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FUNCTIONAL AND MOLECULAR INTERACTIONS BETWEEN AQUAPORINS AND Na,K-ATPase

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Abstract—The water channel aquaporin 4 (AQP4) is abundantly expressed in astrocytes and provides a mechanism by which water permeability of the plasma membrane can be regulated. Astrocytes play a key role in the clearance of both potassium (K^+) and glutamate released during neuronal activity. Emerging evidence suggests that AQP4 facilitates K^+ clearance by astrocytes and contributes to recovery of neuronal excitability. Here we report that AQP4 can assemble with its regulator metabotropic glutamate receptor 5 (mGluR5) and with Na,K-ATPase; the enzyme responsible for active K^+ transport and for establishing the electrochemical gradient across the cell plasma membrane. We have, by use of pull down assays in rat brain tissue, identified the segment in the AQP4 NH_2-terminus containing the amino acid residues 23–32 as the site for interaction with Na,K-ATPase catalytic subunit and with mGluR5. Mutagenesis studies revealed that the AQP4 amino acids K27 and W30 are of key importance for interaction with both Na,K-ATPase and mGluR5. To confirm that interaction also occurs within intact cells, we have performed fluorescence resonance energy transfer (FRET) studies in primary astrocytes derived from rat striatum. The results indicate close proximity of wild type AQP4 and Na,K-ATPase in the plasma membrane of rat astrocytes. FRET efficiencies observed with the mutants AQP4 K27A and AQP4 W30A were significantly lower, highlighting the importance of these residues for the interaction between AQP4 and Na,K-ATPase. We conclude that AQP4/Na,K-ATPase/mGluR5 can form a macromolecular complex/transporting microdomain in astrocytes. This complex may be of functional importance for the regulation of water and K^+ homeostasis in the brain, as well as for neuron-astrocyte metabolic crosstalk.

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Abbreviations: AQP4, aquaporin 4; CTS, cardiotonic steroids; EAAT, excitatory amino acid transporter; EDTA, ethylene diamine tetraacetic acid; EPO, erythropoietin; GST, Glutathione S-transferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mGluR5, metabotropic glutamate receptor 5; OAPs, orthogonal arrays of proteins; pbFRET, photobleaching fluorescence resonance energy transfer.

Key words: astrocyte, water channel, metabotropic glutamate receptor, microdomain, metabolism, interaction.

The role of water channels—aquaporins—for bulk transport of water in epithelial cells is well understood (King et al., 2004; Tait et al., 2008). Emerging evidence suggests that aquaporins expressed in non-epithelial cells may also be involved in a variety of other physiological processes, including cell migration and tissue hydration (Saadoun et al., 2005; Auguste et al., 2007; Bloch and Manley, 2007). The predominant water channel in the brain is aquaporin 4 (AQP4), which is abundantly expressed in astrocytes. The physiological significance of AQP4 mediated water transport in astrocytes is still not completely understood. Here we propose that water transport via AQP4 may have a local modulatory effect on ionic gradients. The ion pump Na,K-ATPase plays a pivotal role for setting the sodium (Na^+) and potassium (K^+) ion gradients across the cell plasma membrane. Fine-tuned regulation of Na^+ and K^+ gradients are of utmost importance for the physiological function of the astrocyte and for its crosstalk with neurons. Astrocytes are responsible for the clearance of K^+ from the extracellular space after neuronal activity. Neuronal activity is also associated with release of glutamate, which is taken up by astrocytes via Na^+ coupled transport. Fluctuations in intracellular Na^+ concentration ($[Na^+]_i$) have been postulated to have a modulatory effect on the metabolic processes in the astrocyte (Bernardinelli et al., 2004). Here we present novel data which indicates that AQP4 and Na,K-ATPase are partners in the same protein complex and that AQP4 also interacts with metabotropic glutamate receptor 5 (mGluR5), which is a regulator of AQP4 activity. We discuss the role of AQP4, Na,K-ATPase and mGluR5 in astrocytes and their function in a transporting microdomain.

EXPERIMENTAL PROCEDURES

Materials

All chemicals, unless specifically mentioned, were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Expression plasmids

All constructs were cloned using Gateway cloning strategy (Invitrogen Corporation, Carlsbad, CA, USA). AQP4 DNA fragments were amplified using AmpliTaq GOLD (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The constructs and PCR primers are specified in Table 1 (Supplementary). DNA fragments were obtained either through PCR protocol or through direct annealing of oligonucleotides (for AQP4 NH_2-terminus N 23-32 and N 13-22 constructs). Fragments were cloned in a pENTR/D-TOPO vector

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and then subcloned into expression vectors pDEST-15 (Invitrogen Corporation, Carlsbad, CA, USA) to obtain glutathione S-transferase (GST) fusion proteins. AQP4 GST fusion peptides were expressed in the bacterial strain BL21-AI and purified on GST sepharose for consequent use in pull down assays. Venus fluorescent protein destination vector was generated from pEGFP-C vector (Clontech Laboratories, Palo Alto, CA, USA) using vector cassette (Invitrogen Corporation, Carlsbad, CA, USA) according to a Gateway® Vector Conversion protocol (Invitrogen Corporation, Carlsbad, CA, USA) by replacing the coding region for EGFP by Venus coding region. Full-length AQP4 coding region was cloned into the Venus destination vector. All point mutations were generated by QuickChange II XL (Stratagene, La Jolla, CA, USA). The construct N 1-22 was obtained from N 1-32 by introduction of stop codon. The nucleotide sequences of all constructs were confirmed by automated sequencing and subsequent bioinformatics analysis using Lasergene software (DNASTAR, Madison, WI, USA).

Co-immunoprecipitation

All experiments were approved by the local committee on Ethics for Animal Experimentation, Stockholm, Sweden, and carried out in accordance to the Swedish national law on the ethical use of animals. Efforts were made to minimize the number of animals used and their suffering. Tissue from rat cerebellum was homogenized in RIPA buffer: 10 mM Tris, 150 mM NaCl, 0.5% NP-40, 0.25% Na^+ dexoycholate, 1 mM EDTA, pH 7.4, containing 20 μg/mL protease inhibitor cocktail (Complete, Roche Applied Science, Basel, Switzerland). The homogenate was centrifuged at 2000 g at 4 °C for 10 min. The supernatant was precleared with Protein G Sepharose™ 4 Fast Flow (GE Healthcare Bio-sciences AB, Uppsala, Sweden) at 4 °C for 2 h on a rotator. Anti-AQP4 antibody (6 μg), or equal amount of nonspecific rabbit immunoglobulin was added to the precleared lysate (1.75 mg protein) and incubated at 4 °C for 3 h on a rotator. The precipitates were transferred to Protein G Sepharose and incubated at 4 °C on a rotator overnight. After incubation, the Protein G Sepharose beads were washed three times with RIPA buffer and the proteins were eluted by heating (80 °C for 15 min) in 50 μl of 2×SDS−PAGE sample buffer and analyzed by immunoblotting.

Pull down assay

All steps were performed at 4 °C if not denoted otherwise. Cerebellum from 20 day old male Sprague–Dawley rat was homogenized in ice cold TBS buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.25% m/v Na-deoxycholate, 1% v/v Triton X-100, containing 20 μg/ml protease inhibitor cocktail (Complete, Roche Applied Science, Basel, Switzerland)) and centrifuged 10,000 g for 30 min. The same procedures were used to prepare a protein lysate from a culture of the *E. coli* strain DH5α grown for 18 hours. AQP4 GST fusion peptides were expressed in the bacterial strain BL21-AI and purified on glutathione S-transferase sepharose (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Each sample of AQP4 GST fusion peptide, immobilized on 30 μl of glutathion-sepharose beads, was further incubated for 18 h with 2 mg of the cerebellum lysate and 2 mg of the protein lysate from the DH5α strain (to reduce unspecific binding). After incubation, the precipitates were washed six times with TBS buffer followed by elution in 1% SDS solution, heated at 37 °C for 30 min and analyzed by immunoblotting.

Antibodies

The following antibodies and dilutions were used: Na,K-ATPase α1 mouse monoclonal antibody (05-369 Millipore Corp., Bedford, MA, USA) dilution 1:10,000 for Western blot and 1:100 for pbFRET; Na,K-ATPase α2 (Millipore Corp., Bedford, MA, USA) 1:2000; mGluR5 rabbit polyclonal antibody (06−451 Millipore Corp., Bedford, MA, USA) 1:2000; AQP4 rabbit polyclonal antibody (AB3594, Millipore Corp., Bedford, MA, USA) 1:2000; secondary Cy3 conjugated goat anti mouse antibody (Jackson Immuno Research, Pittsburgh, PA, USA) 1:500; excitatory amino acid transporter 2 (EAAT2) (GLT-1) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:200; rabbit immunoglobulin (I8140, Sigma-Aldrich Corporation, St. Louis, MO, USA).

Cell culture

Primary cultures of rat astrocytes from striatum were prepared as described (Gunnarson et al., 2008) and used for water permeability studies, photobleaching fluorescence resonance energy transfer (pbFRET) and $[Na^+]_i$ recordings. Cells were grown at 37 °C in 5% CO_2 air in full medium (65% v/v DMEM 31885, 25% v/v Neurobasal medium 21103, 1 μM L-glutamine, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO Laboratories, Grand Island, NY, USA)) for 10 days (12 days for the water permeability studies).

Transfection

Transfection of primary astrocytes was performed for the pbFRET assay. Prior to transfection, the cell medium was substituted with Neurobasal medium 21103 without serum or antibiotics. Transfection was performed with Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA) at the time the cells were subconfluent, using 6.4 μg DNA and 12.6 μl of Lipofectamine 2000 for each 30 mm plate. Six hours after transfection a Venus fluorescent signal could be detected by fluorescence microscopy and cells were used for pbFRET.

Photobleaching fluorescence resonance energy transfer

For the pbFRET assay we used AQP4 as donor and Na,K-ATPase α1 as acceptor. Fluorescence was introduced by using a fluorescent protein, Venus, fused to AQP4 and immunofluorescent labeling of Na,K-ATPase with the fluorescent dye Cy3.

Rat primary astrocytes were transiently transfected with Venus fusion proteins of either wild type AQP4 (AQP4 WT), mutant AQP4 K27A or mutant AQP4 W30A. Six hours after transfection the cells were processed for immunofluorescence. The cells were fixed with −20 °C cold methanol in room temperature for 15 min, followed by incubation with blocking solution containing 5% v/v normal goat serum and 5% (m/v) non fat dry milk diluted in TPBS (KCl 107 mM, NaCl 3.4 mM, Na_2HPO_4 10 mM, KH_2PO_4 17.6 mM, Triton X 100 0.3% v/v, $MgCl_2$ 1 mM, $CaCl_2$ 0.4 mM) for 1 h. Cells were then incubated for 1 h with Na,K-ATPase α1 primary antibody in blocking solution, followed by 1 h incubation with secondary Cy3-conjugated antibody and mounted in Immu-Mount (Thermo electron corporation, Pittsburg). Specificity of the Na,K-ATPase α1 immunostaining was assessed by omitting the primary Na,K-ATPase α1 antibody in control samples. FRET experiments were performed using a Zeiss LSM 510 Meta confocal microscope with a 63×/1.4 NA objective. Donor fluorescence (Venus) was excited with 488 nm and detected with a BP505-530 filter. Acceptor fluorescence (Cy3) was excited with 543 nm and detected with a LP560 filter. All images were recorded with a pinhole setting of 1 AU. FRET was measured using the acceptor photobleaching protocol (Bastiaens et al., 1996) where the acceptor was irreversibly photobleached in a selected region of the cell by a combination of 543 and 633 nm excitation. FRET efficiency was calculated as the relative increase in donor signal after bleaching the acceptor.