Published in final edited form as:

Mol Cell Neurosci. 2008 January ; 37(1): 1–10. doi:10.1016/j.mcn.2007.08.007.

AQUAPORIN-4 INDEPENDENT Kir4.1 K⁺ CHANNEL FUNCTION IN BRAIN GLIAL CELLS

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Abstract

Functional interaction of glial water channel aquaporin-4 (AQP4) and inwardly rectifying K⁺ channel Kir4.1 has been suggested from their apparent colocalization and biochemical interaction, and from the slowed glial cell K⁺ uptake in AQP4-deficient brain. Here, we report multiple lines of evidence against functionally significant AQP4/Kir4.1 interactions. Whole-cell patch-clamp of freshly isolated glial cells from brains of wild-type and AQP4 null mice showed no significant differences in membrane potential, barium-sensitive Kir4.1 K⁺ current or current-voltage curves. Single-channel patch-clamp showed no differences in Kir4.1 unitary conductance, voltage-dependent open probability or current-voltage relationship. Also, Kir4.1 protein expression and distribution were similar in wild-type and AQP4 null mouse brain and in the freshly isolated glial cells. Functional inhibition of Kir4.1 by barium or RNAi knock-down in primary glial cell cultures from mouse brain did not significantly alter AQP4 water permeability, as assayed by calcein fluorescence quenching following osmotic challenge. These studies provide direct evidence against functionally significant AQP4-Kir4.1 interactions in mouse glial cells, indicating the need to identify new mechanism(s) to account for altered seizure dynamics and extracellular space K⁺ buffering in AQP4 deficiency.

Key terms

AQP4; Kir4.1; water channel; potassium channel; astrocyte; glia; epilepsy

INTRODUCTION

Several lines of indirect evidence support the possibility of functionally significant interactions between Kir4.1 K⁺ channels and aquaporin-4 (AQP4) water channels in glial cells. Kir4.1 and AQP4 protein colocalize on plasma membranes in supporting cells in the central nervous system that are closely associated electrically excitable cells, including glial cells in brain, which are associated with neurons, Müller cells in retina, which are associated with bipolar cells, and Hensen's/Claudianus' cells in cochlea, which are associated with hair cells (Nagelhus et al., 2004; Takumi et al., 1998). Electron microscopy has shown similar Kir4.1 and AQP4 distributions in subvitreous and perivascular membranes of Müller cells (Nagelhus et al., 1999). Immunoprecipitation has shown AQP4 and Kir4.1 interaction by co-associations with

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the multi-protein dystrophin-glycoprotein complex (DGC) in brain and retina (Connors et al., 2004; Connors and Kofuji, 2006). From these observations it is believed that Kir4.1 and AQP4 are key constituents of a multi-molecular complex in glial cells that includes α -syntrophin, Dp71, dystrobrevin, α -dystroglycan and β -dystroglycan (Amiry-Moghaddam et al., 2004; Kofuji and Newman, 2004), and that this physical linkage is functionally important.

There is also physiological evidence that functional Kir4.1-AQP4 coupling, if it occurs, may be important in neuroexcitation. AQP4 gene deletion in mice reduces seizure susceptibility and increases seizure duration (Binder et al., 2004a; 2006), greatly reduces auditory evoked potentials responses (Li and Verkman, 2001) and mildly reduces electroretinogram potentials (Li et al., 2002). Cellular K^+ reuptake from brain extracellular space is impaired in AQP4 null mice in models of neuroexcitation, including cortical spreading depression (Padmawar et al., 2005) and electrical seizure induction (Binder et al., 2006). A similar seizure phenotype and delay in K^+ uptake was found in α -syntrophin knockout mice that manifest altered glial cells AQP4 localization (Neely et al., 2001). It was proposed that defective Kir4.1-facilitated cellular K^+ uptake in AQP4 deficiency could account for these phenotype findings (Amiry-Moghaddam et al., 2003), though alternative possibilities, such as extracellular space expansion in AQP4 deficiency (Papadopoulos et al., 2005a; 2005b), have been proposed.

Here, we tested the possibility of functionally significant interactions between glial cell Kir4.1 and AQP4. Kir4.1 K^+ channel function was assayed by whole-cell and single-channel patch-clamp in freshly isolated mouse glial cells, and AQP4 water channel function was measured by calcein fluorescence quenching in glial cell cultures. Effects of AQP4 on Kir4.1 function were measured by comparing glial cells from wild-type vs. AQP4 null mice, and effects of Kir4.1 on AQP4 function were measured by comparing glial cell cultures after barium inhibition of Kir4.1 and Kir4.1 RNAi knock-down. Our experiments provide direct evidence against functionally significant Kir4.1-AQP4 interactions in mouse glial cells, indicating the need to identify alternate mechanism(s) to account for the neuroexcitation phenotypes associated with AQP4 deficiency.

METHODS

AQP4 null mice

AQP4 null mice were generated by targeted gene disruption as described (Ma et al., 1997). Weight-matched male mice in a CD1 genetic background were used. Investigators were blinded to genotype information in all experiments. Protocols were approved by the University of California San Francisco Committee on Animal Research.

Glial cell isolation

Astroglial cells were freshly isolated from hippocampus of wild-type and AQP4 null mice using a modification of a reported protocol (Kimmelberg et al., 2000). Postnatal 21–28 day old mice were anesthetized with intraperitoneal 2,2,2-tribromoethanol and then decapitated. The skull was opened within 1 min, and the brain was removed and transferred to ice-cold sucrose-aCSF containing (in mM): 206 sucrose, 2.8 KCl, 1.25 NaH_2PO_4 , 2 MgSO_4 , 1 MgCl_2 , 1 CaCl_2 , 26 NaHCO_3 and 10 glucose, pH 7.4 (equilibrated with 95% O_2 -5% CO_2). Hippocampi were dissected from the chilled brain hemispheres (on ice). Transverse hippocampal slices (500 μm) were cut and kept submerged for 30 min in aCSF (in mM): 124 NaCl, 5 KCl, 2 MgSO_4 , 2 CaCl_2 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , 10 D-glucose and 1 Na-pyruvate. Slices were then transferred into aCSF containing 24 U/ml papain and 0.24 mg/ml cysteine (as enzyme activator), and incubated for 30 min at 22 °C. To label astroglial cells for later identification, the partially digested slice fragments were incubated with sulforhodamine 101 (SR101, 1 μM , Invitrogen) in aCSF for 1 h at 22 °C (Nimmerjahn et al., 2004). Slice fragments were then

trituted and added onto poly-lysine-coated coverglasses in standard extracellular solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES and 10 dextrose, pH 7.4). Astroglial cells were identified by their red fluorescence when excited at 580 nm.

Glial cell culture

Astroglia were cultured from hippocampus of wild-type and AQP4 null neonatal mice as described (Solenov et al., 2004). Briefly, brains from 1-day-old postnatal mice were removed, hippocampi were isolated, minced with forceps, and incubated for 15 min in Hanks Balanced Salt Solution (HBSS) containing 0.25% trypsin and 0.1% DNase. Dissociated cells were centrifuged, resuspended in Dulbecco's Modified Eagle Medium (DME H-16) containing 15 mM glucose, 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin, seeded on 75 cm² flasks at a density of 300,000 cells/cm², and grown at 37 °C in a 5% CO₂ incubator with twice weekly medium change. At confluence (day 10), primary astroglia were shaken at 200 rpm for 18 h at 37 °C to remove microglia (Giulian and Baker, 1986). Cultures were then allowed to recover for at least 1 day in growth medium prior to experiments. Astroglial cells were then dissociated by trypsinization and reseeded onto the 18-mm diameter round coverglasses for water transport measurements and electrophysiology. Astroglial cell purity was >95% as assessed by immunofluorescence staining for glial fibrillary acidic protein (GFAP).

RNAi knock-down

Custom Stealth™ RNAi for Kir4.1 was obtained from Invitrogen (Carlsbad, CA). After plating on 18-mm round coverglasses as described above, cells were transfected using RNAiMAX (Invitrogen) according to manufacturer's instructions. RNAi (100 nM, Kir4.1 and non-specific control) were mixed with RNAiMAX (Invitrogen), diluted 1:50 in Opti-MEM (Invitrogen), and incubated for 20 min at room temperature for complex formation. The mixture was added to the cells, the medium was replaced at 6 h, and cells were incubated under normal growth conditions. Kir4.1 protein expression and osmotic water permeability were measured at 72–96 h after RNAi transfection.

Whole-cell patch-clamp

Whole-cell patch-clamp recording was done at room temperature (20–22 °C) using an Axon 200B amplifier (Molecular Devices, Union City, CA). All recordings were done within 4 h after plating. Standard external recording solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES and 10 dextrose, pH 7.4, adjusted with NaOH. In some experiments, BaCl₂ (final concentration 100 μM) was added to the bath solution. Exchange of perfusion solutions was done using a DAD-8VC superfusion drug application system (ALA Scientific Instruments, Westbury, NY). Microelectrodes with resistance of 3–5 Mohms were fabricated with P-97 Micropipette Puller (Sutter Instruments, Novato, CA). The intracellular (pipette) solution contained (in mM): 10 NaCl, 130 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 10 HEPES, and 3 Na₂ATP, pH 7.2, adjusted with NaOH. Data was acquired using a Digidata 1440A digitizer with Clampex 10.0 software (Molecular Devices). Data was sampled at 5 kHz and low-pass filtered at 5 kHz. Series resistance and capacitance compensation were not used during recordings. Data were analyzed by Clampfit 10.0 and Sigmaplot.

Single-channel patch-clamp

Cell-attached single-channel patch recordings were done at room temperature. Pipettes were pulled from borosilicate tubing (Sutter Instruments, Novato, CA). The tips of the pipettes were coated with sticky wax (Pourette Candlemaking Supplies, Seattle) and fire polished with a MF-830 Microforge (Narishige, Japan). Pipettes were filled with a solution containing (in mM): 145 KCl, 0.2 CaCl₂, 1 MgCl₂, 5 EGTA and 10 HEPES, pH 7.3, adjusted with KOH.

The bath solution contained (in mM): 145 KCl, 5 EGTA, 1 MgCl₂ and 10 HEPES, pH 7.4, adjusted with KOH. Data was sampled at 5 kHz using an Axon 200B amplifier and low-pass filtered at 1 kHz. Data were analyzed using Clampfit 10.0 software.

Immunocytochemistry

For immunostaining of isolated cells (as prepared for electrophysiological measurements) and astroglial cell cultures, adherent cells were fixed in 10% formalin (Sigma) for 15 min at room temperature, blocked with 1% bovine serum albumin (BSA), incubated with primary 1:200 rabbit anti-AQP4 antibody (Ab) (Alomone Labs, Jerusalem, Israel), 1:100 goat anti-Kir4.1 Ab (Santa Cruz Biotechnology, Inc.), or 1:500 mouse anti-GFAP (Sigma), and detected with Texas Red-conjugated donkey-anti rabbit, FITC conjugated donkey-goat mouse or AMCA conjugated donkey-anti mouse secondary antibodies (1:200 for both; Jackson ImmunoResearch Laboratories, Inc.). Nuclei were counterstained blue with 4,6-diamidino-2-phenylindole (DAPI). Sections were visualized with an inverted fluorescence microscope equipped with a high-resolution Spot color cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI).

Western blotting

Mice brain tissues were isolated and homogenized in 250 mM sucrose, 1 mM EDTA, 2 µg/ml aprotinin, 2 µg/ml pepstatin A, 2 µg/ml leupeptin, and 100 µg/ml serine protease inhibitor (Sigma). Homogenates were centrifuged at 1500 g for 5 min and the supernatant was collected and stored at -80°C. Protein concentration was measured by the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 10% or 12% NOVEX-NuPAGE 10% BT SDS-PAGE gels (10 µg protein/lane, Invitrogen). For astroglial cell cultures, cells were treated with 0.04% EDTA in PBS for 20 min, and suspended cells were collected and plasma membrane proteins were extracted as above. Proteins were electrotransferred to a polyvinylidene difluoride membrane and incubated with rabbit anti-AQP4 or anti-Kir4.1 antibody (1:1000 and 1:400, respectively; Alomone Labs) or anti-β-actin antibody (1:2000; GE Healthcare, Piscataway, NJ) followed by horseradish peroxidase-linked anti-rabbit IgG (1:10,000; GE Healthcare), and visualized by enhanced chemiluminescence (Roche Diagnostics, Indianapolis, IN). Band intensities were quantified by NIH Image, normalizing to β-actin immunoreactivity.

Osmotic water permeability

Cell plasma membrane water permeability was measured by a calcein-quenching method as described previously (Solenov et al., 2004). Astroglial cells on coverglasses were fluorescently labeled by incubation for 15 min with calcein-AM (Molecular Probes; 10 µM) at 22 °C. After rinsing in PBS (pH 7.4), the coverglass was mounted in a custom perfusion chamber with solution exchange time less than 200 ms at 50 ml/min perfusion rate. The time course of cytoplasmic calcein fluorescence was measured in response to cell swelling produced by the two-fold dilution of the extracellular bathing solution with water. Calcein fluorescence was measured continuously using a Nikon Diaphot inverted epifluorescence microscope equipped with halogen light source, calcein filter set (480-nm excitation, 490-nm dichroic mirror, 535-nm emission filter), photomultiplier detector, and 14-bit analog-to-digital converter.

RESULTS

Kir4.1 and AQP4 expression in brain astroglia

Kir4.1 and AQP4 expression were measured in brains of wild-type and AQP4 null mice. Immunoblot analysis with AQP4 antibody showed a band at 32 kDa in wild-type mice, which was absent in AQP4 null mice (Fig. 1A). Kir4.1 expression was seen as two bands, a 105 kDa

band, corresponding to the dimeric form of the channel, and a 50 KDa band, representing the monomer (Fig. 1B, left). Both bands were similar in hippocampus and brain cortex from wild-type and AQP4 null mice as seen qualitatively and by quantitative analysis after normalization to β -actin expression (Fig. 1B, right).

Freshly isolated astroglial cells from brain hippocampus were obtained by enzymatic digestion as described under Methods. Immunofluorescence of isolated cells from wild-type mouse brain showed GFAP immunoreactivity in all AQP4-positive cells (Fig. 1C). No AQP4 immunoreactivity was seen in cells from AQP4 null mouse brain. Kir4.1 and AQP4 were present in a subset of the cells, with greatest immunoreactivity seen at the cell plasma membrane and with some staining seen within cells (Fig. 1D). To quantify Kir4.1 and AQP4 expression, one hundred randomly selected cells per coverglass were examined, and data from six coverglasses from 3 mice were averaged. We found that $20 \pm 4\%$ of all cells in the freshly isolated cell mixture expressed Kir4.1 and $19 \pm 3\%$ expressed AQP4, with all AQP4-positive cells also Kir4.1 positive. Of the AQP4 null cells, $22 \pm 4\%$ of cells were Kir4.1-positive, similar to the percentage in wild-type cells.

Inwardly rectifying K⁺ channel currents in freshly isolated astroglial cells

Astroglial cells were labeled with SR101, a red fluorescent dye that has been used to label astroglial cells in *in vivo* imaging and *in vitro* brain slice preparations (Nimmerjahn et al., 2004). SR101 is selectively taken up by protoplasmic astrocytes and is stable within cells over several hours. In our protocol, about 20% of the freshly isolated cells in the cell mixture showed bright red fluorescence labeling, which is consistent the percentage data above. The SR101-positive cells in general showed the typical morphological characteristics of astroglial cells including small soma ($<10 \mu\text{m}$) and radiating processes (D'Ambrosio et al., 1998; Kimelberg et al., 2000) (Fig. 2A, lower right). Whole-cell currents were characterized in SR101-positive cells isolated from wild-type mouse brain. These cells never exhibited action potentials after injection of large depolarizing currents, indicating that they were not neurons. Under whole-cell voltage-clamp conditions (-80 mV holding potential), a series of voltage steps from -180 mV to $+60 \text{ mV}$ with 10 mV increment produced inward and outward currents as shown in Fig. 2A. The currents were strongly inhibited by $100 \mu\text{M}$ Ba^{2+} added to the bath solution. The Ba^{2+} -sensitive current showed weakly inward rectification (Fig. 2B) with a reversal potential of $-80.6 \pm 2.4 \text{ mV}$ ($n=8$), in agreement with the calculated K⁺ reversal potential of -82.8 mV . Increasing extracellular K⁺ concentration to 10 mM and 20 mM (by replacing NaCl) increased the inward currents and shifted the reversal potential to $-63.0 \pm 2.1 \text{ mV}$ and $-49.2 \pm 1.7 \text{ mV}$, respectively, in agreement with the calculated reversal potentials of -65.2 and -47.6 mV (Fig. 2B). We conclude that the Ba^{2+} -sensitive currents in the SR101-positive cells from wild-type mice is carried mainly by K⁺ ions. Similar data were obtained from the ramp stimuli recordings. As shown in Fig. 2C, after subtraction, the Ba^{2+} -sensitive currents showed inward rectification with a reversal potential of -83.1 mV . All of these characteristics are consistent with those reported for Kir4.1 channel-mediated currents in brain astrocytes and retina Müller cells (Kimelberg et al., 2000; Poopalasundaram et al., 2000).

Characterization of Kir4.1 channels in astroglial cells wild-type and AQP4 null mice

As a first assessment of whether AQP4 expression alters Kir4.1 K⁺ channel function, resting membrane potential (RMP) and whole-cell K⁺ currents were measured in freshly isolated astroglial cells from hippocampus of wild-type and AQP4 null mice. With a KCl-containing intracellular solution in the pipette, the distribution of RMPs was bimodal (Fig. 3A), in agreement with previous data obtained from cultured CA1 hippocampal astrocytes and astrocytes from hippocampal slices (D'Ambrosio et al., 1998; McKhann et al., 1997). The RMP distribution fitted with a bimodal Gaussian distribution with peaks at -63 mV and -43 mV for wild-type cells ($n=34$), not significantly different from -64 mV and -44 mV for AQP4

null cells (n=28) (Fig. 3A). Ba²⁺-sensitive Kir4.1 currents were found only in the cell population with the more negative RMP. For this cell population (21 cells from wild-type and 17 cells from AQP4 null mice), there was no significant difference in current-voltage relationships in wild-type vs. AQP4 null cells (Fig. 3B). We also used a voltage-ramp protocol to characterize Kir4.1 K⁺ currents. Fig. 3C summarizes the data, showing weak inward rectification with no significant differences in wild-type vs. AQP4 null astroglial cells.

The single-channel properties of Kir4.1 in freshly isolated astroglial cells were measured by the cell-attached patch-clamp technique with pipette and bath solutions containing 145 mM K⁺. Three different types of K⁺ channels were seen. At depolarized potentials (> +40 mV), large-conductance (~200 pS) K⁺ channel currents were sometimes seen, which have been identified as Ca²⁺-activated K⁺ channels (Gebremedhin et al., 2003). Also, a K⁺ channel with linear current (60–100 pS) at both depolarized and hyperpolarized potentials was sometimes recorded, with characteristics similar to the reported TREK-2 channel (Gnatenco et al., 2002) (data not shown). However, a single population of inwardly rectifying K⁺ currents was seen consistently in ten successful cell-attached patch recordings for each type, with ~40% of recordings showing single channels. Representative single-channel recordings at different holding potentials are shown in Fig. 4A. The currents through this channel were greater in the inward than outward directions, and opened in bursts. The unitary conductances (in the inward direction) were 20 ± 3 pS (n=4, wild-type) and 21 ± 4 pS (n=3, AQP4 null). Single-channel current-voltage analysis showed no significant differences in wild-type vs. AQP4 null astroglial cell (Fig. 4B). The channel opened in bursts with relatively high open probabilities at negative holding potentials, as summarized in Fig. 4C. Inhibition by Ba²⁺ from the extracellular surface was examined by recording channel activity in the cell-attached configuration with pipette solution containing 100 μM Ba²⁺. In contrast to the reduced unitary current amplitudes, Ba²⁺ produced a voltage-dependent, discrete block (Fig. 4C). The block was present at hyperpolarized potentials, with Ba²⁺ producing a decrease in channel open probabilities (Fig. 4D). These results support the assignment of this channel as Kir4.1, as do the open probabilities of 0.8–1.0 at –60 to –100 mV (see Discussion). There was no significant difference in these channel characteristics in astroglial cells isolated from wild-type vs. AQP4 null mice.

Together, these data indicate that astroglial cell Kir4.1 K⁺ channel function and expression is independent of AQP4 water channel expression. To further confirm this conclusion in a different cell preparation and to be able to determine effects of Kir4.1 on AQP4 water channel function, we established a model of primary cultured astroglial cells.

Kir4.1 expression and function in wild-type and AQP4 null astroglial cell cultures

Immunofluorescence showed that confluent astroglial cells cultured from wild-type and AQP4 null mice had similar expression of GFAP in intracellular fibers, with > 95% of cells positive for GFAP (Fig. 5A, left panel). More than 90% of the wild-type astroglial cells in culture coexpressed AQP4 and Kir4.1 protein (middle and right panels), with no detectable AQP4 expression in the AQP4 null cells. Similar Kir4.1 protein expression was seen in the cultured cells from wild-type and AQP4 null mice, with diffuse expression on the plasma membrane. By immunoblot analysis, cultured astroglial cells from wild-type mice showed AQP4 immunoreactivity at 32 kDa (Fig. 5B, left), which was absent in the cells from AQP4 null mice. The two bands corresponding to Kir4.1 were similar in the cultured cells from wild-type and AQP4 null mice (Fig. 5B, right).

Single channel recordings showed a unitary conductance of 21.7 pS, similar to that recorded in the isolated astroglial cells (Fig. 5C). Whole-cell patch-clamp showed similar, inwardly rectifying, Ba²⁺-sensitive Kir4.1 currents from wild-type and AQP4 null astroglial cells (Fig.

5D). These results in cultured astroglial cells support the conclusions from freshly isolated cells that AQP4 expression does not influence the function or expression of Kir4.1.

AQP4 water permeability following Kir4.1 inhibition and knock-down

RNAi was used to knock down Kir4.1 in the cultured astroglial cells. Knock-down was optimized after testing several RNAi sequences, concentrations, transfection conditions, and incubation times. Immunoblot analysis showed reduced Kir4.1 protein expression at day 3 (Fig. 6A left), with reduction in both the bands at 50 and 105 kDa. In several separate cultures Kir4.1 protein expression was reduced by ~ 50% (Fig. 6A, right). Kir4.1 function in the knock-down astroglial cells was studied by whole-cell patch-clamp by measurement of current responses to a series of voltage steps. Fig. 6B (right) shows strong reduction of the inwardly rectified currents in the Kir4.1 RNAi-treated astroglial cells (without effect on outward, delayed K⁺ currents). Inward current densities summarized in Fig. 6B (right) show a reduction in Kir4.1 RNAi-treated cells from 48 ± 13 pA/pF (control, S.E., n=7) to 4 ± 2 pA/pF (Kir4.1 RNAi, S.E., n=9). Few functional Kir4.1 channels were seen 3 days after Kir4.1 RNAi transfection.

Plasma membrane osmotic water permeability was measured by a calcein fluorescence-quenching method, which quantifies the kinetics of cell volume change following a rapidly imposed osmotic gradient. Osmotic water permeability was much lower in astroglial cells cultured from brains of AQP4 null than wild-type mice (Fig. 6C), in agreement with previous data (Saadoun et al., 2005; Solenov et al., 2004). There was no significant effect on astroglial cell water permeability of Kir4.1 RNA treatment, nor was there an effect of Kir4.1 inhibition by 100 μ M Ba²⁺. These results indicate that AQP4 water channel function in astroglial cells is not affected by the Kir4.1 K⁺ channel expression or function.

DISCUSSION

The purpose of this study was to test the hypothesis, based on indirect morphological, biochemical and physiological evidence, that glial cell AQP4 and Kir4.1 interact functionally. We found, by multiple criteria, no significant differences in Kir4.1 K⁺ channel function in freshly isolated glial cells from wild-type vs. AQP4 null mice, including resting cell membrane potential, whole-cell current analysis and single-channel analysis. Kir4.1 K⁺ channel function also did not differ in astroglial cell cultures from wild-type vs. AQP4 null mice. Further, we found no significant differences in AQP4 water permeability in primary glial cell cultures after barium inhibition of Kir4.1 function or RNAi knock-down of Kir4.1 expression. These results mandate the identification of alternative mechanisms to explain altered seizure and cortical spreading depression dynamics in AQP4 null mice, as well as the slowed K⁺ reuptake from the extracellular space following neuroexcitation (Binder et al., 2004a; 2006; Padmawar et al., 2005). The conclusions here are in agreement with our recent study showing unimpaired Kir4.1 K⁺ currents in retinal Müller cells from AQP4 knock-out mice (Ruiz-Ederra et al., 2007). However, in that study it was not possible to study effects of Kir4.1 inhibition/knock-down on AQP4 water permeability. Further, demonstration of unimpaired Kir4.1 function in retinal Müller cells does not have compelling physiological relevance because the visual phenotype in AQP4 null mice was very mild (Li et al., 2002), contrasting with the substantially more robust brain neuroexcitation phenotypes (Binder et al., 2004a; 2006). Possible differences in composition and/or assembly of the proposed dystrophin complex in glial vs. retinal Müller cells have been proposed to account for the different phenotypes.

Functional measurements of Kir4.1 were done on freshly isolated astroglial cells, well before possible phenotype changes associated with cell culture might occur. The astroglial cells were isolated by enzymatic digestion from brain slices as reported before (Kimelberg et al., 2000; Zhou and Kimelberg, 2001). The isolated astroglial cells retained numerous processes as they

display in intact brain. Several different types of K⁺ currents have been characterized in this model, including Kir4.1 channels (Kimmelberg et al., 2000). Viable astroglial cells were identified by SR101 staining, as reported for their labeling in *in vivo* imaging and *in vitro* brain slice preparations (Jourdain et al., 2007; Nimmerjahn et al., 2004).

We obtained a bimodal distribution of resting membrane potentials from the isolated astroglial cells, as found previously in hippocampus slices (D'Ambrosio et al., 1998). There are discrete classes of astroglial cells in hippocampus that differ in morphology and electrophysiological properties (D'Ambrosio et al., 1998). Kir4.1 channels are nearly fully open at resting potential, and as reported before, are expressed in about one-half of the astroglial cells (Higashi et al., 2001). The bimodal distribution of resting membrane potentials thus accounts for the differences in Kir4.1 expression. This interpretation is supported by our data showing that inwardly rectified K⁺ currents were never seen in the depolarized cell population.

Patch-clamp analysis indicated characteristic Kir4.1 K⁺ channel currents in the freshly isolated glial cells, including weak inward rectification, as found for Kir4.1 currents recorded from Müller cells (Ishii et al., 1997; Kofuji et al., 2000; Ruiz-Ederra et al., 2007), Kir4.1-transfected cells (Tada et al., 1998) and cultured astroglia (our result). Also, from single channel analysis, the Kir4.1 channel in freshly isolated glial cells showed a single population with 20–25 pS unitary conductance at negative potentials, and 0.8–1 open probability, in agreement with recordings from Müller cells and Kir4.1-transfected cells (Ishii et al., 1997; Ruiz-Ederra et al., 2007). However, neither the function nor the expression of Kir4.1 differed in wild-type vs. AQP4 null mice.

Measurements of AQP4 water permeability were done in primary glial cell cultures from neonatal brain cortex, as we have established in prior studies on effects of AQP4 deletion on glial cell water permeability (Solenov et al., 2004). Water permeability was measured by a calcein quenching method, which is based on the sensitivity of cytoplasmic calcein to cell volume. Glial cell cultures rather than isolated glial cells were used for these studies to allow for Kir4.1 RNAi knock-down and to give adequate signal-to-noise for accurate measurement of osmotic water permeability, which was not possible in freshly isolated, coverglass-immobilized glial cells. We found substantially more rapid osmotic equilibration in glial cell cultures from wild-type than from AQP4 null mouse brain; however, neither Kir4.1 inhibition by barium nor knock-down by RNAi affected AQP4 water permeability, indicating that the expression and function of AQP4 is independent of Kir4.1.

We conclude from these functional studies that altered Kir4.1 function in AQP4 deficiency does account for the mouse phenotype findings of altered seizure and cortical spreading depression dynamics in AQP4 deficiency, or of slowed K⁺ reuptake from brain extracellular space following neuroexcitation (Binder et al., 2006; Padmawar et al., 2005). Perhaps altered extracellular space volume or dynamics, which is supported by photobleaching measurements of macromolecular diffusion (Binder et al., 2004b; Papadopoulos et al., 2005a), is in part responsible for the mouse phenotype findings. Alternatively, other glial cell or neuronal ion transporters, whose expression or function are altered in AQP4 deficiency, may account for the phenotype findings.

Acknowledgments

Supported by grants DK35124, EY13574, EB00415, HL73856, HL59198 and DK72517 from the National Institutes of Health, and Research Development Program and Drug Discovery grants from the Cystic Fibrosis Foundation.

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Figure 1. Kir4.1 and AQP4 expression in mouse brain and isolated astroglial cells

A, AQP4 immunoblot in wild-type (+/+) and AQP4 null (-/-) mouse hippocampus. β-actin immunoblot shown for same samples. B, (left) Kir4.1 and β-actin immunoblot in brain hippocampus and cortex. (right) Kir4.1 expression normalized to β-actin expression (SE, n=3, differences not significant). C, Immunofluorescence of AQP4 (red) and GFAP (blue). Bar, 10 μm. D, Immunofluorescence of AQP4 (red) and Kir4.1 (green) in isolated cells from mouse hippocampus (nuclei stained blue with DAPI). Bar, 10 μm.

Figure 2. Whole-cell currents in isolated astroglial cells in wild-type mice

A, Representative recordings in extracellular solutions containing 5 or 20 mM KCl, or 5 mM KCl + 100 μM Ba²⁺. Voltage steps were applied from a holding potential of -80 mV to de- and hyper-polarizing potentials between -180 and +60 mV (250 ms, 10 mV increments). Inset at lower right shows SR-101-stained astroglial cells and unlabeled pyramidal neuron (overlays of phase-contrast and fluorescence) (top) and fluorescence micrograph of a freshly isolated astrocyte during recording (bottom). B, Current-voltage (I-V) curves of whole-cell currents for a single astroglial cell at different [K⁺]_o (5, 10 and 20 mM). Where indicated 100 μM Ba²⁺ was present in the extracellular solution. Data representative of 7 preparations. C, Whole-cell current elicited with a ramp protocol (left) with and without 100 μM Ba²⁺ and deduced I-V curve of Ba²⁺-sensitive current (right). Representative of 8 preparations.

Figure 3. Comparable whole-cell K⁺ currents in astroglial cells from wild-type and AQP4 null mice
 A, Distribution of the resting membrane potentials (V) of SR101-positive cells isolated from wild-type (n=34, top) and AQP4 null (n=28, bottom) mice. Fit shown to bimodal Gaussian distributions. B, Mean whole-cell current-voltage curves in the hyperpolarized subpopulation of astroglial cells mice (S.E., n=17-21). Differences not significant. C, Mean voltage-ramp currents (S.E., n=8 per group). Differences not significant. Inset. Currents recorded by stepping potential from -80 to -160 mV for 30 ms, followed by a ramp to +40 mV over 300 ms.

Figure 4. Single-channel patch-clamp of Kir4.1 K⁺ channels in freshly isolated astroglial cells
 A, Single-channel currents from cell-attached membrane patches of wide-type and AQP4 null astroglial cells with 145 mM K⁺ in the pipette. Holding potentials indicated to the left of each trace (c, channel closed level; o, channel open level). B, Unitary current-voltage data (S.E., n=4). Differences not significant. Dashed line shows linear regression of the inward current with inward single-channel conductances of 20 ± 3 pS (wild-type) and 21 ± 4 pS (AQP4 null). C, Open probability (P_o) (S.E., n=6). Differences not significant. Where indicated, 100 μM Ba²⁺ was present in the pipette solution. Data are from one set of experiments representative of three. D, Increased Kir4.1 channel closed time in the presence of 100 μM Ba²⁺ (holding potential -100 mV).

Figure 5. Characterization of astroglial cell cultures

A, (left) GFAP immunofluorescence of confluent wild-type and AQP4 null astroglial cell cultures (green). Bar, 10 μ m. (right panels) AQP4 (red) and Kir4.1 (green) immunofluorescence. Nuclei counterstained blue using DAPI. Bar, 100 μ m. B, AQP4 and Kir4.1 immunoblot, with β -actin used to normalize expression. Representative of 3 sets of culture. C, Single-channel current traces from cell-attached membrane patches at indicated holding potentials. Unitary current-voltage data shown, with dashed line giving unitary conductance 21.7 pS for inward currents. Data representative of 6 sets of preparations. D, Voltage steps applied from a holding potential of -80 mV to de- and hyper-polarizing potentials between -140 and $+40$ mV (250 ms, 10 mV increments). Ba^{2+} -sensitive Kir4.1 currents computed from difference before and after addition of $100 \mu\text{M}$ Ba^{2+} (S.E., $n=5$). Differences not significant.

Figure 6. Kir4.1 inhibition or knock-down does not affect AQP4 water permeability

A, (left) Kir4.1 immunoblot of wild-type astroglial cell cultures at 3 days after treatment with Kir4.1 or control RNAi. β -actin used to normalize expression. (right) Reduced Kir4.1 expression after RNAi (S.E, $n=3$). B, Whole-cell currents in control and Kir4.1 RNAi-treated wild-type astroglial cells. (left) Currents shown in response to voltage steps from a holding potential of -80 mV to de- and hyper-polarizing potentials between -160 and $+60$ mV (250 ms, 10 mV increments). (right) Reduced current density (pA/pF) in Kir4.1 RNAi-treated astroglial cells in response to voltage step from -80 mV to -160 mV (S.E., $n=8$). C, Osmotic water permeability. (left) Time course of calcein fluorescence in response to exchange between isosmolar and hypoosmolar (150 mOsm) solutions. Data shown for wild-type cell cultures under control conditions, and after Kir4.1 RNAi treatment or $100 \mu\text{M Ba}^{2+}$. Data for AQP4 null cells shown for comparison. (right) Summary of relative water permeability (S.E; $n=8$, $*P < 0.001$). Differences not significant among wild-type cultures.