

Deletion of Aquaporin-4 Renders Retinal Glial Cells More Susceptible to Osmotic Stress

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The glial water channel aquaporin-4 (AQP4) is implicated in the control of ion and osmohomeostasis in the sensory retina. Using retinal slices from AQP4-deficient and wild-type mice, we investigated whether AQP4 is involved in the regulation of glial cell volume under altered osmotic conditions. Superfusion of retinal slices with a hypoosmolar solution induced a rapid swelling of glial somata in tissues from AQP4 null mice but not from wild-type mice. The swelling was mediated by oxidative stress, inflammatory lipid mediators, and sodium influx into the cells and was prevented by activation of glutamatergic and purinergic receptors. Distinct inflammatory proteins, including interleukin-1 β , interleukin-6, and inducible nitric oxide synthase, were up-regulated in the retina of AQP4 null mice compared with control, whereas cyclooxygenase-2 was down-regulated. The data suggest that water flux through AQP4 is involved in the rapid volume regulation of retinal glial (Müller) cells in response to osmotic stress and that deletion of AQP4 results in an inflammatory response of the retinal tissue. Possible implications of the data for understanding the pathophysiology of neuromyelitis optica, a human disease that has been suggested to involve serum antibodies to AQP4, are discussed. © 2010 Wiley-Liss, Inc. © 2010 Wiley-Liss, Inc.

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Aquaporin (AQP) water channels are critically involved in the maintenance of ionic and osmotic balance in the central nervous system. There are at least 13 different members of the AQP protein family that mediate bidirectional movement of water and small solutes such as glycerol across membranes, in response to osmotic gradients and differences in hydrostatic pressure (Verkman and Mitra, 2000). In the sensory retina, various AQPs are expressed in a cell-specific manner (Frigeri

et al., 1995; Nagelhus et al., 1998; Iandiev et al., 2005, 2006a, 2007). Among them, AQP1 was shown to be localized to photoreceptors and distinct amacrine cells, whereas AQP4 is expressed by retinal astrocytes, Müller glial cells, and the vascular endothelium (Nagelhus et al., 1998, 1999; Iandiev et al., 2005).

Neuronal activity is associated with rapid ion shifts between intra- and extracellular spaces, which may cause osmotic imbalances in the neural tissue. It has been shown that intense neuronal activity in the brain and in the retina is accompanied by a decrease in the osmolarity of the extracellular fluid, because the decrease in sodium exceeds the increase in potassium by a factor of two (Dietzel et al., 1989; Dmitriev et al., 1999). A major functional role of glial cells in the central nervous system and in the sensory retina is the control of osmotic and ionic homeostasis, mediated by transglial ion and water transport (Kofuji and Newman, 2004; Nagelhus et al., 2004; Bringmann et al., 2006). Imbalances in the extrac-

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ellular potassium concentration are buffered by retinal glial cells predominantly via passive currents through inwardly rectifying potassium (Kir) channels, especially Kir4.1 (Kofuji et al., 2000; Kofuji and Newman, 2004; Bringmann et al., 2006). The colocalization of Kir4.1 and AQP4 in distinct membrane domains of retinal glial cells has led to the suggestion that the transglial potassium transport is associated with a concomitant water flux through AQPs (Nagelhus et al., 1999).

In addition to osmotic imbalances, intense neuronal activity in the retina is also accompanied by a shrinkage of the extracellular space, which results predominantly from a swelling of neuronal cell bodies and synapses because of activation of ionotropic glutamate receptors associated with a net uptake of sodium chloride (Uckermann et al., 2004a). To avoid deleterious activity-dependent decreases in the perisynaptic space that may result in neuronal hyperexcitability (Dudek et al., 1990; Chebabo et al., 1995), glial cells should not swell, or even decrease their volume, when the neurons undergo an activity-dependent swelling.

Osmotic swelling of glial cells is a major cause of edema in pathological conditions such as ischemia, trauma, and inflammation (Pannicke et al., 2004, 2005; Kimelberg, 2005). Inflammation is a concomitant phenomenon of retinal diseases and injuries. Inflammatory mediators are up-regulated in the diseased retina (Wurm et al., 2006; Hollborn et al., 2008), and pharmacological inhibition of inflammatory responses suppresses osmotic glial cell swelling in several injury models (Wurm et al., 2006, 2008; Rehak et al., 2009). Whereas the localization of Kir4.1 was altered in the diseased retina, expression of AQP4 remained largely unchanged (Pannicke et al., 2004; Liu et al., 2007; Rehak et al., 2009). Thus, it is presently unknown how AQP4 is involved in cell swelling mediated by inflammatory processes.

The aim of the present study was to determine whether AQP4 is involved in the homeostasis of the cellular volume of retinal glial cells under hypoosmotic conditions. We compared the osmotic swelling properties of Müller glial cells in retinal tissues from wild-type and AQP4-deficient (AQP4 null) mice. Furthermore, we assessed whether deletion of AQP4 is associated with alterations in the expression of membrane channels and inflammatory proteins in the retina.

MATERIALS AND METHODS

Materials

Papain was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Chloromethyltetramethylrosamine (Mitotracker Orange) was from Molecular Probes (Eugene, OR). *N*⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) was from Tocris (Bristol, United Kingdom). DNase I, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and all other substances used were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. For immunohistochemistry, the following antibodies were used: rabbit anti-AQP1 (1:200; Chemicon, Temecula,

CA; catalog No. AB3065), rabbit anti-AQP4 (1:200; Sigma-Aldrich; A5971), rabbit anti-Kir4.1 (1:200; Alomone, Jerusalem, Israel; APC-035), mouse anti-glial fibrillary acidic protein (GFAP; 1:200; G-A-5 clone; Sigma-Aldrich; G3893), Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova, Hamburg, Germany; 111-165-144), and Cy2-coupled goat anti-mouse IgG (1:200; Dianova; 115-225-068). Nuclei were stained with TO-PRO-3 (Invitrogen, Karlsruhe, Germany).

Animals

All experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC and were approved by the local authorities. AQP4 null mice (*n* = 12) from a CD1 genetic background (Ma et al., 1997) and wild-type CD1 mice (*n* = 10) were used (age 3–4 months). Animals had free access to water and food in an air-conditioned room on a 12-hr light-dark cycle and were sacrificed with carbon dioxide.

Preparation of Retinal Slices

Pieces of freshly isolated retinas (5 × 5 mm) were placed, with the photoreceptor side down, onto membrane filters (mixed cellulose ester, 0.45 μm pore size; Schleicher & Schuell MicroScience, Dassel, Germany). By using a custom-made cutter equipped with a razor blade, the retinal tissue on the filter was cut radially into 1-mm-thick slices. The slices (still attached to the filter) were mounted in a chamber in such a way that all retinal layers were visible on the radial section area.

Preparation of Freshly Isolated Retinal Glial Cells

Pieces of isolated retinas were incubated in papain (0.2 mg/ml)-containing calcium- and magnesium-free phosphate-buffered saline, pH 7.4, for 30 min at 37°C, followed by several washing steps with saline. After short incubation in saline supplemented with DNase I (200 U/ml), the tissue pieces were triturated by a wide-pore pipette, to obtain isolated glial cells. Müller cells could be identified immunocytochemically (data not shown; see Fort et al., 2008). The cells were stored at 4°C in serum-free minimum essential medium until use within 1.5 hr after cell isolation.

Glial Cell Swelling

All experiments were performed at room temperature (20–23°C). The filter stripes with the retinal slices were transferred to recording chambers and kept submerged in extracellular solution. The chambers were mounted on the stage of an upright confocal laser scanning microscope (LSM 510 Meta; Zeiss, Oberkochen, Germany). Retinal slices were loaded with the vital dye Mitotracker Orange (1 μM); it has been shown that this dye stains selectively the somata of Müller glial cells in the inner nuclear layer of retinal tissues (Uckermann et al., 2004b). After an incubation time of 3 min, the slices were continuously superfused with extracellular solution at a flow rate of 2 ml/min. To monitor volume changes of retinal glial cells in response to hypotonic challenge, the somata of glial cells in the inner nuclear layer of retinal slices were focussed. Recordings were made with an Achroplan

63×/0.9 water immersion objective. The pinhole was set at 172 μm ; the thickness of the optical section was adjusted to 1 μm . Mitotracker Orange was excited at 543 nm with an HeNe laser, and emission was recorded with a 560 nm long-pass filter. Images were obtained with an x-y frame size of 256 × 256 pixel (73.1 × 73.1 μm). To ensure that the maximum soma area was precisely measured, the focal plane was continuously adjusted during the course of the experiments.

Solutions

A gravity-fed system with multiple reservoirs was used to perfuse the recording chamber continuously with extracellular solution; test substances were applied by rapidly changing the perfusate. The bathing solution in the recording chamber was totally changed within ~ 2 min. The extracellular solution consisted of (mM) 136 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 11 glucose, adjusted to pH 7.4 with Tris. The hypoosmolar solution (60% of control osmolarity) was made up by adding distilled water. The sodium-free extracellular solution was made by replacing NaCl with choline chloride. Barium chloride (1 mM) was added to iso- and hypoosmolar solutions; the slices were preincubated in barium-containing solution for 10 min. Blocking substances were preincubated for 15–45 min, and agonists were applied simultaneously with the hypoosmolar solution.

Whole-cell patch-clamp records

The whole-cell currents of freshly isolated Müller glial cells were recorded at room temperature using the Axopatch 200A amplifier (Axon Instruments) and the ISO-2 computer program (MFK, Niedernhausen, Germany). The signals were low-pass filtered at 1, 2, or 6 kHz (eight-pole Bessel filter) and digitized at 5, 10, or 30 kHz, respectively, using a 12-bit A/D converter. Patch pipettes were pulled from borosilicate glass (Science Products) and had resistances between 4 and 6 M Ω when filled with a solution containing (mM) 10 NaCl, 130 KCl, 1 CaCl₂, 2 MgCl₂, 10 EGTA, and 10 HEPES, adjusted to pH 7.1 with Tris. The recording chamber was continuously perfused with extracellular solution that contained (mM) 135 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 1 Na₂HPO₄, 10 HEPES, and 11 glucose, equilibrated to pH 7.4 with Tris. To evoke potassium currents, de- and hyperpolarizing voltage steps of 250-msec duration, with increments of 10 mV, were applied from a holding potential of -80 mV. The amplitude of the steady-state inward potassium currents was measured at the end of the 250-msec voltage step from -80 to -140 mV. The membrane capacitance of the cells was measured by the integral of the uncompensated capacitive artifact (filtered at 6 kHz) evoked by a hyperpolarizing voltage step from -80 to -90 mV in the presence of extracellular barium chloride (1 mM). The resting membrane potential was measured in the current-clamp mode.

Immunohistochemistry

Isolated retinas were fixed in 4% paraformaldehyde for 2 hr. After several washing steps in buffered saline, the tissues were embedded in saline containing 3% agarose (w/v), and 70- μm -thick slices were cut by using a vibratome. For double

labeling, the slices were incubated in 5% normal goat serum plus 0.3% Triton X-100 in saline for 2 hr at room temperature and, subsequently, in a mixture of primary antibodies overnight at 4°C. After washing in 1% bovine serum albumin, secondary antibodies were applied for 2 hr at room temperature. Control slices were stained without primary antibodies. Images were taken with the LSM 510 Meta (Zeiss).

RT-PCR

After the mice had been killed, eyes were removed, and the retinas were taken and frozen immediately in liquid nitrogen. Later, total RNA was extracted from the thawed tissue with Trizol reagent (0.5 or 1.0 ml; Gibco BRL, Grand Island, NY). The quality of the RNA was analyzed by agarose gel electrophoresis. The A₂₆₀/A₂₈₀ ratio of optical density was measured by using the GeneQuantpro device (Pharmacia, Uppsala, Sweden), and was between 1.9 and 2.1 for all RNA samples, indicating sufficient quality (Chomczynski and Sacchi, 1987).

After treatment with DNase I, cDNA was synthesized from 1 μg total RNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany). PCR was performed using the Taq PCR Master Mix kit (Qiagen, Valencia, CA) and the primer pairs described in Table I. One microliter of the first-strand mixture and 0.5 μM of each gene-specific sense and antisense primer were used for amplification in a final volume of 20 μl . Amplification was performed for 40 cycles with the PTC-200 Thermal Cycler (MJ Research, Watertown, MA). Each cycle consisted of 30 sec at 94°C, 60 sec at 58°C, and 1 min at 72°C.

The relative mRNA level in the retinal tissue from AQP4 null mice was determined in comparison with the level in the tissue from wild-type mice. Semiquantitative real-time RT-PCR was performed with the Single-Color Real-Time PCR Detection System (Bio-Rad, Munich, Germany) using the primer pairs described in Table I. The PCR solution contained 1 μl cDNA, specific primer set (0.2 μM each), and 10 μl of iQ SYBR Green Supermix (Bio-Rad) in a final volume of 20 μl . The PCR parameters were initial denaturation and enzyme activation (one cycle at 95°C for 15 min); denaturation, amplification and quantification, 45 cycles at 95°C for 30 sec, 58°C for 20 sec, and 72°C for 45 sec; melting curve, 55°C with the temperature gradually increased (0.5°C) up to 95°C. The amplified samples were analyzed by standard agarose gel electrophoresis. The mRNA expression was normalized to the level of *Actb* mRNA. The changes in mRNA expression were calculated according to the $2^{-\Delta\Delta\text{CT}}$ method (CT, cycle threshold), with $\Delta\text{CT} = \text{CT}_{\text{target gene}} - \text{CT}_{\text{actb}}$ and $\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{treatment}} - \Delta\text{CT}_{\text{control}}$.

Data Analysis

To determine the extent of glial soma swelling, the maximal cross-sectional area of Mitotracker Orange-stained cell bodies in the inner nuclear layer of retinal slices was measured off-line using the image analysis software of the laser scanning microscope. Bar diagrams display the cross-sectional area of glial somata that was measured after a 4-min perfusion with the hypoosmolar solution, in percentage of the soma

TABLE I. Oligonucleotides Used for PCR Experiments

Gene and accession No.	Protein	Primer sequence (5'-3') ^a	Product size (bp)
<i>Arh</i> NM_007393	β-Actin	(s) GACGGCCAGGTCATCACTAT (as) CTTCTGCATCCTGTCTCAGCAA	216
<i>Gfap</i> NM_010277	Glial fibrillary acidic protein	(s) GCCACAGTAACATGCAAGA (as) GCTCTAGGGACTCGTTCCGTG	187
<i>Il1β</i> NM_008361	Interleukin-1β	(s) GCCATCCTCTGTGACTCAT (as) AGGCCACAGGATATTTTGTCCG	230
<i>Il6</i> NM_031168	Interleukin-6	(s) AGTTGCCCTCTGGGACTGA (as) TCCACGATTTCCACAGAAAC	159
<i>Tnfr</i> NM_013693	Tumor necrosis factor-α	(s) CGTCAGCCGATTTGCTATCT (as) CGGACTCCGCAAAGTCTAAG	206
<i>Nos1</i> NM_008712	Neuronal nitric oxide synthase	(s) AGCACCTACCAGCTCAAGGA (as) ATAGTGATGGCCGACCTGAG	209
<i>Nos2</i> NM_010927	Inducible nitric oxide synthase	(s) CACCTTGAGTTCACCCAGT (as) ACCACTCGTACTTGGGATGC	170
<i>Nos3</i> NM_008713	Endothelial nitric oxide synthase	(s) GACCTCACCGCTACAACT (as) CTGGCCTTCTGCTCAATTTTC	209
<i>Alox5</i> NM_009662	Arachidonate 5-lipoxygenase	(s) CTACGATGCACCGTGGATG (as) GTGCTGCTTGAGGATGTGAA	235
<i>cPlat2</i> NM_008869	Cytosolic phospholipase A ₂	(s) GTTTGTTTCATGCCAGACCT (as) ATCCCCGACTCATAACAGTGC	239
<i>Ptgs1</i> NM_008969	Cyclooxygenase-1	(s) GTGGCTATTTCTGCAGCTC (as) CAGTGCCTCAACCCCATAGT	209
<i>Ptgs2</i> NM_011198	Cyclooxygenase-2	(s) AGAAGGAATGGCTGCAGAA (as) GCTCGGCTTCCAGTATTGAG	194
<i>App1</i> NM_007472	Aquaporin-1	(s) GTCCAGGACAACGTTGAAGT (as) ACACACTGGGCGGATGATGA	173
<i>App4</i> NM_009700	Aquaporin-4	(s) AGCAATTGGATTTCCGTTG (as) TGAGCTCCACATCAGGACAG	203
<i>Kirj10</i> NM_001039484	Kir4.1 potassium channel	(s) GCCCTAGGACTGATGAGCTG (as) AACAGTAGGGAGCAGCCAGA	261

^as, Sense; as, antisense.

area measured before hypotonic challenge (100%). Bar diagrams of the PCR data display the relative increase of the mRNA compared with the wild-type control. The data from patch-clamp experiments are given as absolute current amplitudes, membrane potentials, and membrane capacitances. Statistical analysis for all kind of data was done in SigmaPlot (SPSS Inc., Chicago, IL) and the Prism program (GraphPad Software, San Diego, CA); significance was determined by Mann-Whitney U test for two groups and Kruskal-Wallis test followed by Dunn's comparison for multiple groups. Data are expressed as mean ± SD (electrophysiological data) and mean ± SEM (PCR and cell swelling data), respectively.

RESULTS

Expression of Water and Potassium Channels

Real-time RT-PCR analysis was performed to compare the expression of water and potassium channels in the retinal tissues of wild-type and AQP4 null mice. Data obtained with RT-PCR and real-time RT-PCR analysis showed that (in contrast to wild-type tissue) the retina of AQP4 null mice did not contain transcripts for AQP4 (Fig. 1). Deletion of AQP4 was associated with a significant decrease in the expression of AQP1 (to ~13% of the control) and with a slight increase in the expression of the major potassium channel of retinal glial cells, Kir4.1 (~1.7-fold), in the retina. Because AQP1 is expressed by photoreceptors, the decrease of AQP1 in AQP4 null mice should have no direct influence on the swelling of Müller cells described below.

Expression of GFAP and Inflammatory Proteins

Deletion of AQP4 has been shown to be associated with a decrease in GFAP (Zhou et al., 2008). We found that the expression of GFAP was decreased in the retina of AQP4 null mice to about 50% compared with the tissue of wild-type animals (Fig. 1). Deletion of AQP4 was also associated with alterations in the level of transcripts encoding distinct inflammatory proteins. The retina of AQP4 null animals contained significant more mRNA for interleukin (IL)-1β (~2-fold), IL-6 (~6-fold), and inducible nitric oxide synthase (iNOS; ~12-fold) than the retina of wild-type animals (Fig. 1). In contrast, the expression of cyclooxygenase-2 was strongly depressed (Fig. 1), whereas expression of cyclooxygenase-1 was reduced to about 50%. However; expression of tumor necrosis factor-α, neuronal nitric oxide synthase, endothelial nitric oxide synthase, arachidonate 5-lipoxygenase, and cytosolic phospholipase A₂ was not significantly altered compared with wild-type control (Fig. 1). The data suggest that deletion of AQP4 is associated with alterations in the retinal gene expression of distinct inflammatory factors and enzymes.

Retinal Localization of AQP4 and AQP1

The retinal distribution of water channel proteins was determined by immunohistochemical staining of retinal slices. AQP4 protein was localized throughout the whole retinal tissue of wild-type mice, with enrichments

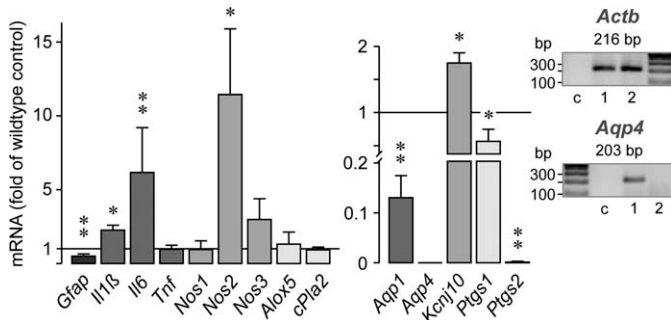


Fig. 1. Gene expression of inflammatory genes, and of water and potassium channels, in the retina of AQP4 null mice in comparison with the tissue of wild-type mice. The mRNA levels of the following genes were determined using real-time RT-PCR and normalized to the level of β -actin (*Actb*): arachidonate 5-lipoxygenase (*Alox5*), aquaporin-1 (*Aqp1*), aquaporin-4 (*Aqp4*), cytosolic phospholipase A₂ (*cPla2*), glial fibrillary acidic protein (*Gfap*), interleukin-1 β (*Il1b*), interleukin-6 (*Il6*), Kir4.1 potassium channel (*Kcnj10*), neuronal nitric oxide synthase (*Nos1*), inducible nitric oxide synthase (*Nos2*), endothelial nitric oxide synthase (*Nos3*), cyclooxygenase-1 (*Ptgs1*), cyclooxygenase-2 (*Ptgs2*), and tumor necrosis factor- α (*Tnf*). *Aqp4* was expressed in none retina investigated from AQP4 null mice and in all tissues investigated from wild-type animals. *Ptgs2* was expressed at a very low level in two of five retinas from AQP4 null mice but in all tissues investigated from wild-type animals. Right: Expression of *Actb* and *Aqp4* in the retina of a wild-type (1) and an AQP4 null animal (2), as determined by RT-PCR. The controls (c) were done by adding double-distilled water instead of cDNA as template. The diagrams display mean values with SEM of four or five independent experiments. Significant difference between tissues from wild-type and AQP4 null animals: * $P < 0.05$, ** $P < 0.01$.

at both limiting membranes and around the blood vessels (Fig. 2A). A high density of AQP4 protein was also found in the ganglion cell and nerve fiber layers and in both plexiform layers (Fig. 2A). No specific immunostaining of AQP4 was found in the retina of AQP4 null mice (Fig. 2B). The distribution of AQP1 immunolabeling was similar in slices from wild-type and AQP4 null animals (Fig. 2C,D). In the murine retina, AQP1 is predominantly localized to photoreceptor cells; in addition, AQP1-expressing erythrocytes within the vessels were stained (Fig. 2C,D). No nonspecific labeling was observed following incubation with secondary antibodies alone (inset in Fig. 2).

Retinal Localization of Kir4.1 and GFAP

To reveal whether AQP4 deficiency results in retinal gliosis, we immunostained retinal slices against GFAP and Kir4.1. Alterations in the retinal distribution of Kir4.1 had been observed in rat models of retinopathies (Pannicke et al., 2004, 2005). The Kir4.1 immunolabeling displayed a similar distribution in retinal slices from wild-type and AQP4 null mice. Kir4.1 labeling was localized predominantly to both limiting membranes and to perivascular glial membranes (Fig. 2E,F). Up-regulation of GFAP is a common response of retinal glial (Müller) cells to pathological stimuli (Bringmann et al.,

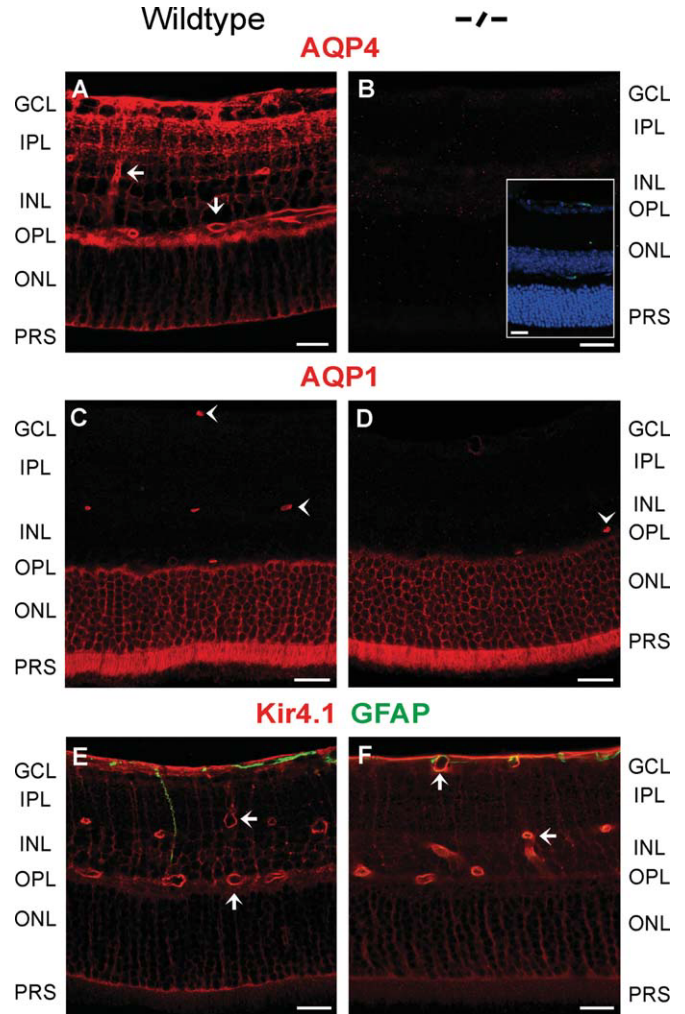


Fig. 2. Immunolabeling for several proteins in retinal slices from wild-type (A,C,E) and AQP4 null (-/-) mice (B,D,F). **A,B:** Staining against AQP4 revealed the typical pattern with strong immunoreactivity at the vitreous border and around blood vessels in the wild-type retina. Specific AQP4 immunostaining was absent in the slice from the AQP4 null animal. **Inset:** Negative control (incubation with both secondary antibodies in the absence of primary antibodies) revealed no specific staining. Cell nuclei were stained with TO-PRO-3 (blue). **C,D:** No difference was found in the immunostaining of AQP1. **E,F:** Immunolocalization of the potassium channel Kir4.1 and of the filament protein GFAP were not altered in the AQP4 null mice. Arrows, perivascular staining; arrowheads, AQP1-positive red blood cells within the vessels. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRS, photoreceptor segments. Scale bars = 20 μ m.

2006). In retinal slices from wild-type and AQP4 null mice, GFAP labeling was largely restricted to the innermost retinal layers, i.e., nerve fiber and ganglion cell layers, suggesting that GFAP was predominantly expressed by retinal astrocytes (Fig. 2E,F). The inner stem processes of a few Müller cells were also immunopositive for GFAP (Fig. 2E). The absence of GFAP

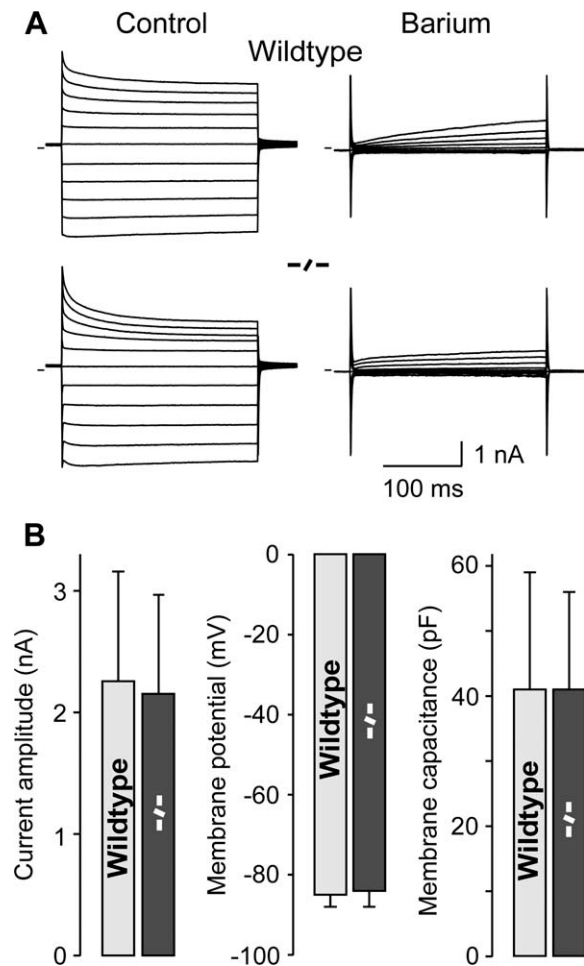


Fig. 3. Potassium currents of isolated retinal glial cells from wild-type and AQP4 null ($-/-$) mice. **A**: Representative traces of whole-cell potassium currents recorded in the absence and presence of Kir channel-blocking barium ions (0.3 mM). Voltage steps were applied from a holding potential of -80 mV to de- and hyperpolarizing potentials between -180 and $+20$ mV (250 msec, 20-mV increment). Small bars at left indicate zero-current levels. **B**: Amplitude of the inward potassium currents (left), resting membrane potential (middle), and cell membrane capacitance (right). The bars display data obtained from 17–33 cells.

immunolabeling from the majority of Müller cells and the unaltered retinal distribution of Kir4.1 largely rule out the possibility that AQP4 deficiency results in Müller cell gliosis in the absence of any pathology. No non-specific labeling was observed following incubation with secondary antibodies alone (inset in Fig. 2).

Potassium Currents of Retinal Glial Cells

It has been shown that the potassium currents of retinal glial cells are not altered in AQP4 null mice compared with cells of control mice (Ruiz-Ederra et al., 2007). To test whether this is also the case in the animals used in the present study, we recorded the potassium currents of freshly isolated retinal glial cells in the ab-

sence and presence of barium, a known blocker of glial Kir channels (Newman, 1989). As shown in Figure 3A, the whole-cell potassium currents were qualitatively not different in cells from wild-type and AQP4 null animals. The amplitude of the inward potassium currents, the resting membrane potential, and the membrane capacitance also were similar in cells from wild-type and AQP4 null mice (Fig. 3B). The unaltered membrane capacitance, which is proportional to the plasma membrane area of the cells, eliminates the possibility of cellular hypertrophy of cells from AQP4 null mice. In addition, we did not find any indication for a difference in the incidence of other types of currents such as the transient (A-type) potassium current and the voltage-gated sodium current between cells from wild-type and AQP4 null mice (not shown).

Osmotic Swelling Properties of Retinal Glial Cells

The swelling of Müller cell somata was investigated by superfusion of freshly isolated retinal slices with a hypoosmolar solution (containing 60% of control osmolarity). Under isotonic conditions, the soma areas were not different between glial cells from wild-type ($49.9 \pm 1.0 \mu\text{m}^2$, $n = 78$) and AQP4 null animals ($48.9 \pm 1.1 \mu\text{m}^2$, $n = 75$; $P > 0.05$). Hypotonic exposure for 4 min did not evoke a significant swelling of glial cell bodies in retinal slices from wild-type mice (Fig. 4A,B). Thus, the degree of swelling after 4 min was taken for comparison with other conditions. However, glial somata in slices from wild-type mice displayed a rapid and strong swelling within 4 min of hypotonic exposure in the presence of barium chloride (1 mM; Fig. 4A,B). Thus, pharmacological experiments to study the swelling behaviour of Müller cells in wild-type retinæ were done in the presence of barium ions (see Figs. 5, 6). In contrast to wild-type cells, glial cells in retinal slices from AQP4 null mice displayed a strong swelling of their cell bodies under hypotonic conditions already in the absence of barium (Fig. 4A). The amplitude of glial swelling in slices from AQP4 null mice was similar in the absence and presence of barium (Fig. 4B), whereas the time course of swelling was different: glial somata swelled with a delay of approximately 1 min in the absence of barium as compared with barium-containing conditions (Fig. 4A). The soma area measured 1, 1.5, and 2 min after beginning of hypotonic exposure was significantly ($P < 0.05$) greater in the presence than in the absence of barium ($n = 9-10$). The data suggest that retinal glial cells from AQP4 null animals are more sensitive to osmotic stress than glial cells from wild-type animals.

Involvement of Inflammatory Mediators and Oxidative Stress in Glial Swelling

Inflammatory lipid mediators and oxidative stress were suggested to be involved in the mediation of osmotic glial cell swelling. First, arachidonic acid, prostaglandin E_2 , or hydrogen peroxide was applied to slices from wild-type mice. These substances caused a swelling

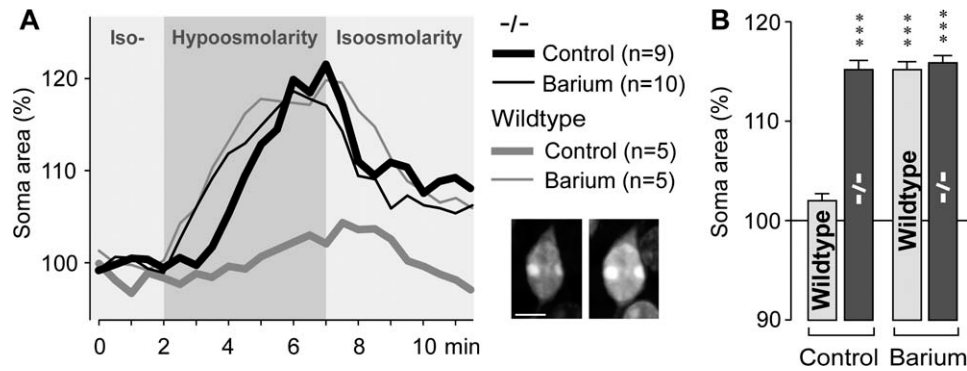


Fig. 4. Osmotic swelling properties of retinal glial cells from wild-type and AQP4 null ($-/-$) mice. Retinal slices were superfused with a hypotonic solution (60% of control ionic strength) in the absence (control) and presence of barium chloride (1 mM), and the cross-sectional area of glial somata in the inner nuclear layer was recorded. **A:** Time-dependent alteration of the soma area. Hypotonic challenge induced swelling of glial cell bodies in retinal slices from AQP4 null animals but not in slices from wild-type animals. Glial cell bodies in slices from wild-type and AQP4 null mice swelled in the presence of

barium. The images display original records of a dye-filled glial soma in a slice from a wild-type animal, obtained before (left) and after (right) a 4-min hypoosmotic exposure in the presence of barium. **B:** Mean (with SEM) soma areas of retinal glial cells, which were measured after a 4-min perfusion with a hypoosmolar solution ($n = 49-83$ cells per bar). Data are expressed in percentage of the soma size recorded before hypoosmotic challenge (100%). Significant difference vs. wild-type control: $***P < 0.001$. Scale bar = 5 μm .

of Müller cells under hypotonic conditions (Fig. 5A). The barium-evoked swelling described above was significantly inhibited under the following conditions: 1) when the formation of arachidonic acid was blocked in the presence of the selective inhibitor of the phospholipase A_2 4-bromophenacyl bromide; 2) in the presence of an inhibitor of the cyclooxygenase indomethacin; and 3) in the presence of dithiothreitol, a cell-permeable reducing agent (Fig. 5B). The barium-evoked osmotic swelling of retinal glial cells was also abrogated in the presence of an extracellular sodium-free solution (Fig. 5B), suggesting that the swelling was caused by intracellular sodium overload.

Similarly to the effects in wild-type tissue, glial cell bodies in retinal slices from AQP4 null mice swelled in the presence of arachidonic acid, prostaglandin E_2 , and hydrogen peroxide (Fig. 5A). The amplitude of this swelling was not significantly different from the osmotically induced swelling observed in the absence of these substances; i.e., there was no additive effect. Moreover, the swelling of Müller cells from AQP4 null mice induced in hypotonic solution without barium was abrogated in the presence of 4-bromophenacyl bromide, indomethacin, dithiothreitol, and an extracellular sodium-free solution (Fig. 5B), suggesting that inflammatory processes are involved in the increased susceptibility to osmotic stress.

Glutamatergic–Purinergetic Inhibition of Glial Swelling

Retinal glial cells of the rat possess an autocrine purinergetic signaling mechanism that inhibits experimentally induced osmotic swelling (Uckermann et al., 2006). To determine whether a similar signaling mechanism inhibits the swelling of retinal glial cells from AQP4 null

mice, we tested various receptor agonists. As shown in Figure 6A, administration of glutamate, adenosine 5'-triphosphate (ATP), or adenosine suppressed the osmotic swelling of retinal glial cells from AQP4 null mice. The effects of the receptor agonists were abrogated in the presence of the selective antagonist of adenosine A_1 receptors DPCPX (Fig. 6A). Similar data were obtained with respect to the barium-evoked osmotic swelling of glial cells in retinal slices from wild-type mice (Fig. 6B). The inhibitory effect of glutamate on the swelling of retinal glial cells from AQP4 null (Fig. 6C) and wild-type mice (Fig. 6D) was also abrogated in the presence of a selective $P2Y_1$ antagonist, MRS2179. The data suggest that the effect of glutamate is mediated by transactivation of $P2Y_1$ and A_1 receptors, likely via stimulation of a release of endogenous ATP and adenosine.

DISCUSSION

Mechanisms of Osmotic Glial Cell Swelling

Normal Müller cells are capable of efficient volume regulation and thus maintain a constant soma volume in hypoosmotic solution for at least 4 min (Fig. 4A; Pannicke et al., 2004). Müller cells from AQP4 null animals lose this capability after 1 min of exposure to hypoosmotic solution; if the Kir channels are blocked by barium, both normal and AQP4-deprived cells swell immediately after onset of the osmotic challenge (Fig. 4A). Although these observations support the view that ion and water efflux through Kir and AQP4 channels are involved in normal cell volume control, a constant cell volume can be maintained under certain conditions in the absence of AQP4 or functional Kir channels (Fig. 6). Thus, the mechanisms of pathological glial cell swelling seem to be rather complex.

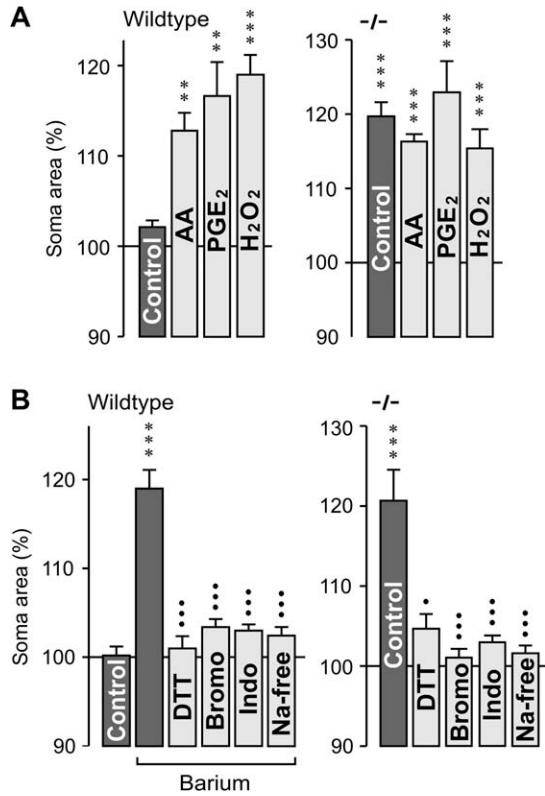


Fig. 5. Oxidative stress and inflammatory mediators are involved in the induction of osmotic glial cell swelling. **A:** Mean (with SEM) soma areas of retinal glial cells from wild-type and AQP4 null (-/-) mice, which were measured after a 4-min superfusion of retinal slices with a hypoosmolar solution. Data are expressed in percentage of the soma size recorded before hypotonic challenge (100%). Hypotonic challenge evoked swelling of glial somata in slices from AQP4 null but not wild-type animals (control). Swelling was observed in all retinal slices investigated in the presence of the following agents: arachidonic acid (AA; 10 μM), prostaglandin E₂ (PGE₂; 30 nM), and H₂O₂ (50 μM). **B:** The osmotic swelling of glial somata in slices from wild-type and AQP4 null (-/-) animals was inhibited in the presence of the following agents: the reducing agent, dithiothreitol (DTT; 3 mM), the selective inhibitor of phospholipase A₂ activation 4-bromophenacyl bromide (Bromo; 300 μM), and the cyclooxygenase inhibitor indomethacin (Indo; 10 μM). The soma swelling was also prevented in the absence of extracellular sodium ions. The solutions contained barium chloride (1 mM) as indicated. The diagrams display data obtained from six to 12 cells per bar. Significant swelling induction compared with nonswelling wild-type control: ***P* < 0.01, ****P* < 0.001. Significant swelling-inhibitory effect: ●*P* < 0.05, ●●*P* < 0.01, ●●●*P* < 0.001.

Oxidative stress and the formation of arachidonic acid and prostaglandins are apparently involved in osmotic swelling of Müller cells (Fig. 5B). It has been shown in various cell systems that the activity of phospholipase A₂ is increased in response to osmotic challenge and oxidative stress, resulting in lipid peroxidation and release of arachidonic acid from the membrane (Landino et al., 1996; Balboa and Balsinde, 2006; Lambert et al., 2006). Free radicals and hydroperoxides also

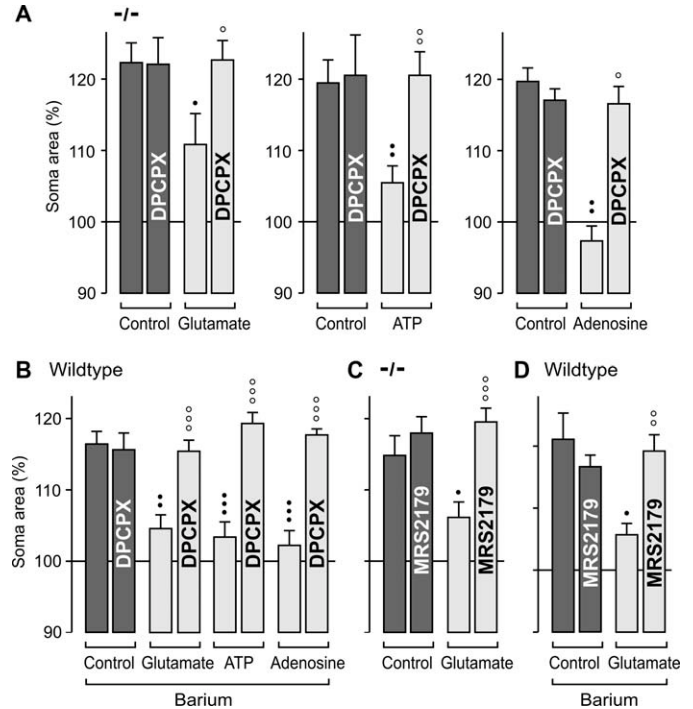


Fig. 6. Activation of glutamate and purinergic receptors inhibits the osmotic swelling of glial somata in retinal slices from wild-type and AQP4 null (-/-) mice. **A,B:** The swelling-inhibitory effects of glutamate (1 mM), ATP (50 μM), and adenosine (10 μM) were abrogated in the presence of the selective antagonist of A₁ adenosine receptors DPCPX (100 nM). **C,D:** The effect of glutamate was prevented by the P2Y₁-selective antagonist MRS2179 (30 μM). The bars display mean (with SEM) cross-sectional areas of glial somata (n = 7–12 per bar), which were measured after a 4-min superfusion of the slices with a hypoosmolar solution, and are expressed in percentage of the soma size recorded before hypotonic challenge (100%). The solutions contained barium chloride (1 mM) as indicated. Significant swelling-inhibitory effect: ●*P* < 0.05, ●●*P* < 0.01, ●●●*P* < 0.001. Significant inhibition of the agonist effect: °*P* < 0.05, °°*P* < 0.01, °°°*P* < 0.001.

stimulate the activities of lipoxygenase and cyclooxygenase (Asano et al., 1987). Arachidonic acid and prostaglandins are potent inhibitors of the sodium pump; inhibition of the pump results in intracellular sodium overload and swelling of cultured cells (Staub et al., 1994; Owada et al., 1999). The swelling of Müller cells was abrogated in the presence of a sodium-free solution (Fig. 5B), suggesting that it was caused by intracellular sodium overload associated with a water influx. Furthermore, arachidonic acid is known to block membrane channels such as volume-regulated anion and outwardly rectifying potassium channels (Lambert, 1991; Bringmann et al., 1998), which may mediate a compensatory efflux of osmolytes such as amino acids, chloride, and potassium ions.

Oxidative stress involved in glial swelling may be induced by nitric oxide produced by iNOS, which was expressed at an elevated level in the retina of AQP4 null

animals compared with the wild-type tissue (Fig. 1). It has been shown, for example, that peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis (Landino et al., 1996). Although we did not determine the cellular localization of iNOS, it is known that Müller cells increase its expression under pathological conditions (Jacquemin et al., 1996; Kobayashi et al., 2000; Abu-El-Asrar et al., 2001). Thus, an up-regulation of iNOS may result in increased oxidative stress in glial cells favoring cellular swelling under hypotonic conditions.

As previously described (Ruiz-Ederra et al., 2007), the glial potassium currents and the retinal distribution of Kir4.1 were not different between wild-type and AQP4 null mice. Accordingly, the presence of Kir channel-blocking barium ions evoked a rapid swelling in cells from AQP4 null and wild-type mice (Fig. 4A,B). This suggests that Kir channels play a role in preventing glial swelling, insofar as an efflux through these channels may compensate the osmotic gradient across the membrane (Pannicke et al., 2004). Inhibition of this potassium efflux will induce swelling because the osmotic gradient causes mechanical stress resulting in the activation of iNOS and phospholipase A₂. Based on the fact that Kir4.1 and AQP4 are colocalized in distinct membrane domains, it has been suggested that currents through Kir4.1 are associated with water movement through AQP4 (Nagelhus et al., 1999). Indeed, AQP4 deficiency or mislocation may result in impaired potassium clearance by glial cells (Amiry-Moghaddam et al., 2003; Binder et al., 2006). Similarly, the Kir channel-mediated potassium conductance in cells from AQP4 null mice should not allow for sufficient volume control, because it is not accompanied by the corresponding water channels; in fact, these cells swell (Fig. 4A,B). However, there was a significant delay in the onset of swelling of approximately 1 min (Fig. 4) in cells from AQP4 null animals. It can be speculated that, during this time, an early cell volume homeostasis is mediated by a potassium efflux through Kir4.1; however, the concomitant water flux across the membrane seems to be insufficient in cells from AQP4 null animals, resulting in delayed cell swelling. Although determining the precise mechanisms of osmotic swelling requires further investigation, our data demonstrate that an influx of Na⁺ ions (as revealed by the effect of Na⁺-free solution), a decrease of the K⁺ conductance (mainly the decrease of compensatory outward currents through Kir4.1 channels), and a lack of AQP4 cause Müller cell swelling under hypotonic conditions.

Inhibition of Osmotic Glial Cell Swelling

Although deletion of AQP4 results in mildly abnormal retinal function (Li et al., 2002), it is not associated with retinal gliosis or degeneration. Moreover, Da and Verkman (2004) even described a neuroprotective effect of AQP4 deletion in a model of transient retinal ischemia. The absence of gliosis after

AQP4 deletion is indicated by the unaltered distribution of GFAP and Kir4.1, and the unaltered potassium conductance and membrane capacitance of glial cells. The absence of retinal gliosis and degeneration suggests that alternative mechanisms of glial volume homeostasis may compensate for the deficit (and maintain retinal integrity) in AQP4 null mice. We found that activation of glutamatergic or purinergic receptors prevents the osmotic swelling of Müller cells from AQP4 null animals (Fig. 6A). Because the effect of glutamate is inhibited by P2Y₁- and A₁-selective blockers (Fig. 6A,C), we suggest that murine Müller cells possess an autocrine signaling mechanism that inhibits osmotic swelling similar to that previously described for rat glial cells (Uckermann et al., 2006). Apparently, glutamate induces a release of endogenous ATP and adenosine from the cells, and autocrine activation of purinergic receptors is a downstream mediator of glutamate action. The presence of this signaling mechanism may explain the absence of degenerative changes in the retina of AQP4 null mice. Although intense neuronal activity is associated with hypoosmotic extracellular conditions (Dietzel et al., 1989; Dmitriev et al., 1999), glutamate released from excited neurons may evoke purinergic receptor activation in Müller cells and, thus, may limit glial swelling. Membrane stretch is a known inducer of ATP release from Müller cells (Newman, 2001). Osmotically induced membrane stretch resulting in a release of ATP may be involved in the glial cell volume homeostasis in wild-type animals.

Inflammatory Response of the Retinal Tissue

We show here that deletion of AQP4 is associated with a distinct inflammatory response of the retina. This low-level retinal inflammation is characterized by an up-regulation of the inflammatory proteins IL-1 β , IL-6, and iNOS and a down-regulation of cyclooxygenase-2, whereas other inflammatory factors such as tumor necrosis factor- α are unaltered in their expression (Fig. 1). The reason for the altered expression of inflammatory proteins is unclear but could be related to osmotic stress conditions or to alterations in protein-protein interaction followed by changes in gene expression. It was shown, for example, that AQP4 and NOS are clustered by dystrophin (Thomas et al., 1998; Liu et al., 1999) and that the AQP4-containing macromolecular complex in glial membranes includes other proteins such as glutamate transporters (Hinson et al., 2008). Whether the down-regulation of cyclooxygenase-2 is an adaptation to avoid overproduction of prostaglandins, which induce glial swelling in the presence of an osmotic gradient (Fig. 5), remains to be determined. Up-regulation of proinflammatory proteins may also represent a neuroprotective response to osmotic stress. Different inflammatory factors such as IL-6 and NO have been shown to have neuroprotective effects (Kashii et al., 1996; Sanchez et al., 2003).

Possible Implications for Neuromyelitis Optica

It has been demonstrated that autoantibodies against AQP4 are involved in neuromyelitis optica (NMO), an uncommon, life-threatening demyelinating disease that produces transverse myelitis and optic neuritis (Lennon et al., 2005; Takahashi et al., 2007). These antibodies induce endocytosis of AQP4 and other proteins that exist as a macromolecular complex in glial membranes (Hinson et al., 2008). This results in a down-regulation and a possible lack of functional AQP4, which is an analogy to AQP4 null mice. Thus, the present data demonstrating that deletion of AQP4 results in swelling of Müller cells under hypotonic conditions (Fig. 4A,B) may help in better understanding NMO. This inflammatory demyelinating disorder, which attacks primarily optic nerves and spinal cord but also other neural tissues including the retinal nerve fiber layer (Wingerchuk et al., 2007; Plant, 2008), is characterized by astrocytic damage and by decreases in AQP4 and GFAP (Misu et al., 2007). Complement-activating serum antibodies to AQP4 that form focal vascular deposits were suggested to play a pathogenic role in NMO (Lennon et al., 2005; Roemer et al., 2007; Takahashi et al., 2007). In addition to fibrous astrocytes in the optic nerve, AQP4 is highly expressed by Müller cells (Nagelhus et al., 1998, 1999) and is enriched in glial membranes contacting retinal ganglion cells and nerve fibers (Fig. 2). Thus, it can be speculated that an antibody-induced dysfunction of glial water transport resulting in cytotoxic swelling of optic nerve astrocytes and Müller cells may contribute to glial damage and tissue edema. Glial swelling associated with a decrease in the extracellular volume may affect retinal ganglion cells via inducing hyperexcitability (Dudek et al., 1990; Chebabo et al., 1995), possibly contributing to glutamate toxicity, which has been implicated in NMO (Hinson et al., 2008). In addition to the malfunction (or lack) of AQP4, the presence of an osmotic gradient across glial membranes seems to be required for the induction of swelling. Hypoosmotic stress is a common characteristic of the neural tissue in periods of intense neuronal activity (Dietzel et al., 1989; Dmitriev et al., 1999), and a decrease in the blood osmolarity, e.g., in cases of hyponatremia and hypoalbuminemia, may contribute to a swelling of perivascular glial processes. A distinct susceptibility of the retinal nerve fiber layer could be explained by the enrichment of AQP4 in this layer (Fig. 2) and by the particular sensitivity for pathogenic stimuli of glial cells that surround the superficial retinal vessels (Iandiev et al., 2006b). However, the pathogenic role of AQP4 in NMO remains to be determined in further investigations.

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