

# Diabetes Alters Osmotic Swelling Characteristics and Membrane Conductance of Glial Cells in Rat Retina

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The development of edema in the diabetic retina may be caused by vascular leakage and glial cell swelling. To determine whether diabetic retinopathy alters the swelling characteristics of retinal glial cells and changes the properties of the glial membrane K<sup>+</sup> conductance, isolated retinas and glial cells of rats were investigated at 4 and 6 months of chemical diabetes. After 6 months of hyperglycemia, application of a hypotonic solution to retinal slices induced swelling of glial cell bodies, a response not observed in control retinas. The osmotic glial cell swelling was blocked by inhibitors of phospholipase A<sub>2</sub> or cyclooxygenase and by a thiol-reducing agent. Glial cells from diabetic retinas displayed a decrease of K<sup>+</sup> currents that was associated with an altered subcellular distribution of the K<sup>+</sup> conductance and a loss of perivascular Kir4.1 protein. The observation that swelling of cells in control retinas was inducible with K<sup>+</sup> channel-blocking Ba<sup>2+</sup> ions suggests a relationship between decreased K<sup>+</sup> inward currents and osmotic cell swelling in diabetic retinas. The data show that glial cells in diabetic retinas are more sensitive to osmotic stress, which is associated with a decrease of K<sup>+</sup> currents, than cells in control retinas. It is suggested that these alterations may be implicated in the development of diabetic retinal edema. *Diabetes* 55:633–639, 2006

**D**iabetic retinopathy is a leading cause of reduced visual acuity and acquired blindness (1). The presence of a macular edema is the main cause of visual impairment in diabetic patients (2). The development of macular edema is thought to be primarily caused by a breakdown of the blood-retinal barrier, resulting in retinal vascular leakage (3,4). However, it has been shown that clinically significant diabetic macular edema occurs only when, in addition to vascular leakage, the active transport mechanisms of the blood-retinal barriers are dysfunctional (5). This observation suggests that a disturbance of the fluid reabsorption from retinal tissue, normally carried out by pigment epithelial

and glial cells (6), is a necessary step in vessel dysfunction and edema formation. In the ischemic brain, the formation of edema is primarily caused by cytotoxic mechanisms, i.e., by swelling of glial cells, and a swelling of glial cells occurs concomitantly in vasogenic edema (7). With regard to the retina, there are good arguments that a swelling of retinal glial (Müller) cells contributes to the development of edema, particularly in cases without significant angiographic vascular leakage (8). A swelling of glial cells in the macula has been suggested to precede the formation of extracellular edema (9). In an animal model of retinal hypoxia, it has been shown that vascular leakage is associated with cellular edema of Müller cells (10). However, the relative contribution of vascular and cytotoxic mechanisms (i.e., vascular permeability and glial cell swelling) to the development of diabetic retinal edema remains to be determined.

Retinal edema develops under ischemic-hypoxic and/or inflammatory conditions (2,11). We have recently shown with rat models of retinal ischemia reperfusion and uveoretinitis that the osmotic swelling characteristics of Müller glial cells are significantly altered under both conditions: acute application of a hypotonic bath solution (a situation that resembles hypoxia-induced cytotoxic edema in the brain) induced swelling of glial cells in slices of postischemic and inflamed retinas but had no effect on the volume of cells in control retinas (12,13). Osmotic glial cell swelling has been linked to the decrease of the main K<sup>+</sup> conductance of the cells (i.e., currents through Kir4.1 channels) (12,14) and to endogenous formation of arachidonic acid in response to osmotic stress (15). An alteration of K<sup>+</sup> channels in cells of postischemic and inflamed retinas disturbs the rapid release of K<sup>+</sup> ions in response to arachidonic acid-induced intracellular Na<sup>+</sup> overload, resulting in cell swelling (15). However, it is not known whether similar changes, which may contribute to cytotoxic edema under pathological conditions, also occur in the diabetic retina. Therefore, the aim of this study was to investigate whether experimental diabetic retinopathy alters the osmotic swelling characteristics of retinal glial cells and whether such changes are accompanied by alterations in K<sup>+</sup> conductance.

## RESEARCH DESIGN AND METHODS

Male Wistar rats ( $n = 15$ ) were handled in accordance with the European Council Directive 86/609/EEC. Animals had free access to water and food in an air-conditioned room on a 12-h light-dark cycle. Diabetes was induced in eight young rats (weighing at least 350 g) with a single intravenous injection of streptozotocin (65 mg/kg body wt diluted in 0.03 mol/l citrate buffer, pH 4.7; Sigma-Aldrich, Taufkirchen, Germany). Seven age-matched animals raised under similar conditions served as controls. The blood glucose levels in each rat were recorded using a point-of-care blood glucose meter (Accu-Chek;

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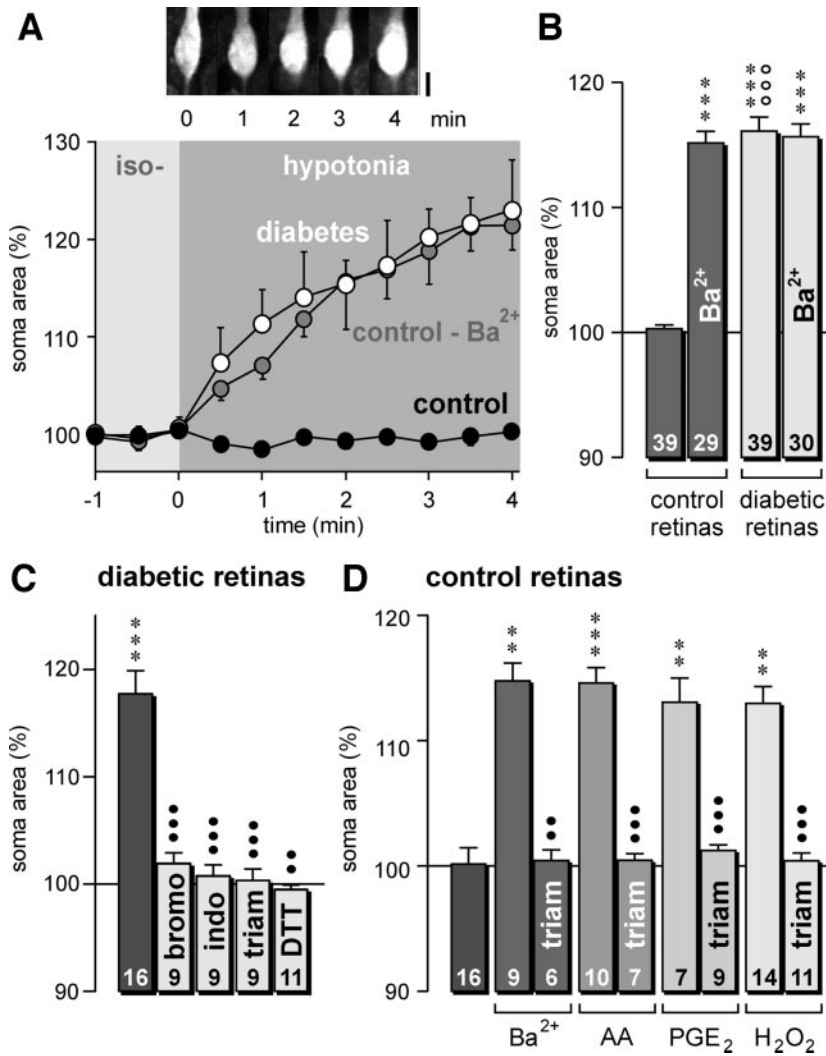
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PLA<sub>2</sub>, phospholipase A<sub>2</sub>.

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**FIG. 1.** Experimental diabetes alters the osmotic swelling characteristics of Müller glial cells in the rat retina. Retinal slices from 6-month diabetic and age-matched untreated control animals were perfused with a hypotonic solution, and the cross-sectional area of glial cell somata in the inner nuclear layer was recorded. **A:** Exposure of the slices to hypotonic solution induced time-dependent soma swelling of glial cells in a diabetic retina but had no effect on the soma volume of cells in a control retina. However, glial cell bodies in control retinas display hypotonic swelling in the presence of Ba<sup>2+</sup> (1 mmol/l) in the bathing solution. The images shown above display an original record of a dye-filled glial cell body in a retinal slice from a diabetic animal, dependent on the time of hypotonic exposure. Scale bar, 5  $\mu$ m. **B:** Mean cross-sectional area of glial cell bodies in retinas from diabetic and control animals during hypotonic conditions, in the absence and presence of Ba<sup>2+</sup> (1 mmol/l). **C:** The osmotic glial cell swelling in diabetic retinas was inhibited in the presence of the following agents: the selective inhibitor of PLA<sub>2</sub> activation, 4-bromophenacyl bromide (bromo; 300  $\mu$ mol/l); the cyclooxygenase inhibitor, indomethacin (indo; 10  $\mu$ mol/l); the corticosteroid, triamcinolone acetonide (triam; 100  $\mu$ mol/l); and the reducing agent, dithiothreitol (DTT; 3 mmol/l). **D:** Triamcinolone acetonide (triam; 100  $\mu$ mol/l) inhibited the glial cell swelling in control retinas, which was evoked by hypotonic challenge in the presence of Ba<sup>2+</sup> (1 mmol/l), arachidonic acid (AA; 10  $\mu$ mol/l), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; 30 nmol/l), or H<sub>2</sub>O<sub>2</sub> (50  $\mu$ mol/l). **B–D:** Values were measured after a 4-min perfusion of the hypotonic solution and are expressed as percent of the control value measured before application of the solution (100%). The data were obtained from six diabetic and five control retinas. Cell numbers are within the bars. Significant difference vs. control (100%): \*\**P* < 0.01; \*\*\**P* < 0.001. Significant difference between cells from control and diabetic retinas: ○○○*P* < 0.001. Significant blocking effects: ●●*P* < 0.01; ●●●*P* < 0.001.

Roche Diagnostics, Mannheim, Germany) at 2 and 5 days after streptozotocin injection and, subsequently, every week during the 1st month and every 2nd week until the end of the study. The blood glucose level in control animals was  $4.8 \pm 0.1$  mmol/l. In streptozotocin-induced diabetic animals, the blood glucose level increased to  $23.0 \pm 1.3$  mmol/l within 2 days after treatment and stabilized at  $27.7 \pm 0.5$  mmol/l during the course of the study. When the blood glucose level exceeded 33.3 mmol/l, insulin (Lantus containing insulin glargine; Aventis, Frankfurt, Germany; 2 units three times per week) was injected subcutaneously as needed to prevent weight loss without preventing hyperglycemia. The body weight of hyperglycemic animals decreased during the first weeks of diabetes to  $305 \pm 14$  g and was  $353 \pm 27$  g at 6 months of hyperglycemia. Within the same time period, the body weight of control animals increased from  $356 \pm 13$  to  $696 \pm 52$  g. At 4 or 6 months after streptozotocin injection, the animals were killed by carbon dioxide inhalation and the retinas removed.

**Müller cell swelling.** All experiments were performed at room temperature. To determine volume changes of Müller glial cells evoked by hypotonic stress in situ, the cross-sectional area of Müller cell somata in the inner nuclear layer of retinal slices was measured. Acutely isolated retinal slices (1 mm thickness) were placed in a perfusion chamber and loaded with the vital dye Mitotracker Orange (10  $\mu$ mol/l; Molecular Probes, Eugene, OR). It has been shown that Mitotracker Orange is taken up selectively by Müller glial cells while neurons remain unstained (16). The dye was dissolved in bathing solution that contained (in mmol/l) 136 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES-Tris, and 11 glucose, adjusted to pH 7.4 with Tris. The hypotonic solution was made by adding distilled water to the extracellular solution. The hypotonic solution and test substances were applied by fast changing of the perfusate. Ba<sup>2+</sup> (1 mmol/l) was preincubated for 10 min, and blocking substances were preincubated for 15 min before hypotonic challenge. Agonists were applied simultaneously with the hypotonic solution. The slices were examined by using a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany). Mitotracker Orange was excited at 543 nm, and emission was

recorded with a 560-nm long-pass filter. During the experiments, the Mitotracker Orange-stained somata in the inner nuclear layer were recorded at the plane of their largest extension. To assure that the maximum soma areas were precisely recorded, the focal plane was continuously adjusted during the course of the experiments.

**Electrophysiological recordings.** Müller glial cells were acutely isolated as previously described (14). The whole-cell currents of Müller cells were recorded using the Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and the ISO-2 computer program (MPK, Niedernhausen, Germany). Patch pipettes were pulled from borosilicate glass and had resistances between 4 and 6 M $\Omega$  when filled with a solution that contained (in mmol/l) 10 NaCl, 130 KCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 EGTA, and 10 HEPES, adjusted to pH 7.1 with Tris. The recording chamber was perfused with solution that contained (in mmol/l) 110 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1 Na<sub>2</sub>HPO<sub>4</sub>, 10 HEPES-Tris, 11 glucose, and 25 NaHCO<sub>3</sub>; the solution was equilibrated to pH 7.4 by continuous bubbling with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

To evoke K<sup>+</sup> currents, de- and hyperpolarizing voltage steps of 250 ms duration were applied from a holding potential of  $-80$  mV. To detect A-type K<sup>+</sup> currents, the whole-cell currents were recorded in the presence of Ba<sup>2+</sup> (0.3 mmol/l) with a voltage step protocol described previously (14). To evaluate the subcellular distribution of the K<sup>+</sup> conductance, a solution containing 50 mmol/l KCl was pressure ejected onto four different membrane regions of isolated cells (cell endfoot, proximal stem process, cell soma, and distal stem process); the evoked currents were recorded at  $-80$  mV from the soma of the cells. Membrane potentials were measured in the current-clamp mode.

**Immunohistochemistry.** Isolated retinas were fixed in 4% paraformaldehyde for 2 h. After several washing steps in buffered saline, the tissues were embedded in saline containing 3% agarose (wt/vol), and 70- $\mu$ m-thick slices were cut with a vibratome. For double labeling, the slices were incubated in 5% normal goat serum plus 0.3% Triton X-100 in saline for 2 h at room temperature and, subsequently, in a mixture of primary antibodies overnight

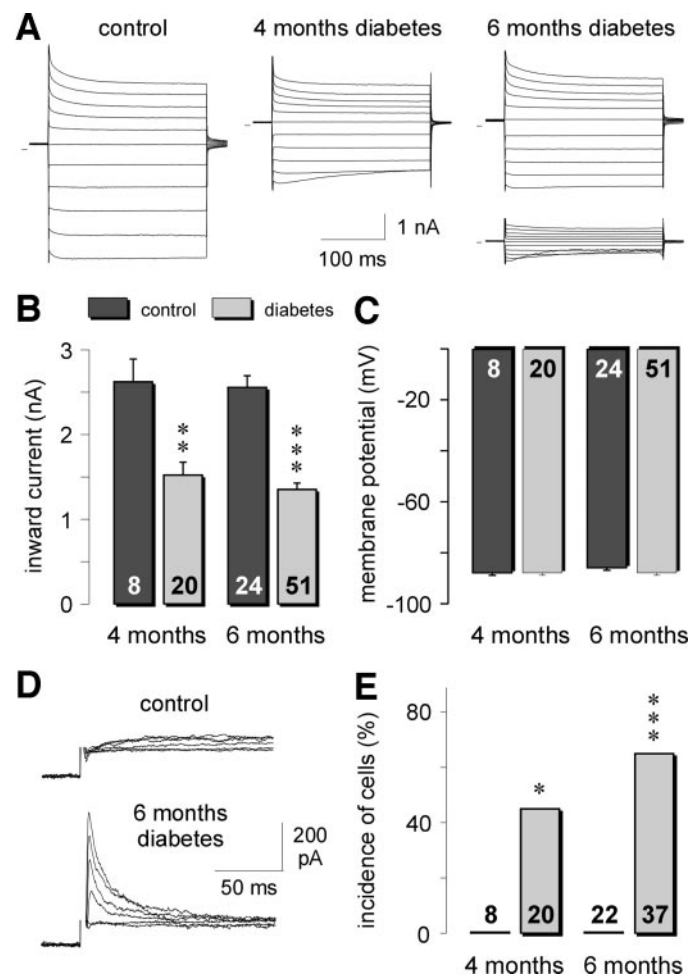
at 4°C. After washing in 1% BSA in saline, secondary antibodies were applied for 2 h at room temperature. Blocking of unspecific binding sites and permeabilization of retinal whole mounts was performed for 3 h in saline containing 5% normal goat serum plus 0.3% Triton X-100. The whole mounts were incubated with primary antibodies for 72 h at 4°C, washed in 1% BSA in saline, and incubated with secondary antibodies for 4 h at room temperature. The following antibodies were used: mouse anti-vimentin (1:500; V9 clone, Immunotech), rabbit anti-Kir4.1 (1:200; Alomone), goat anti-aquaporin-4 (1:50; Santa Cruz), Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova), Cy2-coupled goat anti-mouse IgG (1:200; Dianova), Cy3-coupled donkey anti-sheep IgG (1:200; Dianova), Cy5-coupled donkey anti-rabbit IgG (1:200; Dianova), and Cy5-coupled goat anti-mouse IgG (1:200; Dianova). Blood vessels were stained with Cy3-tagged *Griffonia simplicifolia* agglutinin (GSA) isolectin I-B<sub>4</sub>.

**Data analysis.** To determine the extent of glial soma swelling, the cross-sectional area of Mitotracker Orange-stained cell bodies in the inner nuclear layer of retinal slices was measured using the image analysis software of the LSM. Bar diagrams display the mean cross-sectional areas of glial cell somata that were measured after a 4-min perfusion of the hypotonic solution (shown in percent of the soma area measured before osmotic challenge [100%]). Inwardly directed whole-cell currents were measured at the end of 250-ms voltage steps from -80 to -140 mV. The ratio of the outward-to-inward currents was estimated at the voltage steps from -80 to -60 mV and to -100 mV. The amplitude of the A-type currents was measured at the 120-mV depolarizing voltage step. Statistical analysis was made using the Prism program (Graphpad Software, San Diego, CA); significance was determined by Mann-Whitney *U* test for two groups or by ANOVA followed by comparisons for multiple groups. Data are expressed as means  $\pm$  SEM.

## RESULTS

**Diabetes alters osmotic swelling characteristics of glial cells.** The swelling of the glial cell somata was investigated in acutely isolated retinal slices from 6-month diabetic and age-matched control animals by perfusing the slices with a hypotonic solution (containing 60% of the control osmolarity). Perfusion with an isotonic solution did not change the volume of glial cell bodies in retinal slices from control or diabetic animals (not shown). However, perfusion of the slices from diabetic animals with the hypotonic solution caused an increase of the volume of glial cell bodies by 10–30% (Fig. 1A and B). In contrast, cells in slices from control animals did not increase the volume of their somata under hypotonic conditions (Fig. 1A and B). However, hypotonic swelling of glial cells was also observed in retinas from control animals when the K<sup>+</sup> channels were inhibited by Ba<sup>2+</sup> ions (Fig. 1A and B), suggesting that transmembrane, channel-mediated K<sup>+</sup> currents play a role in preventing cell swelling during anisoosmotic conditions. The data indicate that glial cells in diabetic retinas are more sensitive to osmotic stress than glial cells in control retinas.

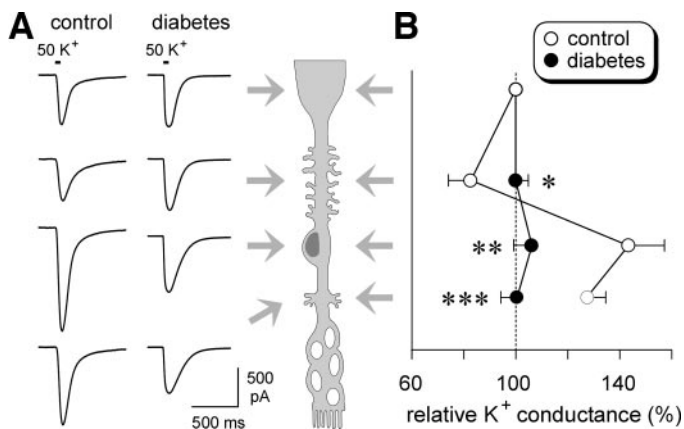
**Involvement of arachidonic acid and oxidative stress in glial cell swelling.** Arachidonic acid and prostaglandins have been implicated in the development of macular edema (11,17). It has been suggested that the osmotic glial cell swelling in retinal slices is caused by arachidonic acid-evoked intracellular Na<sup>+</sup> overload and simultaneous inhibition of channel-mediated K<sup>+</sup> efflux (12,15). To determine whether the osmotic swelling of glial cells in the diabetic retina is caused by formation of arachidonic acid due to activation of the phospholipase A<sub>2</sub> (PLA<sub>2</sub>), the selective PLA<sub>2</sub> inhibitor, 4-bromophenacyl bromide, was tested. As shown in Fig. 1C, inhibition of PLA<sub>2</sub> fully prevented osmotic swelling in diabetic retinas. Moreover, inhibition of the cyclooxygenase by indomethacin blocked the osmotic glial cell swelling in the diabetic retina (Fig. 1C). These data may suggest an involvement of prostaglandins in evoking cell swelling. The glucocorticoid triamcinolone acetonide is commonly used clinically for the rapid



**FIG. 2.** Experimental diabetes causes alteration of the plasma membrane K<sup>+</sup> conductance of Müller glial cells. **A:** Representative traces of whole-cell K<sup>+</sup> currents in cells isolated at 4 or 6 months after induction of diabetes and from a control animal (isolated at 6 months). Voltage steps were applied from a holding potential of -80 mV to de- and hyperpolarizing potentials between -180 and +20 mV (250 ms, 20-mV increment). Small bars at left indicate zero-current levels. **B:** Mean amplitude of the inward K<sup>+</sup> currents. The steady-state currents were measured at the end of the 250-ms voltage step from -80 to -140 mV. **C:** Resting membrane potential, which was measured in current-clamp mode. **D:** Example of fast-transient A-type K<sup>+</sup> currents that were recorded in a cell from a diabetic animal at 6 months after injection of streptozotocin. The control cell displayed no A-type currents. The currents were evoked by 20-mV incremental voltage steps to potentials between -60 and +40 mV, and the different currents that were evaluated as described in RESEARCH DESIGN AND METHODS are shown. **E:** Percentage of glial cells with A-type K<sup>+</sup> currents. The data were obtained from six 6-month diabetic, two 4-month diabetic, and six control retinas. Cell numbers are shown within the bars. Significant differences between cells from diabetic and control animals: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

resolution of diabetic macular edema (18). The hypotonic glial cell swelling in diabetic retinas was fully prevented in the presence of triamcinolone in the bathing solution (Fig. 1C). Whereas cells in control retinas normally did not swell during hypotonic conditions (Fig. 1A and B), they increased their volume in the presence of arachidonic acid or prostaglandin E<sub>2</sub> (Fig. 1D). Triamcinolone inhibited the swelling of cells in control retinas evoked by hypotonic challenge in the presence of Ba<sup>2+</sup>, arachidonic acid, or prostaglandin E<sub>2</sub> (Fig. 1D).

Diabetes is associated with mitochondrial oxidative stress (19–21). To reveal whether acute oxidative stress plays a role in osmotic swelling of glial cells, we tested



**FIG. 3.** Experimental diabetes causes alteration of the subcellular distribution of the plasma membrane K<sup>+</sup> conductance of isolated retinal glial cells. The distribution of the inward K<sup>+</sup> currents was determined by focal ejection of a high-K<sup>+</sup> (50-mmol/l) solution onto four different membrane domains of acutely isolated cells. The control K<sup>+</sup> concentration was 3 mmol/l. **A:** Representative traces of inward currents in cells from a control (*left*) and a 6-month diabetic (*right*) animal. Thick bars indicate the ejection time (50 ms). **B:** Subcellular distribution of the inward K<sup>+</sup> conductance in cells from control and diabetic animals at 6 months after induction of diabetes. The K<sup>+</sup> conductance is normalized to the values obtained at the endfoot membranes (100%). Means ± SE from 16 (obtained from three control rats) and 26 (from four diabetic rats) cells, respectively. Significant differences between cells from diabetic and control animals: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

whether a reducing agent may inhibit the swelling in diabetic retinas and whether application of H<sub>2</sub>O<sub>2</sub> to control retinas may induce glial cell swelling. The osmotic swelling of glial cell bodies in diabetic retinas was fully inhibited in the presence of dithiothreitol, a cell-permeable dithiol-reducing agent (Fig. 1C). In control retinas, H<sub>2</sub>O<sub>2</sub> induced cell swelling under hypotonic conditions, which was not observed in the absence of H<sub>2</sub>O<sub>2</sub> (Fig. 1D). The swelling-evoking effect of H<sub>2</sub>O<sub>2</sub> was diminished in the presence of triamcinolone (Fig. 1D). The data may suggest that both arachidonic acid metabolites and acute oxidative stress are involved in the induction of glial cell swelling under anisoosmotic conditions.

**Diabetes causes a decrease of glial inward rectifier K<sup>+</sup> currents.** It has been shown that altered swelling responses of glial cells in postischemic and inflamed retinas of the rat are associated with diminished transmembrane K<sup>+</sup> currents in the cells (12,13). To determine whether a similar decrease of the glial membrane conductance occurs during experimental diabetic retinopathy, the K<sup>+</sup> currents of acutely isolated Müller glial cells were recorded. Figure 2A displays representative current records in cells that were isolated from a control retina and from diabetic retinas at 4 and 6 months after streptozotocin injection. Depolarization of the plasma membrane from a holding potential of -80 mV evoked outward K<sup>+</sup> currents (upwardly depicted), whereas hyperpolarization evoked inward K<sup>+</sup> currents (downwardly depicted). Obviously, the currents in cells from the diabetic retinas were smaller than those in cells from control retinas, with a considerable variation of the current amplitude in cells from diabetic origin.

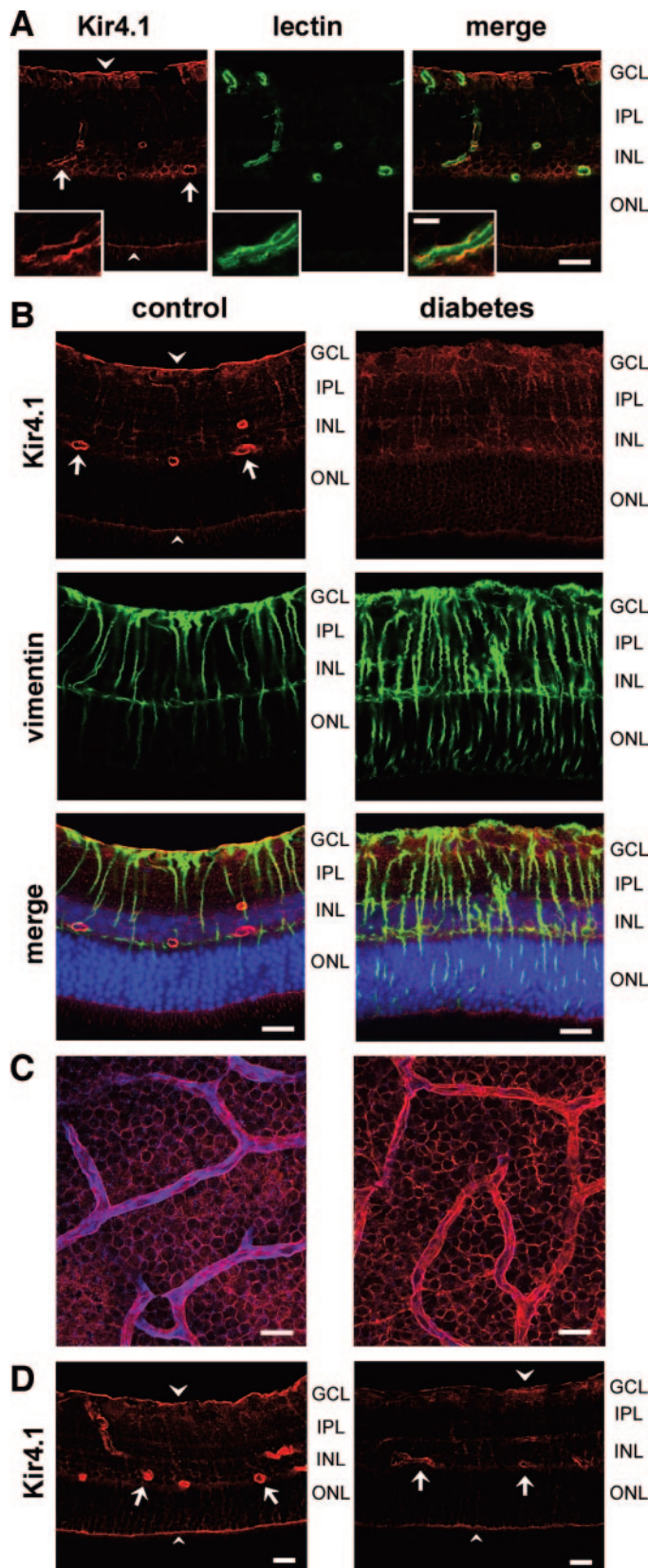
At both 4 and 6 months of hyperglycemia, the mean inward K<sup>+</sup> currents recorded in cells from diabetic animals were significantly decreased compared with cells from control animals (Fig. 2B). The current amplitude was reduced to 58% after 4 months and to 53% after 6 months

of hyperglycemia. There was no alteration of the resting membrane potential in cells from diabetic animals when compared with those from controls (Fig. 2C). Furthermore, there was no difference in the membrane capacitance between cells from 6-month diabetic animals (40 ± 3 pF; *n* = 54) and control animals (40 ± 3 pF; *n* = 27).

Müller glial cells express various subtypes of inward rectifier K<sup>+</sup> channels, which are characterized by different rectification properties (22). The Kir4.1 channel is a weakly rectifying channel, i.e., the inward and outward currents flowing through the channels have similar amplitudes. Other subtypes of inward rectifier K<sup>+</sup> channels found in retinal glial cells, e.g., Kir2.1 (22), are strongly rectifying, i.e., the channels mediate only inward currents and almost no outward currents. Alterations of the ratio of the outward-to-inward currents may, therefore, indicate whether there are selective changes in the occurrence or functional state in the two subpopulations of channels. We found that this ratio was significantly (*P* < 0.001) smaller in cells from 6-month diabetic rats (0.66 ± 0.03; *n* = 51) than in control cells (0.87 ± 0.04; *n* = 24). This relative decrease of the outward currents suggests that weakly rectifying channels such as the Kir4.1 channel are more heavily affected by the disease than strongly rectifying channels.

**Diabetes causes a redistribution of the glial K<sup>+</sup> conductance.** Normally, the K<sup>+</sup> conductance displays an uneven distribution along Müller cell membranes, with the highest conductance at the perisomatic membrane domain in cells of the rat (12,13). However, it has been shown that glial cells in postischemic and inflamed retinas of the rat lose this uneven distribution of the K<sup>+</sup> conductance and display a relatively even distribution along the whole-plasma membrane (12,13). To determine whether a similar alteration occurs in glial cells of hyperglycemic rats, a high-K<sup>+</sup> solution (evoking inward currents) was focally ejected onto four different membrane domains of acutely isolated cells. As shown in Fig. 3A, the relative inward K<sup>+</sup> currents in cells from control retinas were highest at the cell soma and at the inner part of the distal cell process. These parts of the cells are located in situ in the inner nuclear and outer plexiform layers, which both contain retinal blood vessels. The high K<sup>+</sup> conductance of these cellular compartments has been explained by the high amount of Kir4.1 channels arranged in the perivascular membrane domains of glial cells (23). On the other hand, in cells from diabetic retinas, the relative K<sup>+</sup> conductance of the perisomatic membrane was significantly decreased, whereas the relative K<sup>+</sup> conductance at the proximal process was somewhat increased, resulting in an evenly distributed conductance along the entire cells (Fig. 3B). These data may suggest that, particularly, the amount of perivascular Kir4.1 channels was decreased.

**Diabetes causes an increase of glial A-type K<sup>+</sup> currents.** In addition to the decrease of the inward rectifier K<sup>+</sup> currents, glial cells in postischemic and inflamed retinas display another alteration of their K<sup>+</sup> conductance: they possess a voltage-dependent, fast-transient (A-type) outward K<sup>+</sup> current that is not present in cells from control retinas (13,14). A similar alteration was found in cells from diabetic animals (Fig. 2D). Whereas this current could not be observed in any cell from control retinas, it was activated in 45 and 65% of the cells investigated from 4- and 6-month hyperglycemic animals, respectively (Fig. 2E). There was no time-dependent alteration in the A-type current amplitude (386 ± 45 pA, *n* = 8, and 407 ± 78 pA,



$n = 11$ , at 4 and 6 months after induction of diabetes, respectively).

#### Diabetes alters the distribution of Kir4.1 protein.

Among the various inward rectifier  $K^+$  channel genes expressed by Müller cells (24), the Kir4.1 channel has been proposed to be most important for the mediation of spatial-buffering  $K^+$  currents (25). The Kir4.1 protein shows a polarized arrangement in Müller cells; though the protein exists in the whole retinal tissue, it shows a strong enrichment in such membrane domains across which Müller cells in situ are proposed to release excess  $K^+$  into extraretinal fluid-filled spaces, i.e., at perivascular membrane domains and at the inner and outer limiting membranes (Fig. 4A) (22,23,25). In contrast, retinas from diabetic animals (at 6 months after induction of diabetes) displayed a significant alteration of the staining pattern for Kir4.1; the protein was still observed at a low level within the retina, but the prominent concentration at both limiting membranes and around the vessels was markedly decreased (Fig. 4B). The strong decrease of perivascular immunoreactivity was also recognizable in retinal whole mounts stained against Kir4.1 (Fig. 4C). Retinas from animals after 4 months of diabetes showed a partial reduction of Kir4.1 staining at the limiting membranes and around the vessels when compared with control animals (Fig. 4D). The data suggest that the decrease of the  $K^+$  currents in cells from diabetic retinas (Fig. 2B) is correlated with alteration in local distribution of Kir4.1 protein.

#### DISCUSSION

Alterations of the properties of  $K^+$  channels in glial cells have been proposed to be implicated in the development of cytotoxic edema in the retina under ischemic and inflammatory conditions (12,13). Here, we show that functional  $K^+$  channels in retinal glial cells undergo alterations in the course of experimental diabetes and that these alterations are associated with changed swelling characteristics of retinal glial cells.

We found a decrease of the main  $K^+$  conductance in Müller cells of diabetic rats that was associated with an alteration of the subcellular distribution of the  $K^+$  conductance. The altered subcellular distribution of the  $K^+$  conductance may be explained with a decrease of Kir4.1 channels in perivascular membrane domains of Müller cells. This assumption is supported by the observations that 1) the ratio of the outward-to-inward currents decreases during diabetes, indicating that the weakly rectifying Kir4.1 channels are more affected than strongly rectifying channels, and that 2) the prominent occurrence of Kir4.1 around vessels is not found in the diabetic retina.

**FIG. 4.** Experimental diabetes alters the distribution of Kir4.1 protein in the rat retina. The slices (A, B, and D) and whole mounts (C) were derived from age-matched control animals and from diabetic animals at 4 (D) and 6 (B and C) months after streptozotocin injection. A: Kir4.1 immunoreactivity in a control retina. The blood vessels were stained with lectin. The arrows mark perivascular staining of Kir4.1, and the large and small arrowheads indicate staining of the inner and outer limiting membranes, respectively. Insets: A vessel at higher magnification. B: Immunoreactivities for Kir4.1 (red) and vimentin (green). Cell nuclei were counterstained with Hoechst 33258 (blue). C: Immunoreactivities for aquaporin-4 (red) and Kir4.1 (blue); the data were recorded at the plane of the blood vessels within the inner nuclear layer. In the control retina, both immunoreactivities were found around the vessels, whereas in the diabetic retina, the perivascular immunoreactivity for Kir4.1 is decreased. D: Immunoreactivity for Kir4.1 in retinal slices of a control animal and of an animal at 4 months of diabetes. Scale bars, 20 and 5 (insets)  $\mu\text{m}$ . GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

The decrease of perivascular Kir4.1 channels will diminish the  $K^+$  conductance of those membrane domains across which the cells normally release excess  $K^+$  in the process of spatial  $K^+$  buffering (26). This decrease should result in a disturbance of the retinal  $K^+$  homeostasis, contributing (in addition to other factors, e.g., the diabetes-induced dysfunction of glutamate transporters [27]) to neuronal hyperexcitation and glutamate excitotoxicity. A similar alteration of the Kir4.1 protein has been described in retinas of mice with a genetic inactivation of the dystrophin gene product Dp71 (28), which is proposed to be involved in the clustering of Kir4.1 channels in the plasma membrane (29). This alteration in Dp71-null mice was associated with an enhanced vulnerability of retinal ganglion cells to ischemia-reperfusion injury (28).

It has been shown in diabetic patients that in addition to vascular leakage, a dysfunction of the active transport mechanisms of the blood-retinal barriers is a necessary precondition for the formation of a macular edema (5). The abnormalities of the vessel permeability need to be accompanied by ineffective edema-resolving mechanisms in order to cause chronic edema (30). Normally, the fluid reabsorption from retinal tissue into the blood is carried out by pigment epithelial and glial cells (6). It has been suggested that the water movement across the glio-vascular interface is facilitated by aquaporin-4 water channels (31) and coupled to the extrusion of  $K^+$  ions from the glial cells into the blood (23). The idea of a coupled  $K^+$  and water absorption from the retinal tissue by glial cells is supported by the observation that both Kir4.1 and aquaporin-4 proteins are located in perivascular glial membranes (23). A decrease of Kir4.1 channels in perivascular glial cell membranes of the pathologically altered retina may, therefore, result in a disturbance of the water transport across the glio-vascular interface, which should deteriorate the efficacy of glial cell-mediated resolution of vasogenic edema and favor glial cell swelling (6,12).

We have shown here that glial cells in diabetic retinas swell upon hypotonic stress, whereas glial cells in control retinas do not alter their volume under these conditions. As a block of  $K^+$  channels with  $Ba^{2+}$  ions evokes swelling of cells in control retinas under hypotonic conditions, the above-described loss of weakly rectifying  $K^+$  channels appears to play a key role in experimental cell swelling in diabetic retinas. However, it remains to be elucidated how diabetic conditions trigger the observed changes in functional  $K^+$  channels and/or other pathomechanisms involved in edema. We show that both acute oxidative stress and the production of arachidonic acid and prostaglandins induces osmotic cell swelling in diabetic retinas. It has been shown that the retinal content of cyclooxygenase-2 is increased early in diabetes, and it has been suggested that the production of cytotoxic prostaglandins is involved in retinal cell death under hyperglycemic conditions (32). In ischemic retinopathies and in response to oxidant stress, an increased arachidonic acid metabolism within the retina has been described and metabolites of arachidonic acid have been shown to contribute to neurovascular injury (33). An increased activity of PLA<sub>2</sub> and cyclooxygenase in Müller cells of the diabetic retina may be involved in the pathomechanisms causing edema in diabetic retinas. Acute oxidative stress may activate the enzymes of arachidonic acid metabolism, resulting in swelling under hypotonic conditions.

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