Diabetes-induced activation of protein kinase C inhibits store-operated Ca^{2+} uptake in rat retinal microvascular smooth muscle

T. M. Curtis¹, E. H. Major², E. R. Trimble³, C. N. Scholfield²

¹ Department of Ophthalmology and Vision Sciences, The Queen's University of Belfast, Institute of Clinical Sciences, The Royal Victoria Hospital, Belfast, N. Ireland, UK

² Smooth Muscle Group, The Queen's University of Belfast, Medical Biology Centre, Belfast, N. Ireland, UK

³ Department of Clinical Biochemistry, The Queen's University of Belfast, Institute of Clinical Sciences, The Royal Victoria Hospital, Belfast, N. Ireland, UK

Abstract

Aims/Hypothesis. To assess the effects of diabetes-induced activation of protein kinase C (PKC) on voltage-dependent and voltage-independent Ca^{2+} influx pathways in retinal microvascular smooth muscle cells.

Methods. Cytosolic Ca^{2+} was estimated in freshly isolated rat retinal arterioles from streptozotocin-induced diabetic and non-diabetic rats using fura-2 microfluorimetry. Voltage-dependent Ca^{2+} influx was tested by measuring rises in $[\text{Ca}^{2+}]_i$ with KCl (100 mmol/l) and store-operated Ca^{2+} influx was assessed by depleting $[\text{Ca}^{2+}]_i$ stores with Ca^{2+} free medium containing 5 $\mu\text{mol/l}$ cyclopiazonic acid over 10 min and subsequently measuring the rate of rise in Ca^{2+} on adding 2 mmol/l or 10 mmol/l Ca^{2+} solution.

Results. Ca^{2+} entry through voltage-dependent L-type Ca^{2+} channels was unaffected by diabetes. In contrast, store-operated Ca^{2+} influx was attenuated. In microvessels from non-diabetic rats 20 mmol/l D-mannitol

had no effect on store-operated Ca^{2+} influx. Diabetic rats injected daily with insulin had store-operated Ca^{2+} influx rates similar to non-diabetic control rats. The reduced Ca^{2+} entry in diabetic microvessels was reversed by 2-h exposure to 100 nmol/l staurosporine, a non-specific PKC antagonist and was mimicked in microvessels from non-diabetic rats by 10-min exposure to the PKC activator phorbol myristate acetate (100 nmol/l). The specific PKC β antagonist LY379196 (100 nmol/l) also reversed the poor Ca^{2+} influx although its action was less efficacious than staurosporine.

Conclusion/interpretation. These results show that store-operated Ca^{2+} influx is inhibited in retinal arterioles from rats having sustained increased blood glucose and that PKC β seems to play a role in mediating this effect. [Diabetologia (2003) 46:1252–1259]

Keywords Diabetes, retinal arterioles, store-operated Ca^{2+} channels, L-type Ca^{2+} channels, protein kinase C, microvessels.

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Corresponding author: C. N. Scholfield PhD, Smooth Muscle Group, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 6BL, N. Ireland, UK
E-mail: n.scholfield@qub.ac.uk

Abbreviations: DAG, Diacylglycerol; PKC, protein kinase C; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; STZ, streptozotocin; SPP, staurosporine; SR, sarcoplasmic reticulum; MVSM, microvascular smooth muscle; CPA, cyclopiazonic acid; PMA, phorbol myristate acetate; VDCC, voltage-dependent Ca^{2+} channels.

Diabetic retinopathy is the most widespread complication of diabetes mellitus and is a major cause of blindness in the working population of developed countries. While the exact pathogenic basis of this condition remains ill-defined, it is clear that hyperglycaemia is a critical factor in its aetiology [1, 2]. An early effect of hyperglycaemia is a persistent dilatation of the retinal arterioles which increases ocular blood flow and leads to downstream capillary hypertension. This is thought to contribute directly to the development and progression of the disease [3, 4, 5]. The angiopathy which then develops is characterised by structural changes such as pericyte [6] and arteriolar

smooth muscle [7] dropout, microaneurysms [8] and capillary acellularity [9]. The empty tubes of basement membrane that remain are non-perfused [10] and this leads to regions of retinal ischaemia which precipitates a destructive neovascularisation.

As hyperglycaemia seems to be the primary factor in the development of diabetic retinopathy, considerable effort has been directed to the glucose-mediated pathogenic mechanisms that lead to the retinal vascular deterioration. One of the major pathways that could lead to diabetic retinopathy is the aberrant activation of the diacylglycerol (DAG)-protein kinase C (PKC) pathway [11].

In the physiological context, phosphatidylinositol 4,5-disphosphate hydrolysis at the plasma membrane catalysed by receptor-activated phospholipase C becomes an important source of new DAGs that are able to activate PKCs. Receptor stimulation also commonly activates phospholipase D which catalyses phosphatidylcholine hydrolysis. The phosphatidic acid generated is dephosphorylated by phosphatidate phosphohydrolase which produces DAGs. Some DAGs are also produced in the endoplasmic reticulum as intermediates in the biosynthesis of triglycerides (de novo synthesis pathway). DAGs are phosphorylated by DAG kinases or deacylated by DAG lipases, which terminate their action. The cellular effects of DAG mediated PKC activation range from regulation of ion channel gating and permeability, receptor function, cytoskeletal structure, proliferation, apoptosis, cell division and transcription.

Diabetes causes an increase in DAG concentrations in vascular tissues associated with diabetic complications, including the retina [12]. The molecular species of these pathophysiological DAGs are consistent with them being produced by the shunting of excess glucose through the de novo synthesis pathway [13]. These in turn activate a series of enzymes, particularly the α , β and δ isoforms of PKCs [11]. In diabetic rats, PKC β II is preferentially activated in the retina [12] and animal and clinical trials have shown that some of the retinal vascular changes associated with diabetes can be reversed by blockers of PKC β [14]. Prolonged PKC activation in diabetes could also arise through oxidative stress or increased concentrations of free fatty acids [15, 16].

Retinal arteriole diameters are directly regulated by changes in the intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) of the retinal microvascular smooth muscle cells (MVSM). Regulation of $[\text{Ca}^{2+}]_i$ in vascular smooth muscle involves multiple mechanisms including Ca^{2+} influx and efflux and sarcoplasmic reticulum (SR) Ca^{2+} release, and reuptake. Vasoactive factors released from the endothelium and retinal tissue act to modulate retinal arteriole widths by altering the activity of these Ca^{2+} handling mechanisms [17]. Arteriolar relaxation within the diabetic retina could be explained by the enhanced production of vasodilators,

such as nitric oxide [18, 19, 20] or prostaglandins [21] or through a reduced responsiveness of the retinal MVSM cells to vasoconstrictors such as endothelin-1 [22]. The possibility that defects in the Ca^{2+} -homeostatic mechanisms of the retinal arterioles might also contribute has yet to be examined.

Retinal MVSM cells rely upon both voltage-dependent and store-operated Ca^{2+} channels for their Ca^{2+} entry [17]. Unlike the voltage-dependent channels, store-operated Ca^{2+} channels are not activated by changes in membrane potential, but rather through the depletion of Ca^{2+} from the SR following agonist stimulation. The purpose of this study was to examine whether diabetes affects the functioning of both classes of Ca^{2+} channel in rat retinal MVSM cells. PKCs are known to modify the activity of both voltage-dependent and store-operated Ca^{2+} channels in other types of smooth muscle [23, 24, 25] and therefore we also investigated whether PKCs were responsible for the observed changes with diabetes. Our results suggest that diabetes attenuates Ca^{2+} influx in retinal MVSM cells, but that this occurs entirely through the inhibition of store-operated Ca^{2+} channels. Furthermore, PKC β seems to play a major role in mediating this effect.

Materials and methods

Experimental animals. Male Sprague-Dawley rats (200–250 g) were fed on a standard rat/mouse diet (Harlan Teklad TRM). They were injected intraperitoneally with 60 mg/kg of streptozotocin freshly dissolved in 167 mmol/l saline/20 mmol/l citrate buffer, pH 4.6. After 2 weeks the rats were weighed and blood glucose was measured using a meter and test strips. After injection (12 to 14 weeks), the rats were weighed and glucose measured again in duplicate 3 h after food deprivation. The rats were then killed by CO_2 and those that had lost weight over the 12- to 14-week period were rejected. Rats having blood glucose concentrations of less than 10 mmol/l were taken as being non-diabetic and these rats acted as controls for the streptozotocin treatment (mean glucose 5.6 ± 0.13 mmol/l, SEM, $n=24$). Those above this, were deemed to be diabetic (mean glucose 28.0 ± 0.9 mmol/l, SD, $n=30$, $p<0.00001$). In some experiments, non-injected control rats were used. We gave six rats with established diabetes daily subcutaneous injections of insulin (5 units protamine zinc) for 7 days which reduced blood glucose to 3.5 ± 1.1 mmol/l and normalised urine output and weight gain. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the United Kingdom Animals (Scientific Procedures) Act, 1986.

Microvessel preparation. Retinae were removed, lightly triturated using a Pasteur pipette having a fire-polished tip of 0.3 mm internal diameter in low Ca^{2+} (see below) and centrifuged. Fura-2 AM (in 5 μl of DMSO) and detergent (Pluronic) were added to the final precipitate giving final concentrations of 5 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$ respectively in 1 ml of low Ca^{2+} solution. The microvessels were used after 2 h and remained viable for a further 4 h.

The homogenate was diluted with 10 volumes low Ca^{2+} medium and the mixture was again triturated. Of this mixture 1 ml was pipetted into a glass bottomed recording bath at 36°C . Microvessels were anchored down with tungsten wire slips and drug solutions came via a 7-way micro-manifold.

Ca^{2+} microfluorimetry. The preparation was illuminated by 340/380 nm light and fluorescence measured at 510 nm using Cairn software (Kent, UK). R_{max} and R_{min} were obtained using amphotericin (0.2 mg/ml) with 100 mmol/l Ca^{2+} or Ca^{2+} free/1 mmol/l EGTA respectively and these values together with the background reading (estimated using 5 mmol/l Mn^{2+} in Ca^{2+} -free solution) were used in calculating $[\text{Ca}^{2+}]_i$ [26]. Although endothelial cells were still present, they neither loaded fura-2 nor contributed to the MVSM responses [17]. The outer surface of the microvessels were visually sharp and were used to measure the diameter from video recordings.

Solutions and drugs. Normal bathing solution contained (mmol/l): NaCl, 140; KCl, 5; D-glucose, 5; MgCl_2 , 1.3; HEPES, 10; CaCl_2 , 2.0; pH 7.3. For tissues from diabetic rats, all solutions including the low Ca^{2+} solution contained 25 mmol/l D-glucose. For the KCl solution, an additional 95 mmol/l KCl was added to normal solution. The drugs used were obtained from: LY379196, Eli Lilly Indianapolis, USA; Staurosporine, Alomone Laboratories, Jerusalem, Israel; Phorbol 12-myristate 13-acetate (PMA, Sigma, Poole, UK), cyclopiazonic acid (CPA, Alexis Corp., Nottingham, UK and Fermentek, Jerusalem, Israel). When used for prolonged studies, drugs were refreshed every 2 h (1 h for PMA).

Statistical analysis. Data were analysed using GraphPad Prism V3 (GraphPad Software, San Diego). Data was asymmetrically distributed and thus non-parametric statistics were used. Comparisons were made among groups of measurements using the Mann Whitney and Kruskal-Wallis tests. Significance was set at $p < 0.05$ and multiple comparisons were made using Dunn's post test. Data are expressed as a means $\pm$ SEM. At least four rats were used per treatment group and n values within the text correspond to the numbers of microvessels tested within a given protocol.

Results

Quiescent properties of the microvessels. In both diabetic and non-diabetic rats, the smooth muscle cells

Fig. 1. Time-course for $[\text{Ca}^{2+}]_i$ in a retinal arteriole from a STZ-injected non-diabetic rat. Voltage-dependent Ca^{2+} influx was activated with successive additions of 100 mmol/l KCl (filled bars) and this was totally abolished using 1 $\mu\text{mol/l}$ nifedipine (open bar) implicating L-type Ca^{2+} channels in the depolarisation-mediated response

formed a single continuous layer without any gaps that would otherwise suggest cell loss. Microvessels taken from either group of rats had similar diameters ($23.9 \pm 2.3 \mu\text{m}$ in 43 non-diabetic compared to $23.8 \pm 1.5 \mu\text{m}$ in 52 diabetic microvessels). Resting $[\text{Ca}^{2+}]_i$ was similar for each group ($69 \pm 4 \text{ nmol/l}$ for 63 non-diabetic microvessels and $76 \pm 5 \text{ nmol/l}$ for 106 diabetic microvessels) and none of the microvessels showed any spontaneous $[\text{Ca}^{2+}]_i$ oscillations or mechanical responses. These observations are similar to those previously described for retinal arterioles freshly dispersed from normal rat retinae.

Voltage-dependent Ca^{2+} channels. Larger microvessels ($>20 \mu\text{m}$ diameter) possessed voltage-dependent L-type Ca^{2+} channels and these were activated by adding 100 mmol/l KCl causing a rise in cytosolic Ca^{2+} (Fig. 1) and a transient contraction. Microvessels from normal, STZ-injected non-diabetic and diabetic rats produced similar $[\text{Ca}^{2+}]_i$ and contractile responses to high K^+ solution (Table 1).

Store-operated Ca^{2+} influx in retinal MVSM cells. Store-operated Ca^{2+} influx can be routinely measured

Table 1. Effects of diabetes on the voltage-dependent Ca^{2+} entry evoked by 100 mmol/l KCl and contractile responses in rat retinal arterioles. Values headed "normal" are the resting $[\text{Ca}^{2+}]_i$ (nmol/l) for microvessels bathed in normal solution (means $\pm$ SEM) and "test" values are the peak of the $[\text{Ca}^{2+}]_i$ transients from the same microvessels bathed in 100 mmol/l KCl. The "increase" is the ratio of test/normal $[\text{Ca}^{2+}]_i$ minus 1

	Non-injected rats				Non-diabetic rats				Diabetic rats				p
	Normal	Test	Increase	n	Normal	Test	Increase	n	Normal	Test	Increase	n	
$[\text{Ca}^{2+}]_i$	56 ± 5	165 ± 23	1.45 ± 0.3	28	68 ± 6	134 ± 14	1.2 ± 0.2	28	79 ± 5	174 ± 26	1.3 ± 0.3	45	0.53
	% Constriction			n	% Constriction			n	% Constriction			n	
Diameter	14.4 ± 3.1			22	13.7 ± 2.8			27	12.3 ± 2.6			34	0.88

for each microvessel and then these averaged. Microvessel constriction with increased K^+ was monitored by measuring changes in their outer diameter. Results are expressed as the change in diameter of the microvessels as a percentage of the relaxed microvessel. "n" denotes the numbers of microvessels tested and p values were determined using the Kruskal-Wallis test

Fig. 2. Effect of CPA on the rate of $[\text{Ca}^{2+}]_i$ rise when the bathing solution was changed from Ca^{2+} -free solution to one containing 2 mmol/l Ca^{2+} at the point marked by the arrow

using Ca^{2+} microfluorimetry and the mycotoxin, cyclopiazonic acid (CPA), which depletes Ca^{2+} stores (sarcoplasmic reticulum). To demonstrate store-operated Ca^{2+} influx in retinal MVSM cells, microvessels were initially maintained in Ca^{2+} free solution (for 1 min) and the initial rate of rise in $[\text{Ca}^{2+}]_i$ on adding 2 mmol/l extracellular Ca^{2+} was measured (Fig. 2). The Ca^{2+} -free solution was restored and once the drop in $[\text{Ca}^{2+}]_i$ had stabilised, 5 $\mu\text{mol/l}$ CPA was added (still in a Ca^{2+} free solution). A transient increase in $[\text{Ca}^{2+}]_i$ was observed (not shown) and this probably represents store Ca^{2+} leakage (without its re-uptake) which is then extruded from the cell. Following store depletion (determined by the decline of $[\text{Ca}^{2+}]_i$ to the basal value previously established in Ca^{2+} -free solution), 2 mmol/l Ca^{2+} was added. The more rapid rise in $[\text{Ca}^{2+}]_i$ with CPA (Fig. 2) indicated that store-operated Ca^{2+} influx had been stimulated. The rise in Ca^{2+} continued until Ca^{2+} extrusion mechanisms came into operation when $[\text{Ca}^{2+}]_i$ equilibrated at a steady value [when Ca^{2+} extrusion is blocked with carboxyeosin and low (10 mmol/l) Na^+ solution, $[\text{Ca}^{2+}]_i$ continues to rise]. With CPA, the Ca^{2+} influx rate was increased by 5.9 ± 1.2 and 4.0 ± 1.3 fold on going from Ca^{2+} free medium to solutions containing either 2 or 10 mmol/l Ca^{2+} respectively (2 mmol/l Ca^{2+} , $p=0.00001$, $n=20$; 10 mmol/l Ca^{2+} , $p=0.000007$, $n=19$).

Without CPA, the $[\text{Ca}^{2+}]_i$ rise with 2 mmol/l and 10 mmol/l Ca^{2+} was unaffected by diabetes. Hence this step was omitted from the remaining protocols and experiments were confined to the measurement of Ca^{2+} influx rates in microvessels pre-incubated with 5 $\mu\text{mol/l}$ CPA in Ca^{2+} -free solution during the first 10 s of addition of 2 mmol/l or 10 mmol/l Ca^{2+} solutions. The higher non-physiological Ca^{2+} solution (10 mmol/l Ca^{2+}) was used to test for more subtle effects of diabetes.

Diabetes and store-operated Ca^{2+} influx. Store-operated Ca^{2+} influx was substantially reduced in micro-

Fig. 3A, B. Inhibition of store-operated Ca^{2+} influx in rat retinal arterioles induced by diabetes and its reversal by insulin. (A) A protocol similar to Fig. 2 showing the store-operated Ca^{2+} influx induced by CPA in a microvessel from a diabetic and a non-diabetic animal. (B) Histograms showing averaged store-operated Ca^{2+} influx rates for microvessels from non-injected controls, STZ-injected non-diabetics, diabetic rats and insulin-injected diabetic rats. Error bars are SEMs and values above the bars indicate the number of microvessels tested. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$. There was no difference between insulin-treated diabetic rats and non-diabetic rats (2 mmol/l Ca^{2+} $p=0.48$, $n=9,34$ and 10 mmol/l Ca^{2+} $p=0.2$ $n=9,33$)

vessels from diabetic rats (Fig. 3A,B). As additional controls for the STZ treatment, some experiments were repeated in diabetic rats injected with insulin to normalise their blood glucose. The attenuation in store-operated Ca^{2+} influx was reversed in microvessels from these rats (Fig. 3B). To exclude any osmotic effects of the 25 mmol/l glucose, 20 mmol/l D-mannitol (5 mmol/l glucose) was tested on microvessels from non-diabetic rats. Contrary to the effects of diabetes, D-mannitol had no significant effect on Ca^{2+} influx ($p=0.8$ and $p=0.98$ for 2 and 10 mmol/l Ca^{2+} ; $n=8$).

Role of Protein Kinase Cs. Diabetic hyperglycaemia has several metabolic effects including the persistent up-regulation of some Protein Kinase C (PKC) isoforms. To test their role in store-operated Ca^{2+} influx, isolated microvessels from diabetic rats were treated for 2 h with the broad spectrum PKC antagonist, staurosporine (100 nmol/l). With 2 mmol/l Ca^{2+} , the

Fig. 4A–C. Phorbol myristate acetate (PMA; 100 nmol/l) attenuates store-operated Ca^{2+} uptake in control retinal microvessels. **(A)** Store-operated Ca^{2+} influx rates in the presence of CPA induced by 2 mmol/l Ca^{2+} in a normal microvessel and a microvessel pre-exposed to PMA for 10 min. **(B)** Bar chart showing the mean store-operated Ca^{2+} influx rates for control microvessels and microvessels pre-incubated with PMA for 10 min. Error bars are SEMs and values above the bars indicate the number of microvessels tested. *** $p < 0.001$. **(C)** An original recording of $[\text{Ca}^{2+}]_i$ from a rat retinal arteriole on adding 100 nmol/l PMA which reduced $[\text{Ca}^{2+}]_i$, but caused the microvessel to contract (photomicrographs below). Scale bars = 5 μm on photos

store-operated Ca^{2+} influx rate was completely restored to values seen in microvessels from non-diabetic rats (Table 2). With 10 mmol/l Ca^{2+} , the effect seemed to be even greater. Staurosporine had no effect on microvessels from non-diabetic rats with either 2 mmol/l or 10 mmol/l Ca^{2+} ($p = 0.31$ and $p = 0.7$, respectively; paired comparisons with and without staurosporine in the same rats; $n = 10$ & 12).

To further test for a role of PKCs, retinal microvessels from normal rats were exposed to the PKC activator phorbol myristate acetate (PMA; 100 nmol/l),

added 10 min prior to application of 2 mmol/l or 10 mmol/l Ca^{2+} . PMA severely blunted store-operated Ca^{2+} influx in these microvessels (Fig. 4A,B). The microvessels also contracted during the pre-exposure to PMA (in 2 mmol/l extracellular Ca^{2+}), even though $[\text{Ca}^{2+}]_i$ was slightly reduced (Fig. 4C). To discount any effects of PMA unrelated to PKC activation such as direct Ca^{2+} channel blockade, normal microvessels were pre-incubated in 100 nmol/l PMA for 6 h, a time scale over which PKCs are known to 'down-regulate'. In these microvessels, the Ca^{2+} influx rates were normal and the microvessels seemed not to be contracted (2 mmol/l Ca^{2+} $p = 0.6$; 10 mmol/l Ca^{2+} $p = 0.63$; paired comparisons with and without PMA; $n = 11$ & 10).

There are at least 13 different isoforms of PKC, and in the diabetic retina PKC β II is preferentially up-regulated. LY379196 preferentially inhibits the two PKC β isoforms at low concentrations (≤ 100 nmol/l). At 100 nmol/l, it reversed the attenuated store-operated Ca^{2+} influx in diabetic microvessels, but the effect was smaller than that seen with staurosporine (Fig. 5A,B). This difference could arise from an involvement of other PKCs blocked by staurosporine but not by LY379196, or alternatively, an independent

Table 2. Staurosporine (SSP) reverses the attenuated CPA-induced store-operated Ca^{2+} influx in retinal arterioles from diabetic rats. Values (nmol/l/s) are Ca^{2+} influx rates (means

$\pm$ SEM) for "n" microvessels. *** is a $p < 0.001$ and ns is for a $p > 0.05$. Non-diabetic-SSP data corresponds to that for non-diabetic vessels in Fig. 3B

	Diabetic – SSP (1)	Diabetic + SSP (2)	Non-diabetic – SPP (3)	p (1 vs 2)	p (2 vs 3)
2 mmol/l Ca^{2+}	1.78 ± 0.22 $n = 24$	4.94 ± 0.73 $n = 20$	6.69 ± 0.86 $n = 34$	***	ns
10 mmol/l Ca^{2+}	4.59 ± 0.79 $n = 24$	60.6 ± 20.4 $n = 20$	16.4 ± 1.92 $n = 33$	***	ns

Fig. 5A, B. The PKC β antagonist, LY379196 (100 nmol/l), counteracted the effect of diabetes on store-operated Ca^{2+} influx in retinal MVSM cells. **(A)** CPA-induced store-operated Ca^{2+} influx in a microvessel from a diabetic rat and another microvessel from the same rat pre-incubated for 2 h in LY379196. **(B)** A summary histogram showing store-operated Ca^{2+} influx for microvessels from diabetic rats with and without LY379196 pretreatment and untreated microvessels from non-diabetic rats. Error bars as Fig. 3. * $p < 0.05$ and ns indicates a $p > 0.05$

effect of LY379196 on the Ca^{2+} pathway. In some microvessels from non-diabetic rats, LY379196 (100 nmol/l) did slightly reduce store-operated Ca^{2+} influx rates, but the effect was not consistent (2 mmol/l Ca^{2+} $p = 0.12$, 10 mmol/l Ca^{2+} $p = 0.09$; paired comparisons with and without LY379196; $n = 12$ and 13).

Discussion

It is increasingly acknowledged that abnormal $[\text{Ca}^{2+}]_i$ homeostasis contributes to the pathogenesis of diabetic vascular complications. Several studies have found that KCl depolarisation-induced changes in $[\text{Ca}^{2+}]_i$ and contractions are diminished in both macrovascular and microvascular smooth muscle cells from diabetic rats [27, 28, 29, 30, 31] and this is thought to occur through a reduction in the density of voltage-dependent L-type Ca^{2+} channels [32] (VDCC). In our study, the action of high K^+ was maintained in the retinal MVSM cells from diabetic rats suggesting that neither the VDCCs themselves nor their activation require-

ments such as membrane potential were affected. Since our work used the same experimental model (STZ-induced diabetic rats) and diabetic duration (3 months) as the other studies, this difference appears to arise through properties intrinsic to retinal MVSM cells. The present observation is in contrast with a previous study on bovine retinal pericytes where endothelin produced a sustained increase in cell Ca^{2+} that was sensitive to L-type Ca^{2+} channel blockers [33]. This Ca^{2+} increase was attenuated in high glucose. However, it is now clear that a store-operated Ca^{2+} entry pathway is also blocked by L-type Ca^{2+} channel blockers without any depolarisation [34]. It is possible that this store-filling pathway was being attenuated by high glucose rather than the L-type Ca^{2+} channels.

In contrast to the VDCCs, we found that Ca^{2+} entry through store-operated Ca^{2+} channels was decreased in freshly isolated intact retinal MVSM from diabetic rats. This accords with previous observations showing that hyperglycaemia can inhibit store-operated Ca^{2+} influx in other tissues relevant to diabetic complications [23, 35]. The functions of Ca^{2+} entry through store-operated Ca^{2+} channels include: (i) regulation of smooth muscle tone [36]; (ii) refilling of Ca^{2+} stores following agonist stimulation [37, 34] (iii) modulation of numerous Ca^{2+} -dependent enzymes [38, 39, 40] and (iv) control of gene expression through calcineurin-induced dephosphorylation of the transcription factor NF-AT and calmodulin stimulation of various transcription factors [41, 42]. Given this diversity of physiological regulation, inhibition of this pathway in diabetes could severely affect the functional integrity of retinal arterioles. Of particular relevance to the early stages of diabetes, reduced store-operated Ca^{2+} influx could, at least in part, explain the observed dilatation of the retinal arterioles and the resulting increases in ocular blood flow. Nonetheless, in our study no differences were observed in the outer diameters of freshly isolated retinal arterioles from non-diabetic and diabetic rats. However, we found that freshly isolated retinal arterioles are already maximally dilated and cannot be widened further using vasodilators such as acetylcholine or bradykinin [17]. The diabetes-induced inhibition of store-operated Ca^{2+} channels is likely to have more severe consequences for small pre-capillary arterioles than the larger trunk arterioles emanating from the optic disc. This is because the smaller pre-capillary arterioles are more limited in their Ca^{2+} regulation since they lack voltage-dependent Ca^{2+} channels as an alternative pathway of Ca^{2+} influx [17]. This could go some way to explaining why these microvessels seem particularly vulnerable to diabetes.

Our data on the inhibition of store-operated Ca^{2+} channels in diabetic retinal arterioles is consistent with an increased activation of PKC since it was reversed with the generalised PKC blocker staurosporine and mimicked in non-diabetic microvessels by

PKC activation (PMA). These findings are consistent with a similar mechanism for the glucose-induced inhibition of store-operated Ca^{2+} channels in human glomerular mesangial cells [35] and contradict recent studies showing that PKCs open store-operated Ca^{2+} channels in vascular myocytes [25] and glomerular mesangial cells [43]. These differences are explained by heterogeneity of store-depletion/receptor activation pathways and by tissue specificity in PKC isoform activation by diabetes/hyperglycaemia. Thus in diabetic rats, PKC β I in glomeruli and PKC β II in retinae are preferentially activated [44, 12] while Ca^{2+} -store-depletion preferentially translocates PKC α and PKC γ from the cytosol to the membrane [45].

PMA contracted retinal arterioles without any rise in Ca^{2+} in apparent contradiction with our PKC hypothesis of arteriolar dilatation in diabetes. However, PMA is a broad spectrum PKC activator [46] compared to isoform selective activation in diabetes. PKC ϵ appears to mediate Ca^{2+} -independent contractions in vascular smooth muscle [47]. Although PKC ϵ is weakly up-regulated in the diabetic retina [12], its strong activation PMA could explain this contradiction.

We finally sought to determine the PKC isoform involvement in diabetic arterioles. The restoration of store-operated Ca^{2+} influx by LY379196 suggests that PKC β is up-regulated and implies that PKC β could mediate the retinal vasodilatation in diabetes. However, with short durations of diabetes (2–4 weeks) retinal arteriolar constriction has been reported [49] which is also reversed by PKC β inhibition [50]. Thus PKC β effects could be biphasic, the earlier phase possibly occurring via induction of Et-1 [51], or by myofilament protein phosphorylation either directly or through inhibition of myosin-light chain phosphatases [52].

In summary, these results show that store-operated Ca^{2+} influx is inhibited in retinal arterioles from rats with established diabetes by mechanisms which include PKC β upregulation. Decreased store-operated Ca^{2+} influx might contribute to the demise of the retinal MVSM cells and hence the retinal haemodynamic changes in diabetes mellitus.

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References

1. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus (1993) The Diabetes Control and Complications Trial Research Group. *N Engl J Med* 329:977–986
2. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33) (1998) UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 352:837–853
3. Grunwald JE, Brucker AJ, Schwartz SS et al. (1990) Diabetic glycemic control and retinal blood flow. *Diabetes* 39:602–607
4. Kohner EM, Patel V, Rassam SM (1995) Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. *Diabetes* 44:603–607
5. Schmetterer L, Wolzt M (1999) Ocular blood flow and associated functional deviations in diabetic retinopathy. *Diabetologia* 42:387–405
6. Kuwabara T, Cogan DG (1963) Retinal Vascular Patterns. VI. Mural Cells of the retinal capillaries. *Arch Ophthalmol* 69:492–502
7. Gardiner TA, Stitt AW, Anderson HR et al. (1994) Selective loss of vascular smooth muscle cells in the retinal microcirculation of diabetic dogs. *Br J Ophthalmol* 78:54–60
8. Bresnick GH, Davis MD, Myers FL et al. (1977) Clinicopathologic correlations in diabetic retinopathy. II. Clinical and histologic appearances of retinal capillary microaneurysms. *Arch Ophthalmol* 95:1215–1220
9. Engerman RL (1989) Pathogenesis of diabetic retinopathy. *Diabetes* 38:1203–1206
10. Kohner EM, Henkind P (1970) Correlation of fluorescein angiogram and retinal digest in diabetic retinopathy. *Am J Ophthalmol* 69:403–414
11. Koya D, King GL (1998) Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866
12. Shiba T, Inoguchi T, Sportsman JR et al. (1993) Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol* 265:E783–E793
13. Xia P, Inoguchi T, Kern TS et al. (1994) Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 43:1122–1129
14. Frank RN (2002) Potential new medical therapies for diabetic retinopathy: protein kinase C inhibitors. *Am J Ophthalmol* 133:693–698
15. Gopalakrishna R, Jaken S (2000) Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 28:1349–1361
16. McPhail LC, Clayton CC, Snyderman R (1984) A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+} -dependent protein kinase. *Science* 224:622–625
17. Scholfield CN, Curtis TM (2000) Heterogeneity in cytosolic calcium regulation among different microvascular smooth muscle cells of the rat retina. *Microvasc Res* 59:233–242
18. Carmo A, Cunha-vaz JG, Carvalho AP et al. (2000) Nitric oxide synthase activity in retinas from non-insulin-dependent diabetic Goto-Kakizaki rats: correlation with blood-retinal barrier permeability. *Nitric Oxide* 4:590–596
19. Takeda M, Mori F, Yoshida A et al. (2001) Constitutive nitric oxide synthase is associated with retinal vascular permeability in early diabetic rats. *Diabetologia* 44:1043–1050
20. Abu El-Asrar AM, Desmet S, Meerschaert A et al. (2001) Expression of the inducible isoform of nitric oxide synthase in the retinas of human subjects with diabetes mellitus. *Am J Ophthalmol* 132:551–556
21. Johnson EI, Dunlop ME, Larkins RG (1999) Increased vasodilatory prostaglandin production in the diabetic rat retinal vasculature. *Curr Eye Res* 18:79–82
22. Curtis TM, Scholfield CN (1999) Attenuated endothelin responses in retinal microvessels from diabetic rats. *J Physiol* 515P:6P

23. Rivera AA, White CR, Guest LL et al. (1995) Hyperglycemia alters cytoplasmic Ca²⁺ responses to capacitative Ca²⁺ influx in rat aortic smooth muscle cells. *Am J Physiol* 269:C1482–C1488
24. Keef KD, Hume JR, Zhong J (2001) Regulation of cardiac and smooth muscle Ca(2+) channels (Ca(V)1.2a,b) by protein kinases. *Am J Physiol Cell Physiol* 281:C1743–C1756
25. Albert AP, Large WA (2002) Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J Physiol* 544:113–125
26. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450
27. Head RJ, Longhurst PA, Panek RL et al. (1987) A contrasting effect of the diabetic state upon the contractile responses of aortic preparations from the rat and rabbit. *Br J Pharmacol* 91:275–286
28. Fulton DJ, Hodgson WC, Sikorski BW et al. (1991) Attenuated responses to endothelin-1, KCl and CaCl₂, but not noradrenaline, of aortae from rats with streptozotocin-induced diabetes mellitus. *Br J Pharmacol* 104:928–932
29. Carmines PK, Ohishi K, Ikenaga H (1996) Functional impairment of renal afferent arteriolar voltage-gated calcium channels in rats with diabetes mellitus. *J Clin Invest* 98:2564–2571
30. Hattori Y, Kawasaki H, Fukao M et al. (1996) Diminishment of contractions associated with depolarization-evoked activation of Ca²⁺ channels in diabetic rat aorta. *J Vasc Res* 33:454–462
31. Wang R, Liu Y, Sauve R et al. (1998) Hyperosmolality-induced abnormal patterns of calcium mobilization in smooth muscle cells from non-diabetic and diabetic rats. *Mol Cell Biochem* 183:79–85
32. Wang R, Wu Y, Tang G et al. (2000) Altered L-type Ca(2+) channel currents in vascular smooth muscle cells from experimental diabetic rats. *Am J Physiol Heart Circ Physiol* 278:H714–H722
33. McGinty A, Scholfield CN, Liu WH et al. (1999) Effect of glucose on endothelin-1-induced calcium transients in cultured bovine retinal pericytes. *J Biol Chem* 274:25250–25253
34. Curtis TM, Scholfield CN (2001) Nifedipine blocks Ca²⁺ store refilling through a pathway not involving L-type Ca²⁺ channels in rabbit arteriolar smooth muscle. *J Physiol* 532:609–623
35. Mene P, Pugliese G, Pricci F et al. (1997) High glucose level inhibits capacitative Ca²⁺ influx in cultured rat mesangial cells by a protein kinase C-dependent mechanism. *Diabetologia* 40:521–527
36. Wayman CP, McFadzean I, Gibson A et al. (1996) Two distinct membrane currents activated by cyclopiazonic acid-induced calcium store depletion in single smooth muscle cells of the mouse anococcygeus. *Br J Pharmacol* 117:566–572
37. Parekh AB, Foguet M, Lubbert H et al. (1993) Ca²⁺ oscillations and Ca²⁺ influx in *Xenopus* oocytes expressing a novel 5-hydroxytryptamine receptor. *J Physiol* 469:653–671
38. Chiono M, Mahey R, Tate G et al. (1995) Capacitative Ca²⁺ entry exclusively inhibits cAMP synthesis in C6-2B glioma cells. Evidence that physiologically evoked Ca²⁺ entry regulates Ca(2+)-inhibitable adenylyl cyclase in non-excitable cells. *J Biol Chem* 270:1149–1155
39. Cooper DM, Yoshimura M, Zhang Y et al. (1994) Capacitative Ca²⁺ entry regulates Ca(2+)-sensitive adenylyl cyclases. *Biochem J* 297 (Pt 3):437–440
40. McKenzie S, Marley PD (2002) Caffeine stimulates Ca(2+) entry through store-operated channels to activate tyrosine hydroxylase in bovine chromaffin cells. *Eur J Neurosci* 15:1485–1492
41. Crabtree GR, Clipstone NA (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu Rev Biochem* 63:1045–1083
42. Parekh AB, Penner R (1997) Store depletion and calcium influx. *Physiol Rev* 77:901–930
43. Ma R, Pluznick J, Kudlacek P et al. (2001) Protein kinase C activates store-operated Ca(2+) channels in human glomerular mesangial cells. *J Biol Chem* 276:25759–25765
44. Way KJ, Katai N, King GL (2001) Protein kinase C and the development of diabetic vascular complications. *Diabet Med* 18:945–959
45. Ma R, Kudlacek PE, Sansom SC (2002) Protein kinase Calpha participates in activation of store-operated Ca²⁺ channels in human glomerular mesangial cells. *Am J Physiol Cell Physiol* 283:C1390–C1398
46. Liu WS, Heckman CA (1998) The sevenfold way of PKC regulation. *Cell Signal* 10:529–542
47. Horowitz A, Clement-Chomienne O, Walsh MP et al. (1996) Epsilon-isoenzyme of protein kinase C induces a Ca(2+)-independent contraction in vascular smooth muscle. *Am J Physiol* 271:C589–C594
48. Walker LA, Gailly P, Jensen PE et al. (1998) The unimportance of being (protein kinase C) epsilon. *FASEB J* 12: 813–821
49. Takagi C, King GL, Clermont AC et al. (1995) Reversal of abnormal retinal hemodynamics in diabetic rats by acarbose, an alpha-glucosidase inhibitor. *Curr Eye Res* 14:741–749
50. Ishii H, Jirousek MR, Koya D et al. (1996) Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 272:728–731
51. Park JY, Takahara N, Gabriele A et al. (2000) Induction of endothelin-1 expression by glucose: an effect of protein kinase C activation. *Diabetes* 49:1239–1248
52. Weber LP, Seto M, Sasaki Y et al. (2000) The involvement of protein kinase C in myosin phosphorylation and force development in rat tail arterial smooth muscle. *Biochem J* 352 Pt 2:573–582