glucose (1–10 mmol/l Ca^{2+} , respectively, $n = 5$ each, $p < 0.05$).

To confirm that the changes of $[\text{Ca}^{2+}]_i$ occurring upon manipulation of the extracellular Ca^{2+} concentration were indeed due to Ca^{2+} influx, we backed these data with monitoring the quench of fura-2 fluorescence by Mn^{2+} . This divalent cation enters the cell via the same channels as Ca^{2+} , but irreversibly binds the dye decreasing its fluorescence emission [22]. Thus, the change in the slope of fura-2 quenching after addition of AVP (Fig. 3) was attributable to accelerated Mn^{2+} influx via the capacitative pathway. Consistent with our earlier findings, cells grown in HG displayed a more stable fura-2 emission, with

Fig. 3. Effects of 5.5/30 mmol/l glucose on quenching of fura-2 fluorescence by 0.1 mmol/l MnCl_2 in monolayers of cultured rat mesangial cells equilibrated in Ca^{2+} -free media. *Upper panel*, fluorescence decay due to basal Mn^{2+} influx via divalent cation non-selective channels. Note enhanced fluorescence quenching after a transient $[\text{Ca}^{2+}]_i$ elevation induced by 0.1 $\mu\text{mol/l}$ AVP, due to increased Mn^{2+} entry. *Lower panel*, inhibition of AVP-induced Mn^{2+} quenching in cells grown in 30 mmol/l glucose for 5 days. Fluorescence tracings representative of $n = 9$ experiments

Fig. 4. Lack of effect of 30 mmol/l L-glucose on capacitative Ca^{2+} influx in fura-2-loaded monolayers of cultured rat mesangial cells. *Upper panel*, $[\text{Ca}^{2+}]_i$ elevation upon addition of 1–10 mmol/l Ca^{2+} after 0.1 $\mu\text{mol/l}$ arginine vasopressin (AVP) to cells grown in 5.5 mmol/l glucose for 5 days and equilibrated in Ca^{2+} -free solutions. *Lower panel*, identical protocol with 30 mmol/l L-glucose as an isosmotic replacement for D-glucose. Fluorescence tracings representative of $n = 6$ experiments

reduced quenching by Mn^{2+} both basally and after release of intracellular Ca^{2+} stores by AVP.

In order to establish whether the effects of HG on mesangial cells were attributable to a direct biochemical mechanism, or rather to non-specific osmotic effects, we performed a set of experiments employing 30 mmol/l L-glucose as osmotic replacement. Contrary to D-glucose, this non-metabolized isomer had no significant effect on Ca^{2+} influx upon addition of extracellular Ca^{2+} (Fig. 4, Table 1), pointing to a specific, metabolic action of HG.

Fig. 5. Effects of phorbol myristate acetate (PMA) on basal Ca^{2+} influx in fura-2-loaded monolayers of cultured rat mesangial cells equilibrated in Ca^{2+} -free media. *Upper panel*, control experiment, with progressive $[\text{Ca}^{2+}]_i$ elevation upon addition of 1–10 mmol/l Ca^{2+} . *Lower panel*, inhibition of Ca^{2+} influx following pretreatment of the cells with 0.1 $\mu\text{mol/l}$ PMA for 3 min. Fluorescence tracings representative of $n = 6$ experiments

Based on extensive evidence that PKC is activated by a cell culture environment mimicking diabetes, with elevated glucose concentration in the absence of any added insulin beyond that contained in the FBS supplement [10, 11, 14], we studied the effects of PKC activation or suppression on the SOC pathway. As shown in Figure 5 and Table 2, the PKC activator phorbol myristate acetate (PMA) added 3 min prior to application of extracellular Ca^{2+} to monolayers previously equilibrated in Ca^{2+} -free media, blunted both basal and agonist-activated capacitative Ca^{2+} influx in NG cells. However, 24-h pretreatment of HG cells with PMA, a procedure known to down-regulate PKC activity by increased metabolism of the enzyme [13], completely restored Ca^{2+} entry to the levels of NG cells (Fig. 6A–B, Table 3). Nearly identical results were obtained with two structurally unrelated inhibitors of PKC, staurosporine (SSP) and H-7 (Fig. 6C–D, Table 3), thus confirming that HG-induced PKC activity interferes with several ion transport and signalling mechanisms in cultured rat mesangial cells. Both PKC downregulation and SSP had no significant effect on Ca^{2+} influx in NG cells ($160 \pm 46/306 \pm 96$ and $148 \pm 42/317 \pm 55$ nmol/l peak $[\text{Ca}^{2+}]_i$ at 1/10 mmol/l Ca^{2+} , PMA and SSP, respectively, $n = 5$ each).

Discussion

Altering the glucose concentration of the growth media of cultured cells modifies their functional behaviour [23–25]. This concept has been developed in different cell systems derived from target organs for the

Table 2. Effects of the PKC activator, 0.1 $\mu\text{mol/l}$ phorbol myristate acetate (PMA) on resting and arginine vasopressin (AVP)-stimulated capacitative Ca^{2+} influx in monolayers of cultured rat mesangial cells

Culture	1 mmol/l Ca^{2+}	10 mmol/l Ca^{2+}
Resting	135 $\pm$ 22	354 $\pm$ 30
Resting + 0.1 $\mu\text{mol/l}$ PMA	87 $\pm$ 16 ^a	290 $\pm$ 23 ^a
+ 0.1 $\mu\text{mol/l}$ AVP	268 $\pm$ 34	422 $\pm$ 41
+ 0.1 $\mu\text{mol/l}$ PMA	143 $\pm$ 11 ^a	330 $\pm$ 35 ^a

Data are mean $\pm$ SEM nmol/l peak $[\text{Ca}^{2+}]_i$ from $n = 4$ experiments for each condition on monolayers grown in 5.5 mmol/l glucose and equilibrated in Ca^{2+} -free solutions. ^a $p < 0.01$ vs basal or AVP, respectively

Table 3. Reversal of the effects of 30 mmol/l glucose on arginine vasopressin (AVP)-stimulated capacitative Ca^{2+} influx by protein kinase C inhibition in monolayers of cultured rat mesangial cells

Culture	1 mmol/l Ca^{2+}	10 mmol/l Ca^{2+}
30 mmol/l glucose	140 $\pm$ 34 ^a	271 $\pm$ 16 ^a
30 mmol/l glucose + 0.1 $\mu\text{mol/l}$ PMA	218 $\pm$ 50	371 $\pm$ 29
30 mmol/l glucose + 0.1 mmol/l H-7	268 $\pm$ 47	382 $\pm$ 54
30 mmol/l glucose + 10 $\mu\text{mol/l}$ SSP	288 $\pm$ 33	440 $\pm$ 82

Data are mean $\pm$ SEM nmol/l peak $[\text{Ca}^{2+}]_i$ from $n = 4$ experiments for each condition. ^a $p < 0.01$ vs 5.5 mmol/l glucose. PMA, phorbol myristate acetate (24 h preincubation). H-7 and staurosporine (SSP) were added to the bathing medium for 20 min prior to each fluorometric recording

complications of diabetes [26, 27]. Extensive evidence has been provided that mesangial cells of the kidney glomerulus grown in high glucose media express a secretory phenotype, with increased matrix production and a decreased proliferative rate [28–31]. In addition, the cells appear less sensitive to the vasomotor action of peptides and autacoids that activated phospholipase C-coupled receptors [8, 10]. Glomerular hypocontractility in ex vivo preparations is an example of this phenomenon [32, 33]. This is paralleled in most studies by depressed signalling for vasoconstrictors [8, 10], and may explain, at least in part, the glomerular vasodilatation that occurs in diabetes and results in hyperfiltration and microalbuminuria. Work from several laboratories on contractile mesangial cells of the glomerulus or their systemic counterpart, the vascular smooth muscle cell (VSMC), has established that elevated PKC activity may be a common feature of cells grown in high glucose media, that might in turn explain several of the functional changes observed [9–11, 14, 34–38]. This may well be the case of inositol phospholipids/ $[\text{Ca}^{2+}]_i$ signalling, which is well known to be under inhibitory feedback control from PKC [12, 13]. Earlier studies from our group demonstrated that PKC downregulation rapidly reverses the effects of HG on the immediate $[\text{Ca}^{2+}]_i$ responses to vasoactive

Fig. 6 A–D. Effects of protein kinase C (PKC) inhibition by pretreatment with 0.1 $\mu\text{mol/l}$ phorbol myristate acetate for 24 h (PMA, **B**), 0.1 mmol/l H-7 for 20 min (**C**), or 10 $\mu\text{mol/l}$ staurosporine for 20 min (SSP, **D**) on capacitative Ca^{2+} influx in fura-2 loaded monolayers of cultured rat mesangial cells grown in 30 mmol/l glucose media and equilibrated in Ca^{2+} -free solutions. **A**, control experiment. Note inhibited response to AVP and Ca^{2+} influx after culture for 5 days in 30 mmol/l glucose and reversal by PKC inhibition. Tracings are representative of $n = 4$ experiments each condition

agents [10]. As high glucose media and resulting PKC activity did not alter the size of releasable intracellular Ca^{2+} pools, it was concluded that depressed receptor/inositol phosphate coupling was responsible for the functional resistance [8, 10]. This was extensively confirmed by other groups working with cultured VSMC [35, 39]. As evidence surfaced that Ca^{2+} influx is an important component of any $[\text{Ca}^{2+}]_i$ response to vasoconstrictors, in addition to intracellular Ca^{2+} pools, we turned our attention to the so-called ‘capacitative’ Ca^{2+} influx through SOC [15–18], as another relevant ion transport mechanism potentially regulated by ambient glucose level. This novel pathway for Ca^{2+} influx must be kept distinct from voltage-operated or receptor-operated Ca^{2+} channels, which are well documented in a variety of contractile cell types in culture, but are less readily detectable in mesangial cells [15–18]. While the driving force for Ca^{2+} entry across this putative channel is still the normal inward gradient for Ca^{2+} , the signal for its opening is not provided by membrane depolarization or direct binding of the agonist. It is presently believed that capacitative Ca^{2+} influx is actuated by release of Ca^{2+} itself from intracellular stores [16, 40, 41], or by a putative ‘influx factor’ coupled to depletion of the Ca^{2+} stores [42–44]. As it is now apparent that capacitative Ca^{2+} influx is inhibited by PKC in other cell systems [16, 18, 38], we linked our analysis of the functional status of this pathway in high glucose media to manipulation of PKC activity. Consistent with our earlier findings and the recent work by Rivera et al. with VSMC [38], we observed that this type of Ca^{2+} influx is weaker in high glucose cultured cells, and discharge of intracellular Ca^{2+} stores fails

to activate it to the extent seen in cells cultured in normal glucose. Our observation is reminiscent of earlier studies by Williams and Schrier [35, 45], who reported reduced activity of voltage-operated channels in vascular smooth muscle, contributing to the lower amplitude of Ca^{2+} signalling under high glucose conditions. In these works, receptor-operated Ca^{2+} uptake, a process partly related to the recently described SOC, was also depressed [45]. Other studies have documented inhibition of Ca^{2+} influx by insulin-stimulated glucose uptake in VSMC [37]. Recent patch-clamp work has shown that a 27 pS non-selective cation channel is inhibited by growth of rat mesangial cells in 30 mmol/l glucose media [46]. This channel, activated by angiotensin II and thapsigargin, may represent one link between store depletion and Ca^{2+} influx in mesangial cells, although through the intermediation of VOC [46, 47]. Thus, reduced Ca^{2+} entry may be a feature common to several cell types cultured under high glucose conditions, but the mechanism of internalization involved could be different, based on the peculiar features of a given cell population. The weaker Ca^{2+} influx in mesangial cells may indeed be due to the smaller amounts of Ca^{2+} made available from intracellular stores; nevertheless, studies with PMA indicate that Ca^{2+} uptake is also directly downregulated by PKC. Recent work in human mesangial cells from our laboratory confirmed direct control of Ca^{2+} influx by activated PKC, even in the presence of intact Ca^{2+} stores [48]. Interestingly, also the electrophysiology studies by Seal et al. [46] indicated that PKC inhibition restores channel responsiveness to angiotensin II in the presence of high glucose [46]. This finding points to a general inhibitory activity of PKC on multiple Ca^{2+} transport functions, ranging from the above-mentioned release from intracellular stores, to plasma membrane channels [45, 46], to $\text{Na}^+/\text{Ca}^{2+}$ exchange in our previous studies [49]. Such inhibition may represent an endogenous negative feedback, aimed at limiting the state of activation of the cells in the presence of vasoconstrictors, and preventing $[\text{Ca}^{2+}]_i$ from rising to uncontrolled levels.

The mechanism by which PKC activity is upregulated in cells cultured under high glucose conditions is still elusive, although evidence that only the α , β , δ and ϵ isoforms binding to DAG are activated, suggests that de novo synthesis of the latter compound from excess glucose may be involved [14, 39]. As reported in other tissue/cell systems, this metabolic pathway appears to involve the formation of glycerol 3-phosphate, acylation of phosphatidic acid followed by dephosphorylation by a phosphatase [39, 50]. Reversal of the effects of high glucose by three unrelated procedures known to inhibit PKC is strongly suggestive that this enzyme is the main, if not the only, intracellular mechanism of the inhibition of Ca^{2+} signalling. Clearly, osmotic mechanisms cannot be invoked, as

L-glucose did not share the potent effects of D-glucose, similar to mannitol in our earlier work in the same experimental system. Additionally, other studies in various cell preparations have ruled out a role for the hypertonicity of the high glucose solutions [8, 39].

The implications of these findings are relevant to the short-term changes of glomerular haemodynamics occurring in diabetes, but also to complications that involve structural changes of the glomerulus, such as matrix deposition and fibrosclerosis. As multiple cell functions are regulated by $[\text{Ca}^{2+}]_i$ and related signals, it is conceivable that altered refilling of the intracellular Ca^{2+} pools resulting from impaired influx via the capacitative pathway may translate into dysregulated protein synthesis and proliferation. Moreover, further evidence that PKC activation is involved in the functional abnormalities induced by high glucose in vitro, points to novel targets for therapeutic intervention aimed at limiting the complications of diabetes.

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