Journal of Neurochemistry, 2001, **77**, 1157–1167

Effects of insulin-like growth factor-1 on retinal endothelial cell glucose transport and proliferation

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Abstract

Insulin-like growth factor-1 (IGF-1) plays important roles in the developing and mature retina and in pathological states characterized by retinal neovascularization, such as diabetic retinopathy. The effects of IGF-1 on glucose transport and proliferation and the signal transduction pathways underlying these effects were studied in a primary bovine retinal endothelial cell (BREC) culture model. IGF-1 stimulated uptake of the glucose analog 2-deoxyglucose in a dose-dependent manner, with a maximal uptake at 25 ng/mL (3.3 nM) after 24 h. Increased transport occurred in the absence of an increase in total cellular GLUT1 transcript or protein. IGF-1 stimulated activity of both protein kinase C (PKC) and phosphatidylinositol-3 kinase (PI3 kinase), and both pathways were required for IGF-1-mediated BREC glucose transport and thymidine incorporation. Use of a selective inhibitor of the β isoform of PKC, LY379196, revealed that IGF-1 stimulation of glucose transport was mediated by PKC-β; however, inhibition of PKC-β had no effect on BREC proliferation. Taken together, these data suggest that the actions of IGF-1 in retinal endothelial cells couple proliferation with delivery of glucose, an essential metabolic substrate. The present studies extend our general understanding of the effects of IGF-1 on vital cellular activities within the retina in normal physiology and in pathological states such as diabetic retinopathy.

Keywords: diabetic retinopathy, glucose transport, GLUT1, inner blood–retinal barrier, insulin-like growth factor, retinal endothelial cells.

J. Neurochem. (2001) **77**, 1157–1167.

Insulin-like growth factor-1 (IGF-1), a polypeptide hormone that mediates the systemic actions of growth hormone (GH) (Jones and Clemmons 1995), is synthesized by a variety of cell types in the retina, including microvascular endothelial cells, pericytes, Müller cells, ganglion cells, and retinal pigment epithelium (RPE) (Lee *et al*. 1992). In normal retinal physiology, IGF-1 is thought to play a role in the development of the neuroretina in different species (Ocrant *et al*. 1989; Calvaruso *et al*. 1996), and the expression of IGF-1, IGF-1 binding proteins and the IGF-1 receptor in the adult mammalian retina (Waldbillig *et al*. 1988, 1991; Ocrant *et al*. 1989) suggests that IGF-1 participates in processes in the mature retina as well. These processes include interactions between the RPE and photoreceptor in the vision signal transduction cascade (Waldbillig *et al*. 1988, 1991), and analogous to its role in the brain, IGF-1 also appears to act as an anti-apoptotic factor in an experimental model of retinal injury (Kermer *et al*. 2000).

In pathological states characterized by retinal neovascularization, such as diabetic retinopathy (DR), accumulating evidence suggests that IGF-1, along with vascular endothelial growth factor (VEGF), serves as a key mediator in disease progression. Elevated vitreal and serum concentrations of IGF-1 have been documented in patients with proliferative diabetic retinopathy (PDR) (Merimee *et al*. 1983; Grant *et al*. 1986; Meyer-Schwickerath *et al*. 1993; Burgos *et al*. 2000) and are in the range known to stimulate

Received December 8, 2000; revised manuscript received February 23, 2001; accepted February 26, 2001.

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Abbreviations used: BRB, blood–retinal barrier; BREC, bovine retinal endothelial cells; 2DG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; DR, diabetic retinopathy; EGS, endothelial growth supplement; FCS, fetal calf serum; GH, growth hormone; IGF-1, insulin-like growth factor-1; KRP-BSA, Kreb's Ringers phosphate buffer containing 1% bovine serum albumin; 3MG, 3-*O*-methylglucose; PKC, protein kinase C; PI3 kinase, phosphatidylinositol-3 kinase; PDR, proliferative diabetic retinopathy; PMSF, phenylmethylsulfonyl fluoride; RPE, retinal pigment epithelium; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor.

endothelial cell chemotaxis and proliferation *in vitro* and *in vivo* (Grant *et al*. 1987, 1993a,b). In individuals with diabetes diagnosed after the age of 30, elevated serum concentrations of IGF-1 are correlated with an increased frequency of PDR (Dills *et al*. 1991). There has not, however, been universal agreement among clinical studies investigating the relationship of serum IGF-1 concentrations and PDR (Hyer *et al*. 1988; Arner *et al*. 1989; Lee *et al*. 1994). In contrast, pituitary ablation (Poulsen 1953) and growth hormone deficiency (Merimee 1978; Alzaid *et al*. 1994) have been associated with a lower risk of DR, and in experimental models of proliferative retinopathy, mice with decreased circulating GH and IGF-1 due to either the expression of a GH antagonist transgene or the administration of an inhibitor of GH fail to develop retinal neovascularization in response to retinal hypoxia (Smith *et al*. 1997).

IGF-1 is known to have direct mitogenic effects on endothelial cells, including increased endothelial proliferation (King *et al*. 1983; Grant *et al*. 1993b), chemotaxis (Grant *et al*. 1987), and angiogenesis (Grant *et al*. 1987, 1993b), both *in vitro* and *in vivo*. Although the signaling pathways underlying the mitogenic effects of IGF-1 have been described in a variety of cells of non-endothelial origin (Butler *et al*. 1998), there are significant differences in the signaling pathways in which IGF-1 acts in different cell types (Petley *et al*. 1999), and the precise molecular events through which IGF-1 stimulates endothelial cell proliferation have not been elucidated.

Similar to the endothelial cells comprising the blood–brain barrier, the endothelial cells of the retinal microvasculature are characterized by intercellular tight junctions (zonulae occludens) and a lack of fenestrations (Cunha-Vaz 1976). These characteristics of the so-called inner blood–retinal barrier (BRB) prevent the passive diffusion of polar compounds from the blood into the neuroretina (Cunha-Vaz 1976). Delivery of the essential substrate glucose across the inner BRB occurs via a specific facilitated transport process mediated by the sodium-independent glucose transporter GLUT1 (Harik *et al*. 1990; Takata *et al*. 1990; Mantych *et al*. 1993; Kumagai *et al*. 1994). Modulation of glucose transport by changes in inner BRB GLUT1 at this critical interface may therefore have direct effects on neuroretinal function, since the neuroretina, like all neural tissue, is absolutely dependent on a constant supply of glucose for normal metabolism (Cunha-Vaz 1976).

In primary bovine retinal endothelial cell (BREC) cultures, Takagi and coworkers have demonstrated that hypoxia increases endothelial cell glucose transport via up-regulation of GLUT1 mRNA and protein (Takagi *et al*. 1998). We have recently shown that VEGF, a potent retinal endothelial cell mitogen, stimulates glucose transport in BREC via activation of the β isoform of protein kinase C (Sone *et al*. 2000). Unlike the effects of hypoxia, however, this enhancement of glucose uptake is not due to increases in total cellular abundance of GLUT1; rather, it is due to VEGF-induced translocation of pre-existing cytosolic transporters to the endothelial plasma membrane (Sone *et al*. 2000).

The aim of the present study was two-fold: first, to investigate the effects of IGF-1 on retinal endothelial glucose transport and GLUT1 expression, and, second, to determine whether the effects of IGF-1 on retinal endothelial cell glucose transport and proliferation are mediated through similar signal transduction pathways. We report that IGF-1 enhances retinal endothelial cell glucose transport and proliferation via phosphatidylinositol-3 kinase (PI3 kinase) and PKC; however, IGF-1-mediated increases in glucose transport, and not endothelial cell proliferation, are mediated by the β isoform of PKC.

Materials and methods

Bovine retinal endothelial cell (BREC) cultures

BREC cultures were established from fresh calf eyes as described previously (Sone *et al*. 2000) and grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) with 10% fetal calf serum (FCS), endothelial growth supplement (EGS, 100 μg/mL, Boehringer Mannheim, Indianapolis, IN, USA), heparin (88 μg/mL), and antibiotic-antimycotic solution (Sigma, Saint Louis, MO, USA) on fibronectin-coated dishes under 5% CO_2 at 37°C. For all experiments, the BREC cultures were serum-deprived (0.5% FCS) for 16 h prior to the addition of growth factor, as described previously. (Sone *et al*. 2000)

For experiments involving PKC inhibitors, designated cultures were pretreated for 1 h at 37°C with either calphostin C (100 nM), GF109203X (5 μM, both from Sigma) or the β-selective inhibitor, LY379196 (kindly provided by Dr Kirk Ways, Eli Lilly & Co., Indianapolis, IN, USA). LY379196 has selective inhibitory effects on PKC-βI and βII isoforms with ED_{50} values of 0.05 and 0.03 μM, respectively (Sone *et al*. 2000). At 600 nM, LY379196 demonstrates generalized inhibition of all typical PKC isoforms (α, βI, βII and δ); at 30 nM, LY379196 has βI- and βII-selective inhibition (Sone *et al*. 2000). Therefore, concentrations of 600 nM and 30 nM were used for generalized and PKC-β-selective inhibition, respectively. The inhibitory profiles for LY379196 for typical and atypical PKC isoforms and related kinases have been published previously (Sone *et al*. 2000). For experiments involving inhibition of PI3 kinase, designated cultures were pretreated with either wortmannin (100 nM, Sigma) or LY294002 (Calbiochem, San Diego, CA, USA) before addition of growth factor. Because wortmannin is unstable in aqueous solutions (Yano *et al*. 1993), the media of the appropriate cultures were changed every 6 h to fresh media with IGF-1 with or without wortmannin during prolonged incubations. Media from control cultures were changed at the same intervals to fresh media without IGF-1 or inhibitor. Effects of the various inhibitors on cell viability were assessed by visual assessment and comparison of cell morphology between treated and untreated groups at the end of the treatment period and by a MTT-based cell

viability assay (Sigma) on cultures in the presence of maximum concentrations used for each inhibitor for a 24-h period. The cell viability assay was performed according to manufacturer's instructions.

Glucose transport studies

2-Deoxyglucose transport studies

2-Deoxyglucose transport studies were performed according to the methods of Tai *et al*. (1990) with minor modifications. Briefly, the media from the BREC cultures was aspirated and replaced with low-calcium Kreb's Ringers phosphate buffer containing 1% bovine serum albumin (KRP-BSA), and the cultures were incubated at 37°C for 30 min in order to deplete intracellular glucose. After two 5-min washes with KRP-BSA, the cultures were incubated with 0.25 μCi of the glucose analog 2-[1,2-^{3}H(N)]-deoxy-D-glucose ([^{3}H]2DG, specific activity 25 Ci/mmol, Amersham, Piscataway, NJ, USA) in 1 mL of KRP-BSA for 3 min at room temperature. Preliminary studies by our laboratory demonstrated that [^{3}H]2DG uptake was linear in BREC cultures between 2 and 10 min under these conditions (data not shown). To assess non-specific binding, parallel cultures were preincubated with KRP-BSA containing 40 μM cytochalasin-B (Sigma) for 5 min at room temperature, followed by incubation with the [^{3}H]2DG solution containing cytochalasin-B. The assay was terminated by rapid aspiration of the radioisotope solution, followed by repeated washes with ice-cold 0.2 mM phloretin (Sigma) in KRP without BSA. Cells were solubilized in 1% sodium dodecyl sulfate (SDS), 0.1 M NaOH at RT for 30 min. Protein was measured by a Pierce BCA assay (Pierce Chem. Co., Rockford, IL, USA). ^{3}H was measured by liquid scintillation counting on a Tri-Carb Scintillation Counter (Packard Instruments, Downers Grove, IL, USA). Results were expressed in pmol [^{3}H]2DG per mg protein per min. For each experiment, $n = 4–5$ cultures in each group.

3-O-Methylglucose (3MG) transport studies

3-*O*-Methylglucose (3MG) transport studies were performed on BREC cultures as described previously (Sone *et al*. 2000) and were expressed as the zero-trans initial uptake rate of [^{14}C]3MG in pmol per mg protein per s. For each experiment, $n = 5–6$ cultures in each group.

Thymidine incorporation studies

Endothelial cell proliferation was determined by the incorporation of [^{3}H]thymidine, by the method of Yano *et al*. (1999) with minor modifications. Briefly, BREC cultures were seeded in equal density and grown to 60% confluence in fibronectin-coated 35-cm^2 tissue culture dishes. Following overnight serum deprivation, the media was exchanged with fresh medium with or without growth factors and corresponding inhibitors and containing 0.66 μCi/mL [^{3}H]thymidine (Amersham). After 24 h, the medium was aspirated, and cultures were washed with cold PBS. The cells were incubated twice for 10 min in 5% trichloroacetic acid (TCA, Sigma), solubilized in 1 mL 0.1N NaOH, 1% SDS lysis buffer, and ^{3}H was measured by liquid scintillation counting.

Western blotting analysis

Western blotting analysis was performed for GLUT1 on 25-μg aliquots of BREC whole cell lysates as described previously (Sone *et al*. 2000). The anti-GLUT1 antiserum used for western blotting was a rabbit polyclonal antiserum directed against purified human erythrocyte glucose transporter (a kind gift of Dr Christin Carter-Su, University of Michigan) at a concentration of 1 : 10 000.

Northern blotting analysis

Northern blotting analysis was performed on 20 or 25 μg of total RNA extracted from semiconfluent control or IGF-1-treated BREC cultures. Northern blotting for GLUT1 using a 512-kb Pst1 fragment of bovine blood–brain barrier glucose transporter cDNA (provided by Dr Rubén J. Boado, UCLA School of Medicine) was performed as described previously (Sone *et al*. 2000). GLUT1 northern blots were normalized by repeat hybridization with a ^{32}P-labeled mouse actin cDNA, as described previously (Sone *et al*. 2000). Quantification of autoradiograms was performed using NIH Image software and was normalized against the signal for actin.

Assay of PKC activity

Assay of PKC activity was performed in permeabilized cells according to the methods of Heasley and Johnson with modifications (Heasley and Johnson 1989). Briefly, subconfluent BREC cultures were grown in fibronectin-coated 6-well culture dishes, and after serum deprivation, cultures were incubated with and without IGF-1 in the presence and absence of inhibitors for 15 min at 37°C. After incubation, the cells were washed once with DMEM containing 20 mM HEPES (pH 7.4) and incubated in a salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mg/mL glucose, 20 mM HEPES, pH 7.2) containing 25 μg/mL of purified digitonin (Calbiochem, La Jolla, CA, USA), 100 μM ATP, 14 μCi/mL of [γ-^{32}P]ATP (approximately 400 cpm/pmol or 1.8×10^5 μCi/pmol, Dupont-NEN, Boston, MA, USA) and 300 μM of the specific PKC substrate KRTLRR (Bachem, Torrance, CA, USA) for 15 min at 30°C. In previous experiments, PKC activity was linear between 10 and 30 min in BREC cultures under these conditions (data not shown). At the end of the incubation, 40-μL aliquots of the reaction mixture were spotted on Whatman P-81 phosphocellulose discs (Fischer Scientific, Pittsburg, PA, USA) and air-dried for at least 10 min. The discs were washed three times in 75 mM phosphoric acid, followed by one wash in sodium phosphate (pH 7.5). Radioactivity on the filters was measured by scintillation counting, and the activity was normalized for protein concentrations measured in parallel cultures under each experimental condition.

Phosphatidylinositol-3-kinase (PI3 kinase) activity assay

PI3 kinase activity assay was performed on semiconfluent BREC cultures after overnight serum deprivation and treatment with or without IGF-1 (25 ng/mL) at 37°C for 10 min in the presence or absence of the PI3 kinase inhibitor wortmannin, according to the methods of Tsakiridis *et al*. (1995).

Immunoprecipitation of IRS-1

After treatment, the cells were immediately washed twice with ice-cold Buffer A (20 mM Tris, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM sodium orthovanadate, pH 7.5) and lysed in 0.500 mL of Lysis Buffer [Buffer A plus 1% Nonidet NP-40, 10% glycerol, 2 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin and 2 μM E-64 (Sigma)] on ice for 30 min with frequent gentle mixing. The lysates were collected, the proteins measured, and immunoprecipitation for IRS-1 was performed on 500-μg aliquots of lysates with 2 μg of

anti-IRS-1 antibody (Upstate Biotechnologies, Inc. Lake Placid, NY, USA) overnight at 4°C with mixing. Following incubation, 60 μL of a 25% Protein A agarose slurry was added, followed by mixing for 1 h at 4°C. The immunoprecipitates were collected by centrifugation, washed twice each with Wash Buffer I (20 mM Tris, 1% NP-40, 1 mM sodium orthovanadate and protease inhibitors), Buffer II (100 mM Tris, 500 mM lithium chloride, 1 mM sodium orthovanadate and protease inhibitors) and Buffer III (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate and protease inhibitors), and were pelleted by centrifugation.

PI3 kinase assay

The kinase reaction was performed on the immunoprecipitates by the addition of 26 μL of a solution containing final concentrations of 13.8 mM $MgCl_2$, 0.156 mM ATP, 15 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Dupont-NEN) and 40 μg of L-α-phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL, USA) resuspended by sonication in 10 μL of 10 mM Tris and 1 mM EGTA. The reaction was terminated by the addition of 20 μL of 1 M HCl, and lipids were extracted with 160 μL of chloroform : methanol (1 : 1). Aliquots of 75 μL of the aqueous phase were loaded on an oxaloacetate-coated HPTLC silica glass plate (EM Science, Cincinnati, OH, USA), and a 50-μg of phosphatidylinositol 3-phosphate (PI3P) standard (Matreya Inc., Pleasant Gap, PA, USA) suspended in $CHCl_3$ was loaded for comparison. Lipids were separated by chromatography in chloroform : methanol : H_2O : NH_4OH at a 60 : 47 : 11.6 : 2 ratio, after which the plate was dried and wrapped in plastic wrap. Autoradiography was performed at room temperature. The PI3P standard was visualized by ultraviolet light after treating the TLC plate with primulin-acetone, and quantification of the labeled reaction products was performed by scintillation counting of bands excised from the TLC plates.

Protein measurements

Protein concentrations in all experiments were determined by a bicinchoninic acid (BCA) assay (Pierce). For western blot samples, β-mercaptoethanol was added after protein measurement (final concentration of 5%).

Statistical analysis

All results are expressed as mean ± SEM. Comparisons between control and IGF-1 treated cultures for transport assays, western blot analyses, and northern blot analyses were performed by analysis of variance (ANOVA) or Student's *t*-test, as appropriate. All transport and thymidine incorporation assays and PKC and PI3 kinase activity assays were performed a minimum of 2–4 times, and western and northern blot analyses were repeated a minimum of three times on separate cell cultures. $p < 0.05$ was considered statistically significant for all experiments.

Results

Exposure of BREC cell cultures to increasing concentrations of recombinant IGF-1 for 24 h resulted in an increase in 2DG uptake in a dose-dependent manner (Fig. 1a), with a maximal effect at 25 ng/mL (3.3 nM) at 24 h. Time course studies of increasing duration using 50 ng/mL IGF-1 revealed an increase in 2DG uptake at 12 h of exposure, with a maximum uptake at 24 h (data not shown). Because 2-deoxyglucose is both transported and phosphorylated within the cell (Sokoloff *et al*. 1977), the increase in 2DG accumulation measured in the assay could be the result of an increase in hexokinase activity by IGF-1 rather than an IGF-1-stimulated increase in transport *per se*. To examine this possibility, the initial uptake rate of [^{14}C]labeled 3-O-methylglucose (3MG), a glucose analog that is transported into, but not metabolized within, cells was measured. The initial uptake rate of [^{14}C]3MG approximately doubled in response to 25 ng/mL of IGF-1 after 24 h (0.76 ± 0.14 pmol/mg/s in IGF-1-treated cultures versus 0.38 ± 0.09 pmol/mg/s in controls, $p = 0.02$, Fig. 1b). This increase in 3MG uptake was comparable to the increase in 2DG uptake seen in identically treated cultures and confirmed that the increase in 2DG uptake after treatment with IGF-1 reflected an increase in glucose transport in BREC in response to IGF-1.

Exposure to 25 ng/mL caused a modest (25–35%) but significant increase in specific PKC activity in the BREC cultures (Fig. 2a). PKC activation was inhibited by treatment with the general PKC inhibitor GF109203X (5 μM,

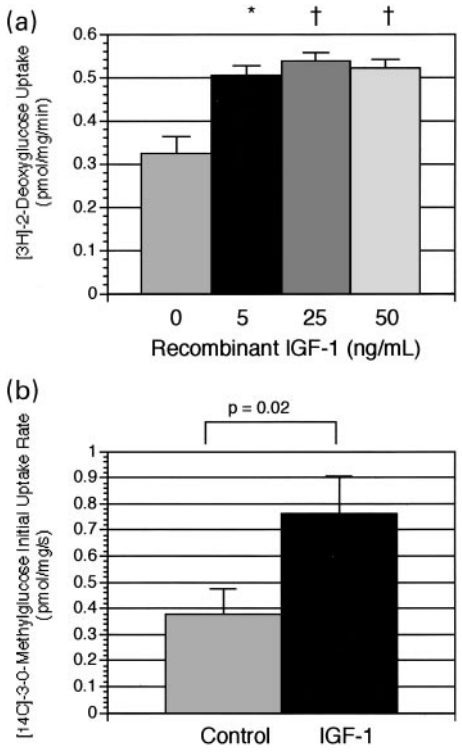

Fig. 1 Effects of IGF-1 on BREC glucose transport. (a) Representative [^{3}H]2-deoxyglucose uptake assay of treatment of BREC cultures with increasing concentrations of recombinant IGF-1. $n = 4–5$ in each group, $^*p < 0.002$ and $\dagger p < 0.0001$ compared with control (0). (b) Representative graph of initial [^{14}C]3-*O*-methylglucose uptake rates of BREC cultures without and with treatment with IGF-1 (25 ng/mL, 24 h.). $n = 6$ in each group, $p = 0.02$ compared with control.

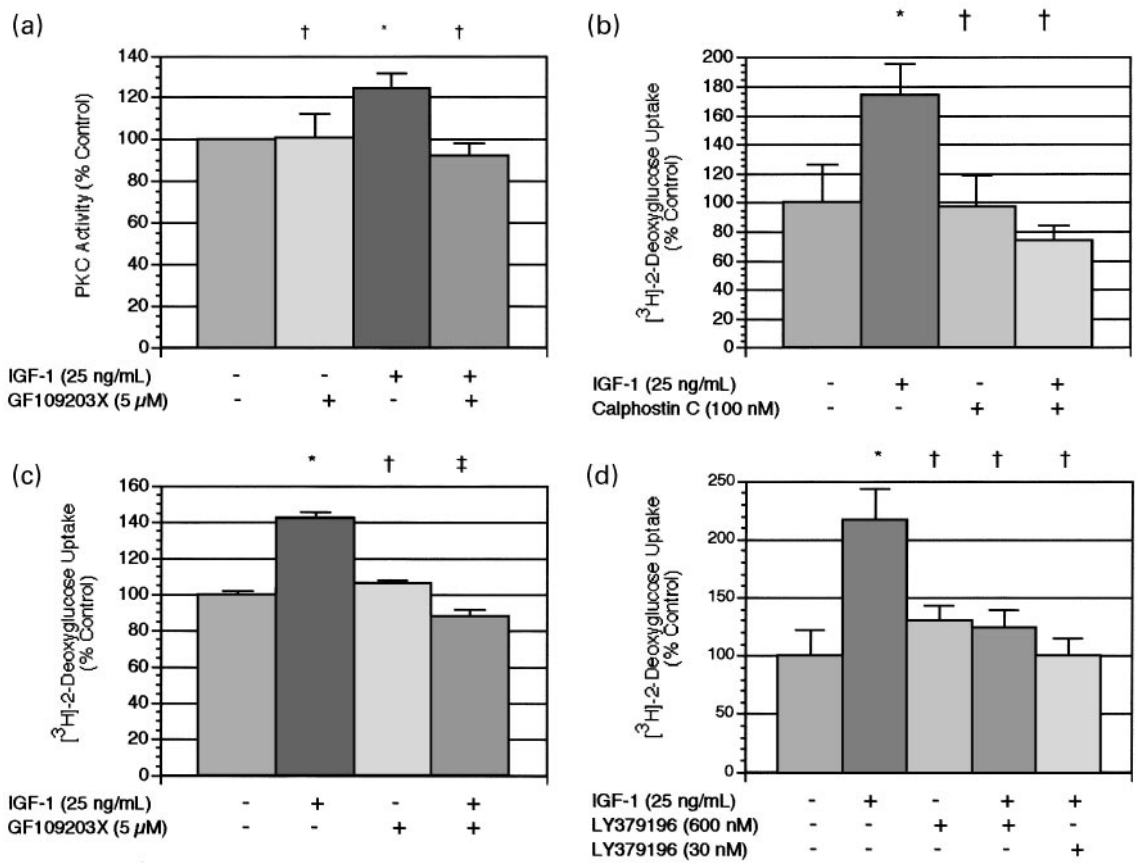

Fig. 2 Effects of IGF-1 on BREC PKC activity. (a) PKC activity assay on permeabilized cells. Semi-confluent BREC cultures were incubated with and without IGF-1 (25 ng/mL) for 15 min in the presence and absence of the general PKC inhibitor GF 109203X (5 μM) and phosphorylation of a PKC-specific substrate was assayed as described in Materials and methods. The results are expressed as a percentage of control PKC activity and represent the mean and SE of four independent experiments with duplicate samples in each experiment. $^*p < 0.02$ compared with control; †p not significant compared with control. (b)–(d) Effects of PKC inhibition on IGF-1-mediated BREC glucose transport. Representative 2-deoxyglucose uptake assays of control (without IGF-1) or IGF-1-treated (25 ng/mL, 24 h) BREC cultures in the presence or absence of general (calphostin C, GF 109203X) or β-selective (LY379196) PKC inhibitors. (b) Calphostin C. $^*p < 0.05$ compared with control; p not significant compared with control. (c) GF 109203X. $^*p < 0.01$ compared with control; †p not significant compared with control: †$p < 0.02$ compared with control. (d) LY379196, at generalized (600 nM) or β-selective (30 nM) PKC inhibitory concentrations. $^*p < 0.01$ compared with control; †p not significant compared with control.

Fig. 2a). Treatment of the cultures with the inhibitor in the absence of IGF-1 had no effect (Fig. 2a). In order to determine whether IGF-1 enhancement of BREC glucose transport was mediated by PKC, a series of experiments employing selective PKC inhibition was performed. Treatment of BREC cultures with the general PKC inhibitors calphostin C (100 nM) and GF 109203X (5 μM) completely abrogated the increase in 2DG uptake in response to IGF-1 (Figs 2b–c). Treatment of control cultures with either inhibitor alone had no effect on 2DG uptake (Figs 2b–c).

The β isoform of PKC has been proposed to be a common effector pathway in the development of diabetic complications, including retinopathy (Ishii *et al*. 1996; King *et al*. 1997). In order to determine whether IGF-1-stimulated BREC glucose transport was mediated by PKC-β, BREC cultures were incubated with IGF-1 in the presence and absence of LY379196 at general PKC and PKC-β-selective concentrations (600 nM and 30 nM, respectively). As is seen in Fig. 2(d), general and PKC-β-selective inhibition completely abolished IGF-1-stimulated increases in 2DG uptake. Treatment of the BREC cultures with the inhibitor alone at either concentration had no effect (data for 30 nM not shown).

In a variety of cell types, the mitogenic effects of IGF-1 are mediated by PI3 kinase (Jones and Clemmons 1995). Exposure of BREC cultures to IGF-1 (25 ng/mL) resulted in a three-fold increase in PI3 kinase activity (Fig. 3a), as evidenced by the increase in ^{32}P-labeled phosphatidylinositol-3-phosphate (PI3P) on thin layer chromatography (Fig. 3a). This increased activity, as well as the basal

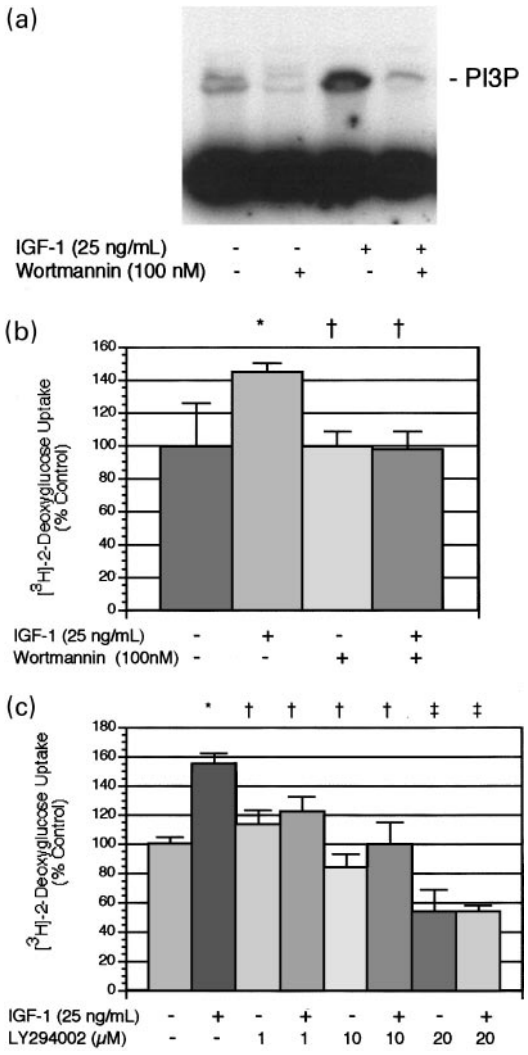

Fig. 3 Effects PI3 kinase on IGF-1-mediated BREC glucose transport. (a) Representative PI3 kinase assay of BREC with and without stimulation by IGF-1 (25 ng/mL) for 10 min in the presence and absence of the PI3 kinase inhibitor wortmannin (100 nM) as described in Materials and methods. The migration of the PI3P standard is noted on the right. (b) and (c) Representative 2-deoxyglucose uptake assays of control (without IGF-1) or IGF-1-treated (25 ng/mL, 24 h) BREC cultures in the presence or absence of the selective PI3 kinase inhibitors wortmannin (b) and LY294002 (c). $^*p = 0.05$, †p not significant compared with control. LY294002 at increasing concentrations (0–20 μM). $^*p = 0.003$ compared with control; †p not significant compared with control; ‡$p < 0.002$ compared with control.

activity, was suppressed by treatment with the PI3 kinase inhibitor wortmannin (100 nM, Fig. 3a). Treatment of cultures with wortmannin (100 nM) completely abolished IGF-1-stimulated glucose transport (Fig. 3b). Furthermore, treatment with increasing concentrations of another, structurally unrelated PI3 kinase inhibitor, LY294002, resulted in a dose-dependent decrease in 2DG uptake (Fig. 3b). Treatment of the cultures with increasing concentrations of LY294002 alone also significantly depressed 2DG uptake (Fig. 3b), an observation that supported the results of the PI3 kinase activity assay (Fig. 3a), which suggested that basal, as well as IGF-1-stimulated, glucose transport was mediated by PI3 kinase.

To determine whether the effects of IGF-1 on stimulating BREC glucose transport were due to an increase in the abundance of the sodium-independent glucose transporter, GLUT1, northern and western blotting analyses for GLUT1 were performed on IGF-1-treated BREC cultures. A 24-h exposure of BREC cultures to IGF-1 at concentrations found to stimulate glucose transport (25 ng/mL) had no effect on the abundance of total cellular GLUT1 protein (Fig. 4a). In addition, quantification of northern blots of total RNA from cultures with or without with IGF-1 for durations of up to 24 h showed no effect of IGF-1 on the abundance of BREC GLUT1 mRNA (Fig. 4b).

As has been previously shown by other investigators (King *et al*. 1983; Grant *et al*. 1993b), IGF-1 increased BREC proliferation, as evidenced by an increase in [^{3}H]thymidine incorporation, in a dose–response manner (Fig. 5a). Inhibition of PKC by treatment of the BREC cultures with GF109203X inhibited IGF-1 stimulated proliferation in a dose–response manner (Fig. 5b). Furthermore, treatment with GF 109203X alone significantly suppressed thymidine incorporation in the absence of IGF-1 treatment, which suggested that PKC mediates both basal and IGF-1-stimulated BREC proliferation. Interestingly, although the β-selective PKC inhibitor LY379196 suppressed thymidine incorporation at concentrations which are known to inhibit all typical PKC isoforms (600 nM, Fig. 5c), treatment of the BREC cultures with IGF-1 in the presence of LY379196 at β-selective concentrations (30 nM) had no effect (Fig. 5c versus Fig. 2c).

With regards to the role of PI3 kinase in mediating BREC proliferation, treatment of the BREC cultures with IGF-1 in the presence of the PI3 kinase inhibitor wortmannin (100 nM) significantly suppressed IGF-1-stimulated increases in thymidine incorporation (Fig. 6a); wortmannin alone had no effect (Fig. 6a). Similarly, exposure of the cultures to IGF-1 in the presence of increasing concentrations of the PI3 kinase inhibitor LY294002 (0.5–20.0 μM) resulted in suppression of both IGF-1-stimulated and basal thymidine incorporation in a dose–response manner (Fig. 6b).

As described above, exposure to IGF-1 stimulated both PKC and PI3 kinase in primary BREC cultures (Figs 2a and 3a). In order to clarify the relationship between these two signaling pathways, PKC and PI3 kinase activity assays were performed in the presence and absence of PI3 kinase and PKC inhibitors, respectively. As seen in Fig. 7(a), pretreatment of BREC with the PI3 kinase inhibitor, wortmannin (100 nM), abolished the ability of IGF-1 to stimulate PKC activity. In contrast, prior exposure of BREC to the

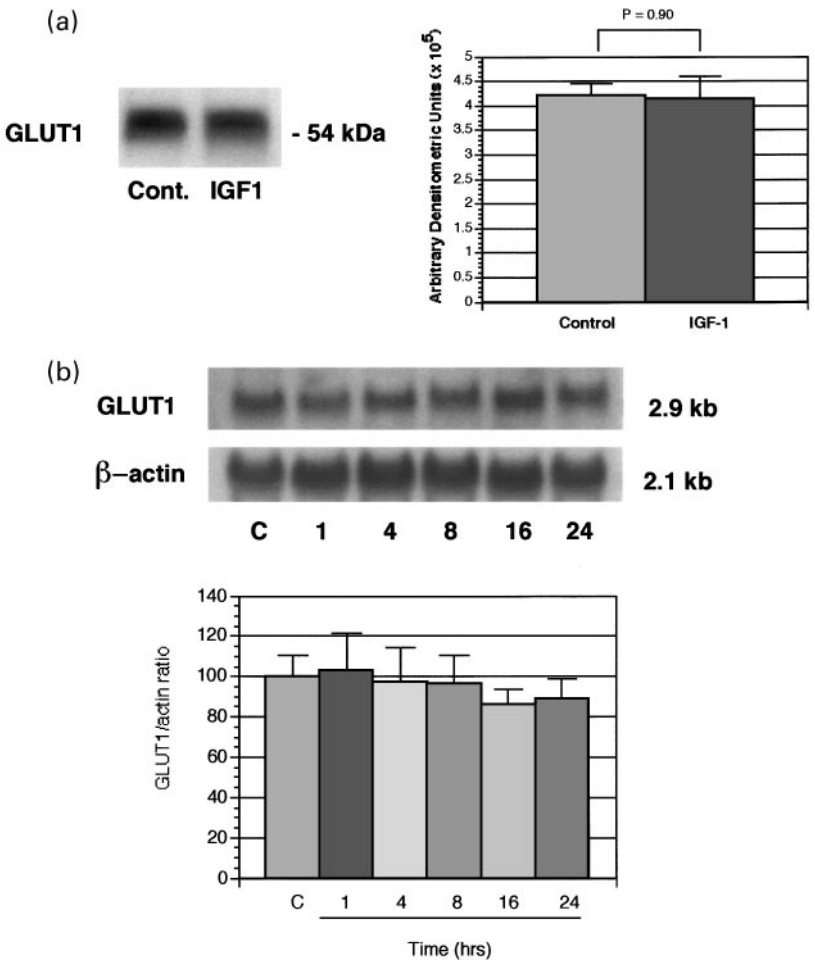

Fig. 4 Effects of IGF-1 on GLUT1 expression in BREC cultures. (a, left) Representative western blot of control cultures (Cont.) and cultures treated with 25 ng/mL IGF-1 for 24 h (IGF1). Western blotting analysis was performed on 25-μg aliquots of solubilized whole cell BREC lysates. Blots were reacted with a polyclonal antiserum raised against purified human erythrocyte glucose transporter and developed using a horseradish peroxidase-conjugated secondary antibody and a chemiluminescence system. (a, right) Representative quantification of western blots for control and IGF-1-treated BREC cultures. $n = 3–6$ in each western blot. Western blotting was performed three times on different cell cultures with comparable results. (b) Representative northern blot of control cultures (C) and cultures treated with IGF-1 (25 ng/mL) for the indicated time periods (in hours). Each GLUT1 northern blot was normalized for actin, and quantification was performed on three northern blots of identically treated cultures.

selective PKC inhibitor, GF 109203X (5 μM), had no effect on PI3 kinase activity (Fig. 7b). These results suggested that PI3 kinase is upstream from PKC in signaling by IGF-1. Wortmannin or GF109203X alone had no effect on PKC or PI3 kinase activity, respectively (data for wortmannin not shown).

There were no morphological differences or differences in cell viability, as assessed by an MTT-based viability assay, between BREC cultures with and without treatment with the maximal concentrations of the inhibitors of PKC (GF109203X and LY379196) or of PI3 kinase (LY294002 and wortmannin) for up to 24 h.

Discussion

Binding of IGF-1 to the IGF-1 receptor (type 1 IGFR), a tyrosine kinase receptor structurally similar to that for insulin, results in receptor dimerization, autophosphorylation and activation of several downstream signal transduction pathways (LeRoith *et al*. 1995). In endothelial cells, IGF-1 may be transported intact across the endothelia and released into the interstitium; however, unlike insulin, a substantial proportion of IGF-1 that is internalized is retained within the endothelial cells, where it may exert biological effects (Bar *et al*. 1988).

In the present study, we demonstrate that IGF-1 increases glucose transport into retinal endothelial cells in culture (Figs 1a and b). This enhancement is similar to that recently reported for VEGF, another growth factor involved in retinal development and in pathological states, such as diabetic retinopathy (Sone *et al*. 2000). Stimulation of glucose transport by IGF-1 is not a non-specific cellular response to growth factors *per se*, since insulin, which is a major mitogenic factor in the developing central nervous system (Knusel *et al*. 1990) and which has a high degree of amino acid homology to IGF-1 (Jones and Clemmons 1995), does not stimulate glucose transport in endothelial cells (Betz *et al*. 1983; Pekala *et al*. 1990). Furthermore, retinal endothelial cell glucose transport is mediated via GLUT1 (Harik *et al*. 1990; Takata *et al*. 1990; Mantych *et al*. 1993; Kumagai *et al*. 1994), which is known to be insensitive to insulin (Mueckler 1994).

IGF-1-stimulated glucose transport into retinal microvascular endothelial cells is mediated by PKC, and in

particular, PKC-β. The requirement of PKC in mediating the effects of IGF-1 on glucose transport into the retinal endothelial cells is demonstrated by the complete inhibition of transport after treatment with a variety of general and PKC-β-selective inhibitors (Figs 2b–d). These effects on glucose transport are similar to those of VEGF, which also stimulates BREC glucose transport via stimulation of the β isoform of PKC (Sone *et al*. 2000). PI3 kinase is also necessary for mediating the effects of IGF-1 on BREC glucose transport, because treatment with two structurally unrelated inhibitors of PI3 kinase, wortmannin and LY294002, abolishes the effects of IGF-1 on 2DG uptake (Figs 3b and c). PI3 kinase and PKC activation by IGF-1 in BREC occur in a sequential, rather than parallel manner, since inhibition of either PI3 kinase or PKC alone results in complete abrogation of IGF-1 stimulated enhancement of BREC glucose transport. As seen in Fig. 7, inhibition of PI3 kinase by wortmannin abolishes the ability of IGF-1 to stimulate PKC activity and, in contrast, inhibition of PKC by GF 109203X has no effect on IGF-1 stimulation of PI3 kinase (Figs 7a and b). Together, these data suggest that PKC is downstream from PI3 kinase in the signaling pathways regulating IGF-1 actions in retinal endothelial cells. In this regard, both insulin and IGF-1 stimulate activity of PI3 kinase and PKC in a sequential manner in rat adipocytes; however, in these cells, PI3 kinase stimulates phosphorylation of another PKC isoform, PKC-ζ (Valverde *et al*. 1996; Standaert *et al*. 1997). IGF-1 is also known to activate other signaling pathways, such as mitogen-activated protein (MAP) kinase, in a variety of cell types (Petley *et al*. 1999); however, it is not known whether other signaling pathways participate in the effects of IGF-1 on glucose transport and proliferation in retinal endothelial cells.

IGF-1 enhancement of BREC glucose transport does not occur via an increase in total cellular GLUT1 glucose transporter expression. At concentrations shown to increase glucose transport (25 ng/mL, Fig. 1a), treatment with IGF-1 for 24 h had no effect on total cellular GLUT1 protein (Fig. 4a), and exposure to IGF-1 for various time periods up to 24 h failed to result in an increase the abundance of GLUT1 mRNA in BREC cultures (Fig. 4b). The increase in glucose transport in the absence of increased total cellular GLUT1 abundance is similar to the effects of IGF-1 on mesangial cell glucose transport (Heilig *et al*. 1997) and is also reminiscent of the effects of VEGF on primary BREC cultures (Sone *et al*. 2000). Exposure to VEGF (50 ng/mL up to 24 h) failed to increase total cellular GLUT1 protein

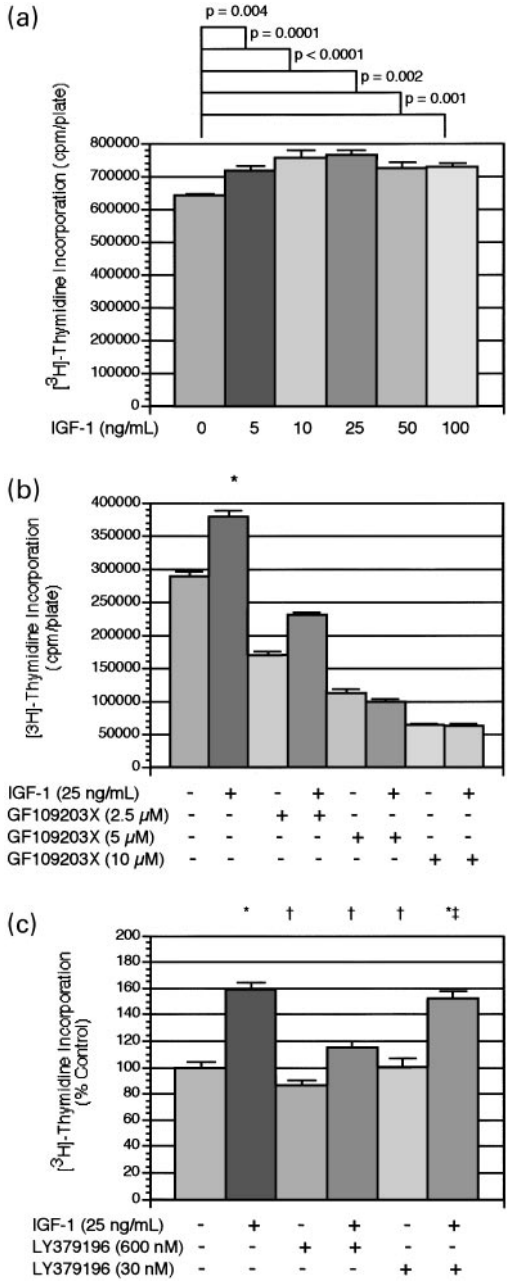

Fig. 5 Thymidine incorporation in BREC cultures. (a) Effects of IGF-1. Representative thymidine incorporation assay in control cultures (0) and in cultures exposed to increasing concentrations of recombinant IGF-1. BREC were plated in identical densities, and after achieving approximately 60% confluence, the cultures were serum deprived overnight and subsequently treated with the appropriate concentration of IGF-1. Incorporation of [^{3}H]thymidine was measured as described in Materials and methods. (b) Effects of generalized PKC inhibition on IGF-1-stimulated thymidine incorporation. BREC cultures treated without (–) or with 25 ng/mL IGF-1 for 24 h were exposed to increasing concentrations of the PKC-specific inhibitor, GF 109203X. *$p < 0.0001$ compared with control. (c) Effects of selective PKC-β inhibition on IGF-1-stimulated thymidine Incorporation. Control (–) and IGF-1-treated (25 ng/mL, 24 h) cultures were exposed to LY379196 at general PKC (600 nM) or PKC β-selective (30 nM) inhibitory concentrations. *$p < 0.0001$ compared with control; †p = not significantly different from control; ‡p = not significantly different compared with IGF-1 alone

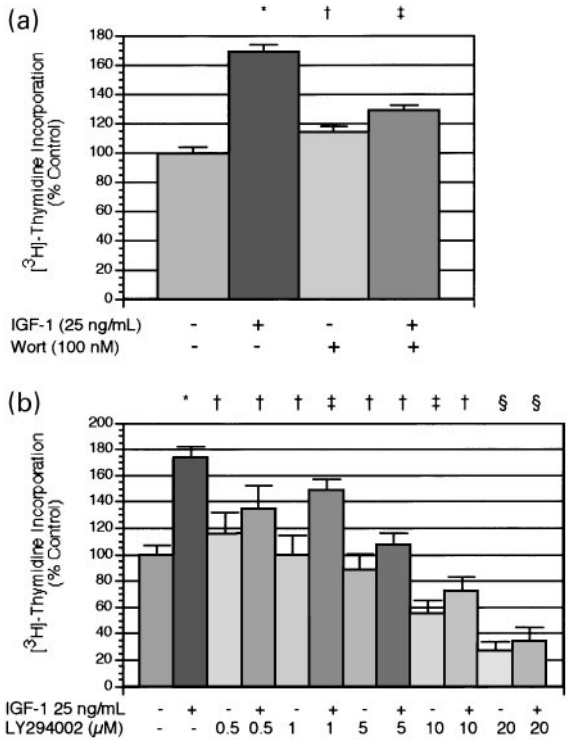

Fig. 6 Effects of inhibition of PI3 kinase on IGF-1-mediated BREC thymidine incorporation. (a) Treatment of control (–) and IGF-1 (+) cultures with the PI3 kinase inhibitor, wortmannin (100 nM). *$p < 0.0001$ compared with control; p not significant compared with control; †$p < 0.01$ compared with control. (b) Treatment of control (–) and IGF-1 (+) cultures with the PI3 kinase inhibitor, LY294002 at increasing concentrations. *$p < 0.0001$ compared with control; †p not significant compared with control; ‡$p < 0.02$ compared with control; §$p < 0.001$ compared with control.

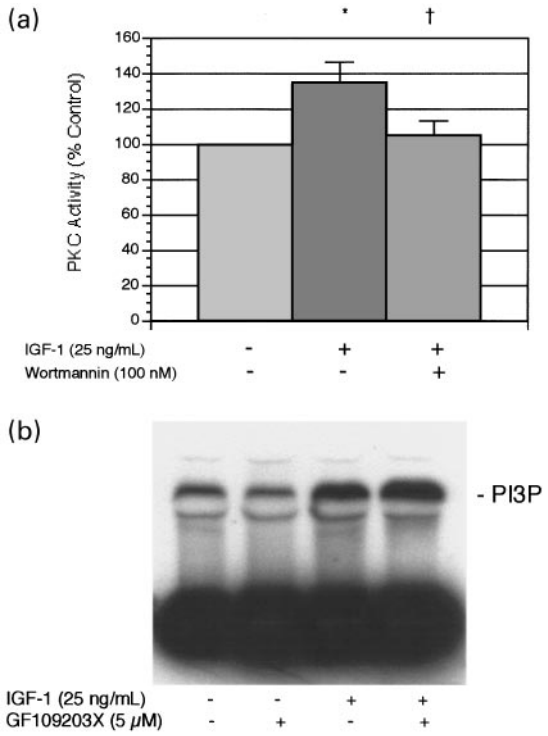

Fig. 7 Effects of IGF-1 on BREC PKC and PI3 kinase activity: effects of cross-inhibition. (a) PKC activity assay on permeabilized control and IGF-1-treated BREC cultures in the presence and absence of the PI3 kinase inhibitor, wortmannin (100 nM). PKC assay was performed as described in Materials and methods. Graph represents the mean and SE of three different experiments, each performed in duplicate. *$p = 0.017$, p not significant compared with control. (b) Representative PI3 kinase assay on control and IGF-1 treated BREC cultures in the presence and absence of the PKC inhibitor, GF 109203X (5 μM). The migration of the PI3P standard is noted on the right. The PI3 kinase assay was performed as described in Materials and methods and was repeated twice on different BREC cultures with identical results.

and transcript, despite an increase in glucose transport of approximately 70% under identical conditions (Sone *et al*. 2000). In the case of VEGF, quantitative western blotting of plasma membrane fractions from control and VEGF-treated BREC cultures revealed a comparable increase in plasma membrane GLUT1 (Sone *et al*. 2000). These observations suggest that VEGF stimulates BREC glucose transport by causing translocation of pre-existing cytosolic transporters to the plasma membrane, where the transporters are available for mediating glucose uptake (Sone *et al*. 2000). Although not directly investigated in the present study, one may speculate that the effects of IGF-1 on increasing glucose transport in BREC cultures involve a process of translocation similar to that of VEGF. This speculation is strengthened by the observation that both VEGF and IGF-1 increase BREC glucose transport to similar magnitudes and do so via activation of PKC-β (Sone *et al*. 2000 and this study). We cannot, however, rule out the possibility that IGF-1 increases the affinity of GLUT1 for its substrate.

Similar to its effects on glucose transport, the mitogenic effects of IGF-1 on retinal endothelial cells are mediated by PKC kinase and PI3 kinase. As in the case of glucose transport, both PKC and PI3 kinase appear to be required for IGF-1-stimulated BREC proliferation, since use of inhibitors for either PKC or PI3 kinase result in abrogation of IGF-1 effects on thymidine incorporation (Figs 5 and 6). Nonetheless, the pathways mediating glucose transport and proliferation in retinal endothelial cells are not identical. While inhibition of the β isoform of PKC abolishes IGF-1-stimulated glucose transport, it has no effect on IGF-1-stimulated thymidine incorporation (compare Figs 2d and 5c). Taken together, these data suggest that while PI3 kinase mediates the effects of IGF-1 on both glucose transport and proliferation in retinal endothelial cells, the effects of IGF-1 on these critical cellular processes are mediated by different isoforms of PKC. Among typical, calcium-dependent PKC

isoforms, α and βII, have been identified in BREC cell cultures, whereas βI and δ have not been detected (Shiba *et al*. 1993). Presumably, the α isoform mediates IGF-1-stimulated BREC proliferation, since generalized inhibition of calcium-dependent isoforms of PKC with LY379196 at 600 nM, but not selective inhibition of the β isoform of PKC at 30 nM, abolishes the effects of IGF-1 on BREC thymidine incorporation (Fig. 5c). The lack of effect of the PKC β-selective inhibitor on IGF-1-stimulated thymidine incorporation in BREC suggests an important difference between the actions of IGF-1 and those of VEGF in stimulating endothelial cell proliferation: while both involve PI3 kinase (Yu and Sato 1999, and this study), the mitogenic actions of VEGF are mediated by PKC-β, while those of IGF-1 are mediated through a non-β PKC isoform (Fig. 5c).

In summary, the present study demonstrates that IGF-1 enhances two essential processes within retinal endothelial cells, i.e. glucose transport and DNA synthesis, via pathways involving PKC and PI3 kinase; however, IGF-1 does so through different PKC isoforms. In normal physiology, the modulation of both endothelial cell proliferation and glucose transport by IGF-1 ensures delivery of an essential metabolic substrate to the developing neuroretina. In addition, we propose that by increasing glucose flux into the retinal endothelial cell, the elevated intravitreal IGF-1 concentrations seen in diabetic retinopathy may increase intracellular glucose and exacerbate its toxic effects on the retinal microvasculature (Kumagai 1999).

Acknowledgements

The authors wish to thank Dr Christin Carter-Su for her kind gifts of anti-GLUT1 antiserum and [^{14}C]3-*O*-methylglucose, and Dr Rubén J. Boado for the bovine BBB GLUT1 cDNA. The PKC-β inhibitor LY379196 was generously supplied by Dr Kirk Ways and James Gillig of Eli Lilly Pharmaceuticals. The authors are indebted to Drs Akira Abe, Romel Somwar and Amira Klip for advice on the PKC and PI3 kinase assays and to Drs C. Carter-Su, F.C. Brosius and E. Feldman for many helpful discussions. This work was supported by National Institutes of Health grant K08 EY000369 (AKK) and the Juvenile Diabetes Research Foundation (AKK). BJD was supported by an Honors Project stipend from the University of Michigan. AKK is supported by the JDRF Center for Complications in Diabetes and is supported in part by National Institutes of Health Grant RPO60DK-20572, which supports the Michigan Diabetes Research and Training Center.

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