Stable transfections

Stable transfections of MDCK cells were done using the neomycin resistance gene on the pDEST47 vector. Cells were transiently transfected using polyethyleneimine (PEI) as previously described. 4 μg of DNA and 12 μL of a 1 mg/mL stock of PEI was used per well of a 6 well culture plate. After 24 hours, cells were trypsinized, resuspended in culture media containing 700 μg/mL G418 and reseeded at varying dilutions, 1:2, 1:5, 1:10 and 1:20 in 4 wells of a 6 well plate. Transient transfection and reseeding was done in triplicate. G418-supplemented media was refreshed every 3 days. After 14 days, resistant colonies were inspected on a widefield fluorescence microscope for GFP expression. GFP-positive colonies were picked using 150 μL cloning cylinders (Corning). Colonies from the lowest dilution were preferentially isolated (e.g. colonies from a 1:10 plate were preferred to those from a 1:5 plate). Marker pen was used to label the plates at the positions of the desired colonies. For each stable transfection, a total of 3 clones were isolated. Plates were washed with calcium-free PBS. Cloning cylinders were placed over the pen-marked positions on the plate(s) and pressed down firmly to seal. 50 μL trypsin-EDTA was added to cylinders and incubated until cells were removable by gentle pippetting. Cells were removed, serially diluted by a factor of 2 (to give 1:2, 1:4, etc) up to 1:32, and seeded into wells of a 96 well plate. Culture media was added to a total volume of 100 μL/well. The lowest dilution that grew to confluence was trypsinized and transferred to a well of a 24 well plate, grown to confluence and transferred to a T25 flask. Upon reaching confluence, 2/3 of these cells were transferred to a T75 flask to grow for frozen stocks, and the remaining cells were seeded in 3 wells of a 24 well plate to be harvested for western blotting for AQP expression using an anti-AQP4 antibody (Abcam, ab128906, diluted 1:5,000). The highest expressing clone for each mutant was chosen for experiments. All steps were performed with 700 μg/mL G418 in culture media. For routine culture after establishment of the stable transfection, 200 μg/mL was used to maintain light selection pressure.

Calcein fluorescence quenching

Cells were plated in cell culture-treated black-walled clear-bottomed 96 well plates (Corning) the day before an experiment. 90 mins before the start of experiments, cells were loaded with calcein using 5 μM calcein-AM (Life Technologies) and 1 mM probenecid (Sigma-Aldrich) in culture media (probenecid is an organic anion transporter antagonist used to prevent calcein leakage). Calcein-AM was prepared as a 5 mM stock in DMSO and stored at -20 °C in foil. Probenecid was prepared as a 175 mM stock in 200 mM NaOH and stored at -20 °C. After 90 minutes at 37 °C, cells were washed twice in HEPES buffered DMEM with 1 mM probenecid. HEPES buffering was to allow the cells to remain buffered in the CO_2-free environment of the plate reader. Cells were incubated for 10 mins at 37 °C to equilibrate with the new media (in a 75 μL volume). A Biotek Synergy HT plate reader was used for all experiments. The plate reader temperature control was set to 37 °C 1 hour before the experiment. The plate reader injection system was not temperature-controlled, so all solutions that were used in the injection system were pre-warmed to 37 °C in a water bath. During the experiment, injected solutions were stored in 15 mL tubes (Greiner) and kept at 37 °C using a heating block. Tubes were covered in parafilm to minimize evaporation. 75 μL of 600 mM mannitol in HEPES-buffered DMEM (total osmolality 300 mOsm) was injected to give a final osmolality of 600 mOsm and an osmotic gradient of 300 mOsm to induce cell shrinkage. Background was measured by

including three wells of calcein-free cells on each plate and subtracting the average fluorescence of these wells from all readings with calcein. Fluorescence data was then normalized to the average of the first 5 readings for each well. Relative fluorescence was converted to relative volume using a standard curve constructed by measuring the equilibrium relative fluorescence and equilibrium relative volume (uisng a Coulter counter) for a variety of extracellular osmolalities. This procedure is described in detail by Fenton et al[1]. Single phase exponetial decay functions were fitted using the Solver tool in Microsoft Excel.

Cell surface biotinylation

Cells were plated in 24-well plates 1 day before experiments. Cell surface amines were biotinylated using a cell impermeable amine-reactive biotinylation reagent (EZ-Link Sulfo-NHS-SS-Biotin, Thermo Scientific). Cells were incubated in 600 µL of 0.5 mg/mL biotinylation reagent in PBS on ice for 30 mins. Unreacted reagent was quenched 3 times in 25 mM glycine in PBS for 5 mins. Cells were lysed in 350 µL tris-triton lysis buffer (1% v/v triton X-100, 100 mM NaCl, 2 mM $MgCl_2$, 25 mM tris pH 7.4) for 45 minutes on ice. The lysate was centrifuged at 21,000 g at 4 °C for 10 minutes to remove insoluble material. Biotinylated proteins in the supernatants were precipitated by incubation in Neutravidin-coated 96-well plates (Pierce) for 2 hours at 4 °C. 100 µL of each lysate was loaded in triplicate with the same amount of total cellular protein (measured by BCA assay) per lysate. Plates were washed three times with 0.05% PBS-tween and blocked with 3% w/v BSA in PBS for 1 hour at RT on an orbital shaker. After washing once with PBS-tween plates were incubated overnight on an orbital shaker at 4 °C with an AQP4 antibody that was verified to have minimal background signal by western blotting (Abcam, ab128906) diluted 1:2,000 in 0.05% PBS-tween. Plates were washed with 3 times with 0.05% PBS-tween and incubated with HRP-conjugated secondary antibody (Santa Cruz, sc-2313) diluted 1:5,000 on an orbital shaker at RT for 1 hour. Plates were washed three times with 0.05% PBS-tween and incubated with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) HRP substrate for 30 minutes on an orbital shaker, wrapped in foil. A volume of 100 µL of each solution (block, antibodies, washes and substrate) was used. Absorbance was measured at 450 nm using a BioTek Synergy HT plate reader. Linearity of the assay was confirmed by measuring the surface expression signal of HEK293 cells transfected with varying amounts (0-1 µg) of AQP4 DNA / 15 mm cell culture well.

Simulations

Simulations were done using Gromacs software, version 4.5.5[2]. The GROMOS96 53A6 parameter set[3] was used for the protein. Proteins were embedded in pre-equlibrated palmitoyloleoylphosphatidylcholine (POPC) bilayers using the inflateGRO tool[4]. Forcefield parameters for lipids were taken from Berger et al[5]. An AQP4 tetramer was generated according to the biological assembly entry in the AQP4 PDB entry 3GD8[6]. N- and C-termini of the protein were truncated in the structure so proteins were simulated with neutral termini. Na^+ and Cl^- ions were added to a final concentration of 100 mM. Equilibration was achieved by steepest gradient energy minimization, 100 ps NVT simulation with 1000 $kJmol^{-1}nm^{-1}$ restraints on protein heavy atoms followed by three 1 ns NPT simulations with 1000, 100 and 10 $kJmol^{-1}nm^{-1}$ restraints on protein heavy atoms followed by followed by 30 ns unrestrained simulation. After this 30 ns, a further 100 ns of data was collected for analysis. A Nosé-Hoover thermostat (0.5 ps, 310 K) was used to maintain constant temperature and 2 Parinello-Rahmann barostats (2 ps, 1 atm) were used to maintain

constant pressure with zero surface tension. 1.4 nm cut-offs were applied for dispersion and short-range electrostatic interactions. Long-range electrostatics were treated using PME. The uncertainty on the channel open probability was roughly estimated by calculating the probability independently for each monomer and using the standard error over the four monomers.

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