Materials- Cell permeable inhibitors were purchased from the following; Demecolcine and Cytochalasin D from Sigma (Poole, Dorset, U.K.), Myr-PKC (19-27) from Fisher (Loughborough, U.K.) and Myr-PKA (14-22) from Merck Chemicals (Nottingham, U.K.). Fluorodish™ dishes were from WPI Ltd (Stevenage, U.K.), Gateway vectors and enzymes from Invitrogen (Paisley, U.K.). Unless otherwise specified, all other chemicals were from Sigma (Poole, Dorset, U.K.) or Fisher (Loughborough, U.K.). Cell culture reagents were from Gibco BRL (Paisley, Renfrewshire, U.K.) or Sigma.

Expression Constructs and Mutagenesis- AQPs were fused with C-terminal GFP using the Invitrogen Gateway™ cloning system according to the instructions provided by the supplier. Sequence-verified AQP cDNAs were the kind gift of K. Hedfalk (University of Gothenburg). For directional cloning of blunt-ended PCR products into an entry vector using the Gateway™ system, four bases (GGGG) were added to the 5′-end of the forward primer followed by the 25bp *att*B1 attachment sequence (underlined). This was followed by five bases (bold) to introduce a Kozak sequence upstream and to keep the sequence in frame with the coding sequence of AQP. Finally 18-25bp of the AQP sequence were added to create the amino-terminal forward primers 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC T**CC ACC** ATG –AQP(18-25bp)-3′. For the reverse primer, four bases (GGGG) were added to the 5′-end followed by the 25bp *att*B2 attachment sequence (underlined) and then one base (bold) was added to keep the sequence in frame with the coding sequence of AQP. Finally 18-25bp of the AQP sequence without the stop codon were added to create the carboxy-terminal forward primers 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT**C**–AQP(18-25bp)-3'. KOD polymerase was used in PCR amplification of the AQP cDNA. Samples were heated to 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 3 min and then 68 °C for 7 min. Purified PCR products were subcloned into the pDONR221™ entry vector (Invitrogen) using the *att*B1 and *att*B2 sites in a reaction with Gateway™ BP Clonase™ enzyme mix (Invitrogen). pDONR221™ vectors containing the required sequences were recombined with the pcDNA-DEST47 Gateway™ vector using *attL* and *attR* reaction with Gateway™ LR Clonase™ enzyme mix (Invitrogen). This created expression vectors with the cycle 3 mutant of the green fluorescent protein (GFP) gene at the C-terminus of the AQP gene of interest, which were subsequently expressed as fusion proteins.

Cell Culture and Transfection- HEK 293 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum in humidified 5% (v/v) CO_2 in air at 37 °C. Cells were seeded into 30mm Fluorodish™ dishes and transfected after ~48 h at ~50% confluency using the Transfast (Promega) transfection protocol with 3μg of DNA/dish.

Confocal Microscopy- AQP-GFP fusion proteins were visualized in live cells enclosed in a full environmental chamber via confocal laser scanning microscopy. Confocal images were acquired with a Leica SP5 laser scanning microscope and a Zeiss Axiovert 200m inverted microscope with a 63× (1.4 NA) oil immersion objective. The nucleus and the plasma membrane were stained with DAPI or 5 μg/ml FM4-64 (Molecular Probes), respectively. Images were acquired using an argon laser (excitation, 488 nm; emission, BP505–530 nm emission filter) for GFP, UV excitation and a BP385–470 nm emission

filter for DAPI, and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for FM4-64. 48 h post-transfection, cells in Fluorodishes™ were incubated with or without 2 mg/ml demecolcine, 2 µM cytochalasin D, 50 µM Myr-PKC (19-27) or 50 µM Myr-PKA (14-22), respectively, for 1 h at 37^0C and 5% CO_2. The volume of inhibitor (in water) added to the cells was <1% (v/v) to ensure no/minimum effect on overall tonicity. Cells were visualized in DMEM which has an inorganic salt concentration of 120 mM, a glucose concentration of 25 mM and an osmolality in the range 322-374 mosM/Kg H_2O. DMEM + H_2O is a dilution of DMEM by a factor of 3 with water.

Image analysis- Protein localization was measured by a line profile (pixel density) traced on each transfected cell. Localization data are representative of three to five cells from at least three independent experiments. Line expression profiles were generated and analyzed with the program ImageJ NIH [http://rsb.info.nih.gov/ij/, 1997–2007] and are indicated in yellow and displayed below each confocal image. To measure the relative increase in plasma membrane localization, the fluorescence intensity over a distance covering the membrane and the immediate cytosol was measured. Three to five line profiles, distributed as regularly as possible, avoiding the nucleus, were performed to obtain an average profile of fluorescence intensity representative of the staining at the perimeter of each cell. The difference between the peak and the plateau of fluorescence was divided by the maximum fluorescence measured along the linescan to give the percentage increase in fluorescence at the membrane. Scale bars are 10 µm.