Elevated cAMP increases aquaporin-3 plasma membrane diffusion

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Running head: Aquaporin-3 diffusion

Keywords: Aquaporin, AQP3, MDCK, single particle tracking, kICS, image correlation spectroscopy, kidney, urine, vasopressin, AVP
Abstract

Regulated urine concentration takes place in the renal collecting duct upon arginine vasopressin (AVP) stimulation, where subapical vesicles containing aquaporin-2 (AQP2) are inserted into the apical membrane instantly increasing water reabsorption and urine concentration. The reabsorped water exits via basolateral AQP3 and AQP4. Upon long-term stimulation with AVP or during thirst, expression levels of both AQP2 and AQP3 are increased, however, there is so far no evidence for short-term AVP regulation of AQP3 or AQP4. To facilitate the increase in trans-epithelial water transport, AQP3 may be short-term regulated via changes in protein-protein interactions, incorporation into lipid rafts and/or changes in steady state turn-over, which could result in changes in the diffusion behavior of AQP3. Thus, we measured AQP3 diffusion coefficients upon stimulation with the AVP mimic forskolin, to reveal if AQP3 could be short-term regulated by AVP. k-Space Image Correlation Spectroscopy (kICS) analysis of time-lapse image sequences of basolateral EGFP-tagged AQP3 (AQP3-EGFP) revealed that forskolin mediated elevation of cAMP increased the diffusion coefficient by 58% from 0.0147 ± 0.0082 µm²/sec (control) to 0.0232 ± 0.0085 µm²/sec (forskolin, p<0.05). Quantum dot conjugated antibody labeling also revealed a significant increase in AQP3 diffusion upon forskolin treatment by 44% (0.0104 ± 0.0040 µm²/sec (control) vs. 0.0150 ± 0.0016 µm²/sec (forskolin, p<0.05)). Immunelectron microscopy showed no obvious difference in AQP3-EGFP expression levels or localization in the plasma membrane upon forskolin stimulation. Thus, AQP3-EGFP diffusion is altered upon increased cAMP, which may correspond to basolateral adaptations in response to the increased apical water reabsorption.
A basic epithelium separates two biological compartments and generates a barrier allowing selective transport of water, ions and nutrients. Water transport across an epithelium is facilitated via specialized water channels called aquaporins (AQP), which are a family of homotetrameric transport proteins. Each monomer contains a water selective pore capable of mediating passive trans-epithelial water transport following an osmotic gradient \((13, 40)\). Several different aquaporins are expressed in the human kidney, where they facilitate urine concentration and regulation of body water homeostasis (for reviews please see \((32, 36)\)). The human kidneys filter approximately 180 liters of blood per day. The filtrate first passes through the proximal tubule, where approximately 90% of the filtered water is constitutively reabsorbed via AQP1, which is present in large quantities in both the apical and basolateral plasma membranes of proximal tubule cells \((35)\). In the collecting duct, fine tuning of urine concentration occurs via regulated water reabsorption mediated by the anti-diuretic hormone AVP \((10, 31)\). In the absence of AVP, apically AQP2 is mainly localized to sub-apical vesicles making the collecting duct water impermeable \((31)\). AVP binding to the basolateral AVP type 2 receptor (AVPR2), initiates a signaling cascade, which via G-proteins activates cyclic adenosine monophosphate (cAMP). This results in activation of protein kinase A (PKA), that phosphorylates AQP2 on serine 256 in the COOH-terminal \((20)\). The phosphorylation on serine 256 results in fusion of AQP2 containing vesicles with the apical plasma membrane inserting AQP2 into the membrane \((9, 14, 31, 37)\). Insertion of AQP2 into the membrane instantly, within minutes, increases water reabsorption across the apical plasma membrane of collecting duct principal cells thereby increasing urine concentration \((31, 50, 51)\). The water reabsorped by AQP2 exits the collecting duct principal cells via basolaterally localized AQP3 and AQP4 \((6, 32, 47)\).

Upon long-term stimulation with AVP (days) or decreased water intake (thirst), expression levels of both AQP2 and the exit pathway AQP3 increase, which raises trans-epithelial water transport capacity of collecting duct principal cells \((4, 6, 12, 28, 39, 47)\), and facilitates increased urine concentration to minimize water loss and dehydration. Apical AQP2 is regulated by short-term AVP stimulation \((31, 51)\), however, there is no evidence for shuttling of AQP3, as no sub-basolateral AQP3 containing vesicles have been demonstrated \((6)\).

Dysregulation of renal aquaporins have been found in numerous diseases associated with changes in body water balance such as pre-eclampsia \((38)\), diabetes mellitus type I \((29, 43)\) as well as chronic \((21)\) and acute renal failure \((7)\). Nephrogenic diabetes insipidus (NDI) is a disease, which is characterized by the inability of the kidneys to concentrate urine appropriately in response to AVP stimulation, resulting
in excessive urine production (for review please see (16, 26)). AQP3 null mice display severe NDI (23), signifying the role of basolateral AQP3 for water exit in renal urine concentration (23). AQP4 null mice do not display NDI, (24), however, isolated inner medullary collecting ducts from AQP4 KO mice displayed a four-fold reduction in water permeability (3) . Thus, AQP3 is hypothesized to be the main exit pathway for water in collecting duct principal cells. Recent advances in imaging of plasma membrane proteins have revealed that regulation of transporters can occur via changes in protein-protein interactions and regulated incorporation into plasma membrane microdomains (5, 41, 42). Such regulation would likely change the diffusion mode of a protein, which can be measured as changes in the diffusion coefficient. We hypothesize that upon short-term AVP stimulation, the rapid increase in transcellular water transport could affect not only apical AQP2 shuttling, but also affect basolateral AQP3, perhaps via such changes in protein-protein interactions and/or microdomain incorporation. We thus aimed to test if the diffusion behavior of AQP3 changed upon short-term vasopressin stimulation, which would suggest a cAMP dependent regulatory mechanism. To measure the average diffusion coefficient of AQP3, we employed kICS analysis of both QD-labeled and EGFP-tagged AQP3 (18). We found that upon forskolin stimulation (increases cAMP via bypassing the AVPR2 receptor), AQP3-EGFP diffusion dramatically increased by 58% from 0.0147 ± 0.0082 µm²/sec (control) to 0.0232 ± 0.0085 µm²/sec (forskolin, p<0.05), indicating for the first time, that indeed the collecting duct exit pathway for water, AQP3, is regulated upon short-term vasopressin stimulation.
Materials and Methods

**DNA constructs, cloning, and Site-directed, Ligase-Independent Mutagenesis (SLIM)**

cDNA of human AQP3 tagged with EGFP was generously provided by Dr. Anita Aperia. A c-myc tag, flanked by alanine at either terminal, was inserted between amino acid asparagine and glutamine into the second extracellular loop of AQP3 by SLIM PCR (2) using the following primers:

Rtail 5'-CGCCAGATCTTCTTCAGAATAAGTTTTTGTTCCGCGTTGGCGAAGTGCCA-3'

Rshort 5'-GTTGTCGGCGAAGTGCCA-3'

Ftail 5'-GCGGAACAAAAACTTATTTCTGAAGAAGATCTGGCGCAGCTTTTTGTTTCGGGC-3'

Fshort 5'-CAGCTTTTTTGGCGGCC-3'

(F denotes forward primer and R denotes reverse primer) (2). Insertion of the c-myc tag was confirmed by DNA sequencing.

**Cell culture**

MDCK GII cells (11, 22) were cultured in DMEM low (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 0.5 U/ml penicillin (Sigma), 0.5 g/ml streptomycin (Gibco), 1 mg/ml kanamycin (Gibco), (1% PSK), and maintained at 37°C and 5% CO2. Transfections were performed using Lipofectamine 2000 (Invitrogen). For selection of stable cell lines expressing AQP3-EGFP and AQP3-myc-EGFP (see below), selection with G418 (Gibco) was carried out. Stable clones were analyzed for expression and localization by microscopy, and protein size was confirmed by western blotting using a rabbit anti-GFP antibody (Abcam). MDCK cells stably expressing the plasma membrane targeting domain of the Lyn kinase coupled to tdRFP (tandem dimeric red fluorescent protein, Lyn-tdRFP) was generously provided by Dr. Soichiro Yamada (University of California, Davis).

**Quantum dot (QD) labeling**

For QD labeling, MDCK GII cells stably expressing AQP3-myc-EGFP were seeded on rat-tail collagen coated coverslips the day before labeling, and grown to 80% confluence. Before labeling, cells were washed twice with phosphate-buffered saline (PBS) followed by 10 min incubation in blocking buffer (3% bovine serum albumin (BSA) in PBS), followed by 10 min incubation with 0.132 μg/μl mouse anti-c-myc antibody (9E10) diluted in blocking buffer. Cells were then rinsed five times with PBS and incubated for 10 min with 10 nM goat F(αb)2 anti-mouse IgG-conjugated QD655 (Invitrogen) in blocking buffer.
Excess QDs were removed by five times gentle washing with PBS. Control experiments where the primary antibody was omitted were performed.

**Image acquisition and drug treatments**

For imaging of AQP3-EGFP, AQP3-myc-EGFP and QD-labeled AQP3-myc-EGFP, coverslips were fitted in a temperature controlled incubation chamber with imaging media (DMEM low (Gibco) without phenol red supplemented with 10% BSA, 1% PSK and 25 mM HEPES). For QD labeled cells, forskolin stimulation was done at two different conditions. First, cells were pretreated with 50 µM forskolin (Sigma) in culture media for 30 min at 37°C before labeling. Alternatively, cells were exposed to forskolin in the live incubation chamber for 15 min following labeling, followed by image acquisition. To depolymerize actin fibers, cells were treated with 1 µg/ml cytochalasin D (Sigma) in culture media and incubated at 37°C for 30 min before labeling. Similarly, to disrupt microtubules, cells were incubated with 10 µM nocodazole (Sigma) in the media at 37°C for 30 min prior to labeling. Regular wide field epifluorescence microscopy was performed at 37°C on a Zeiss Axiovert 200M Inverted Fluorescence Microscope equipped with a 63x oil-immersion lens (NA 1.4) and a Xenon lamp. EGFP was excited using a D480/40 excitation filter and detected through a D535/50 emission filter (Chroma). QDs were imaged using a D560/40 excitation filter, and a D630/60 emission filter (Chroma). Images were collected with a Photometric CoolSnapTM HQ camera from Roper Scientific controlled by MetaMorph acquisition software. For QD imaging, a single EGFP image was obtained followed by acquisition of 500 frames with an integration time of 20 ms at 4.1 Hz. The shutter was kept open throughout the image acquisition. Spinning disk microscopy was performed at 36°C at the basal plasma membrane on a Nikon Ti eclipse inverted microscope equipped with a Yokogawa spinning disk unit and an Andor EMCCD camera (model Andor iXonEM +). Stacks composed of 600 frames were acquired with 30 ms integration time at a resulting frame rate of 9.2 Hz. 491 and 615 lasers were used to excite EGFP and RFP, respectively. Emission filters 525/39 and 593/40 were used. All experiments were repeated minimum 3 times.

**Measurement of diffusion coefficients by kICS analysis**

Image stacks were imported into Image J (45) and crops including no moving membrane, holes or moving cell organelles, were selected. The crops were analyzed using the kICS code in MATLAB (18). For all crops the same settings were used, the maximum number of time lags (t) was set to 6 and the maximum k² value was set to 20. The diffusion coefficients were furthermore imported into Excel, and averaged over all crops from one dataset. For each stimulation an average diffusion plot was generated.
and is shown in figure 1-3, S1 and S2. For all conditions, a minimum of 5 cells and 6 crops were analyzed. Minimum crop area was 32 µm².

**Trajectory construction**

Trajectories of QD labeled AQP3-myc-EGFP were constructed from time-lapse movies of 250 frames acquired at 20 ms integration time, by the use of 2D Particle Tracker Plugin (written by Guy Levy, MOSAIC group, ETH Zurich) (44) in Image J. Linking criteria such as link range and displacement were set to 2 and 5, respectively. A mixture of immobile and mobile trajectories of various lengths were generated after the analysis of single time-lapse stack. Trajectories shorter than 15 frames were filtered out.

**Statistical analysis**

Values are presented as means ± STDEV. Comparisons between groups were made by unpaired t-test. P-values < 0.05 were considered significant.

**Immunoelectron microscopy**

For immunoelectron microscopy, MDCK GII cells, untransfected or stably expressing AQP3-EGFP (see above) were grown at confluence for 4 days on 24 mm diameter, 0.4µm pore size Transwell filters (Corning). Media in the basolateral chamber (DMEM supplemented with 10% FBS and 1% PSK) was changed every day whereas media in the apical chamber (DMEM without FBS and PSK) was changed every second day. On the day of the experiment, DMSO (0.5%, control) or 50 µM forskolin (dissolved in DMSO, 0.5%, Sigma) was added to the media in the basal chamber of transfected cells followed by 30 min incubation at 37°C. Cells were gently washed with 1X PBS two times and fixed on filters for 15 min in 4% paraformaldehyde in 0.01 M PBS. Filters were placed in a “sandwich” containing 12% gelatin/filter/12% gelatin and incubated on ice for a least 10 min for stiffening of the gelatin. Square sections (2 mm x 2 mm) were cut and infiltrated in 2.3 M sucrose in 0.01 M PBS over night followed by incubation again in 2.3 M sucrose in 0.01 M PBS for 1 hour at RT and finally sections were frozen in liquid nitrogen.

Ultrathin cryosections (50-80 nm) were cut on an ultra-cryomicrotome (Reichert Ultracut S, Leica, Wetzlar, Germany) and preincubated in 10 mM PBS with 0.1% skinned milk powder and 50 mM glycine. The sections were incubated overnight at 4°C with primary goat anti-GFP antibody (ab6673,
Abcam, dilution: 1:6400) diluted in 10 mM PBS containing 0.1% skimmed milk powder. The primary antibody was visualized by incubation for 1 h at room temperature with secondary rabbit anti-goat IgG conjugated to 10 nm colloidal gold particles (RAG.EM10, Bio-Cell Research Laboratories, Cardiff, UK, dilution: 1:50) diluted in PBS with 0.1% skimmed milk powder, 1% fishgelatine and 0.06% polyethyleneglycol. Electron micrographs were taken on a Morgagni 268 electron microscope FEI COMPANY (Philips Electron Optics, the Netherlands).
Results

Elevated cAMP levels increased AQP3 diffusion

To investigate if AQP3 is regulated by a rapid increase in cAMP, we first made a stable cell line expressing AQP3-EGFP. As seen in figure 1A, AQP3-EGFP localized to cell-cell contacts as expected in subconfluent cells. This cell line was used to measure the diffusion coefficient of AQP3 upon forskolin stimulation. MDCK cells do not express the AVPR2 receptor, and thus, forskolin is commonly used to bypass the receptor and directly activate the adenylate cyclase and raise cAMP levels. We first measured the diffusion of bulk AQP3-EGFP by kICS analysis. kICS is a recently developed image correlation spectroscopy technique that calculates the correlation in the Fourier-space representation of images in both time and space, and thereby separates contributions due to photophysical fluctuations from those due to transport (1, 18). As a result, kICS can determine the number densities, flow speeds, and average diffusion of fluorescently labeled molecules while being unbiased by complex photobleaching or blinking of the fluorophores. The analysis is best done on image sequences of very thin membranes. Conventional analysis methods only calculate diffusion of the small fraction of the expressed proteins which are labeled with for example QDs at an extracellular epitope-tag, however, kICS analysis of AQP3-EGFP calculates average diffusion of the total pool of AQP3-EGFP. A representative region for kICS analysis is shown in figure 1B. First, diffusion of regular AQP3-EGFP without the c-myc insertion was measured by kICS analysis of image stacks obtained using spinning disk microscopy with focus set on the basal membrane. The diffusion coefficient of steady state AQP3-EGFP was 0.0147 ± 0.0082 µm²/sec (DMSO), which increased to 0.0232 ± 0.0085 µm²/sec (Figure 1C+D, p<0.05) upon forskolin stimulation for 30 min prior to imaging. This indicates that forskolin, and hence cAMP increase, has a major effect on AQP3 diffusion, likely due to changes in AQP3 protein-protein and/or protein-lipid interactions. The solvent for forskolin and drugs mentioned below, DMSO, had no effect on the diffusion of AQP3-EGFP 0.0241 ± 0.0109 µm²/sec (untreated) vs. 0.0174 ± 0.0062 µm²/sec (DMSO) (figure not shown). To test if forskolin affected plasma membrane compartments in general or acted specifically on AQP3, we tested diffusion of Lyn-tdRFP upon forskolin treatment. Lyn is a member of the Src family of protein tyrosine kinases and is incorporated into the inner side of the lipid bilayer. The lipid incorporated domain of Lyn kinase alone (used here) is enough to target tdRFP to membranes. Spinning disk microscopy of the basal membrane and subsequent kICS analyses revealed that Lyn-tdRFP was quite immobile in the plasma membrane compared to AQP3-EGFP. Forskolin stimulation did not
alter Lyn-tdRFP diffusion which was 0.0089 ± 0.0121 µm²/sec compared to DMSO treated 0.0093 ± 0.0088 µm²/sec (figure not shown). Thus, forskolin had no effect on the mobility of Lyn-tdRFP.

To compare our data from kICS analysis of EGFP-tagged AQP3 with conventional QD labeling, a 10 amino acid c-myc tag was inserted into the second extracellular loop for antibody and subsequent QD labeling. C-myc insertion between amino acid 133 and 134 resulted in correct localization of AQP3-myc-EGFP to the plasma membrane (Figure 2A), indistinguishable from wild-type AQP3-EGFP (Figure 1A) in the vast majority of cells. We first tested if the c-myc insertion as well as QD labeling had an effect on AQP3-EGFP diffusion, spinning disk microscopy was also performed on AQP3-myc-EGFP (not labeled with QDs) followed by kICS analysis. Baseline diffusion of AQP3-myc-EGFP was 0.0382 ± 0.0147 µm²/sec (Figure 2B), which is significantly higher than for regular AQP3-EGFP 0.0147 ± 0.0082 µm²/sec. This indicates that even though AQP3-myc-EGFP localized correctly to the plasma membrane, some alterations in the protein, perhaps folding, must occur due to the c-myc tag. When cells expressing AQP3-myc-EGFP were treated with forskolin, a similar increase in diffusion from 0.0382 ± 0.0147 µm²/sec (DMSO) to 0.0581 ± 0.0235 µm²/sec (forskolin) (p<0.05) (Figure 2B+C), revealed that although the steady state diffusion of the AQP3-myc-EGFP increased compared to AQP3-EGFP, forskolin stimulation still increased diffusion of AQP3-myc-EGFP similarly to forskolin stimulated AQP3-EGFP. As with AQP3-EGFP, DMSO in itself had no effect on AQP3-myc-EGFP diffusion, 0.0399 ± 0.0109 µm²/sec (untreated) vs. 0.0393 ± 0.0145 µm²/sec (DMSO) (figure not shown).

To test QD labeling of AQP3-myc-EGFP, live cells were labeled and evaluated (Figure 3A and 3B) by microscopy. As seen in figure 3B, QDs exclusively labeled AQP3 in the plasma membrane. We have shown in a previous study (30) that AQP3-EGFP localized correctly to the basolateral plasma membrane in polarized MDCK cells, however, in subconfluent and hence non-polarized cells, AQP3-EGFP was also observed on the free plasma membrane, which is where we could do measurements of QD labeled AQP3-myc-EGFP. No labeling of endogenous AQP3-myc-EGFP with QDs was observed, however, intracellular aggregates were observed corresponding to endocytosed fractions.

AQP3-myc-EGFP was labeled with QDs either following 30 min forskolin treatment or prior to 15 min forskolin treatment. Time-lapse imaging of QDs on the free plasma membrane was performed and image stacks were analysed using kICS. As seen with AQP3-EGFP diffusion measured on the basal membrane, forskolin stimulation increased diffusion of QD labeled AQP3-myc-EGFP by 35% from 0.0091
± 0.0031 µm²/sec (DMSO) to 0.0123 ± 0.0031 µm²/sec (Figure 3C+D) in samples treated with forskolin before QD labeling, and by 44% from 0.0104 ± 0.0040 µm²/sec (DMSO) to 0.0150 ± 0.0016 µm²/sec (p<0.05) (Figure 3E+F) in samples treated with forskolin following QD labeling. Although there was a tendency for increased diffusion upon forskolin stimulation of QD labeled AQP3-myc-EGFP in the pre-labeling treatment, it was not statistically significant, which could be due to the longer duration from forskolin treatment until imaging (80 min). In contrast, the forskolin mediated increase in diffusion of QD labeled AQP3-myc-EGFP post-labeling was significant and highly similar to forskolin treated AQP3-EGFP, indicating that indeed, forskolin mediated increase in cAMP levels significantly increase AQP3 diffusion in the plasma membrane.

As stated earlier, kICS can measure the average diffusion of AQP3-EGFP either based on the EGFP signal or the QD signal attached to the protein. Based on the QDs, we were able to generate trajectories of AQP3-myc-EGFP (Figure 3 G-J), however the primary aim of this work was to measure the diffusion coefficients, which was achieved by kICS analysis.

**AQP3 diffusion is unaffected by cytoskeleton disruption**

Regulated AQP2 diffusion has been shown to depend on cAMP and an intact actin cytoskeleton, but in contrast to AQP3, forskolin treatment significantly slowed AQP2 diffusion (48). Since AQP3 diffusion was regulated by elevated cAMP, we tested if the actin cytoskeleton could also be involved in regulating AQP3 diffusion. kICS analysis showed that AQP3-EGFP diffusion in the basal membrane were unaltered by cytochalasin D disruption of the actin cytoskeleton, (0.0179 ± 0.0055 µm²/sec of DMSO treated vs. 0.0237 ± 0.0093 µm²/sec for cytochalasin D treated) (Figure 4). Thus, cAMP-dependent AQP3 diffusion seems not to be regulated by the actin cytoskeleton as was seen for AQP2 (48). Moreover, the effect of disruption of the microtubule network on AQP3 diffusion was tested by employing the same imaging conditions as above in combination with nocodazole treatment. As with actin disruption, no statistically significant difference in AQP3 diffusion was observed in the basal membrane (0.0169 ± 0.0052 µm²/sec of DMSO treated vs. 0.0245 ± 0.0120 µm²/sec for nocodazole treated) (Figure 4). Thus, neither the actin cytoskeleton nor microtubules seem to be involved in forskolin mediated regulation of AQP3 diffusion.

**Forskolin did not change plasma membrane localization of AQP3-EGFP**

To test if forskolin treatment induced changes in plasma membrane localization of AQP3-EGFP, immunogold electron microscopy was performed on DMSO and forskolin treated cells grown on transwell filters. As a control for antibody specificity, untransfected cells were included. As seen in
AQP3-EGFP localized to the lateral (Fig 5, panel D) and basal membranes (not shown) with little intracellular (Fig 5, panel B) and apical staining (Fig 5, panel C). Forskolin did not seem to change the plasma membrane localization of AQP3-EGFP (Fig 6, panel B and C), and images were not noticeable different from DMSO treated. No staining of plasma membranes were observed in non-transfected cells (Fig 7, panel B).
Diffusion of AQP2 has been shown to decrease upon forskolin stimulation (48), whereas forskolin stimulation had no effect on diffusion of proximal tubule AQP1 (48). It was hypothesized, that the change in diffusion of AQP2 upon forskolin stimulation could be involved in regulating AQP2 in the membrane, via regulation of endocytosis (15), however, this hypothesis has not been tested. Possible inhibition of AQP2 endocytosis alongside increased insertion of AQP2 in the plasma membrane would aid in facilitating high water reabsorption in response to vasopressin stimulation and thus, increased urine concentration. Investigating AQP3 diffusion, we found that forskolin stimulation resulted in increased AQP3-EGFP diffusion as diffusion coefficients increased from $0.0147 \pm 0.0082 \, \mu\text{m}^2/\text{sec}$ (control) to $0.0232 \pm 0.0085 \, \mu\text{m}^2/\text{sec}$ (forskolin), in contrast to AQP2, which decreased (48). Since AQP2 and AQP3 are differentially regulated, this increase in basolateral AQP3 diffusion, may aid AQP3 facilitated water transport upon short-term vasopressin stimulation. This strongly suggests that the collecting duct exit pathway for water, AQP3, is regulated on short-term basis upon vasopressin stimulation along-side AQP2 regulation. Further studies will reveal if vasopressin results in alteration in microdomain formation, protein-protein interactions or changes in endocytosis rates. The surface area of the basolateral plasma membrane is much larger than the apical due to extensive invaginations and AQP3 is constitutively present in the membrane (49). Thus, the basolateral plasma membrane may not require extensive adaptation to short-term AVP mediated increases in water flux, as is seen in the apical membrane where AQP2 is inserted into the membrane upon elevation of cAMP. Immuno electron microscopy showed no apparent difference in plasma membrane localization of AQP3-EGFP, so the finding that lateral diffusion of AQP3 significantly increases upon forskolin stimulation suggests that indeed adaptation of AQP3 via changes in protein-protein and/or protein-lipid interactions occurs. Future studies will be needed to elucidate the underlying mechanisms for basolateral adaptation to AVP mediated water flow.

The diffusion coefficients measured by Verkman and colleagues, were obtained using FRAP analysis of AQP1 and AQP2 in the membrane of cell-cell contacts (48). FRAP and SPT analyses are widely used to calculate diffusion coefficients of fluorescently labeled and QD-coupled proteins, respectively. In contrast to FRAP analysis, SPT analysis provides not only diffusion coefficients but also reveals diffusion behavior and trajectories of single proteins. However, SPT analysis is a very time-consuming process since hundreds of trajectories need to be analyzed to extract the average diffusion coefficient. Recently, image correlation spectroscopy has been used to calculate diffusion of bulk fluorescently labeled
proteins (18). Using this technique to analyze diffusion of plasma membrane proteins has several advantages over both FRAP and SPT analysis. FRAP analysis will only measure replacements of a mobile pool of proteins and SPT is extremely labor intensive, and moreover, only a fraction of proteins are labeled with for example QDs. Furthermore, labeling with QDs requires insertion of an extracellular tag, which might interfere with protein localization and function, and for some proteins, is not possible. Due to the bulk weight of the QD, protein diffusion is slowed down (27), which might be due to, 1) the added steric of the larger probe which affects diffusion, and 2) the pluri valency of the QD probes induces cross-linking.

We used kICS analysis to measure diffusion coefficients of AQP3-EGFP, which we could compare to diffusion coefficients obtained from unlabeled AQP3-myc-EGFP as well as QD labeled AQP3-myc-EGFP. Interestingly, we observed that inserting the c-myc-epitope into the second extracellular loop of AQP3 caused an increase in steady state diffusion of AQP3-GFP without compromising plasma membrane localization. Apart from possible changes in folding, there could be intracellular/extracellular conditions that enhance the diffusion of the engineered protein. If we had only used conventional QD SPT, we would not have noticed this difference, as we would have no control to test the effect of diffusion when inserting the c-myc tag. Thus, kICS is a powerful tool to measure diffusion coefficients without the need to tag proteins with an extracellular epitope.

Disruption of the cellular cytoskeleton was found to diminish the regulated water transport in toad urinary bladder and kidney collecting ducts in rat (8, 25, 46), however, no changes in AQP3 diffusion was observed upon disruption of the actin or microtubule cytoskeleton, indicating no changes in possible physical interactions. Disruption of the actin cytoskeleton has previously been shown to reverse forskolin mediated decrease in diffusion of AQP2 (48), indicating a role of actin in vasopressin mediated AQP2 regulation. This difference might be due to the fact that AQP2 is regulated via vesicle shuttling, whereas there is no evidence for basolaterally localized AQP3 vesicles.

Thus, elevated cAMP increased basolateral diffusion of AQP3, and thus, likely altered AQP3 interactions with other proteins or lipids in the plasma membrane. Further studies will be needed to reveal the underlying molecular mechanism regulating AQP3 diffusion and adaptation to short-term vasopressin stimulation, which may be a physiological adaptation to the increased water flow mediated by apical AQP2.
Acknowledgements

We thank Dr. Anita Aperia and Dr. Soichiro Yamada for providing the AQP3-EGFP cDNA and Lyn-tdRFP expressing MDCK cells, respectively. This work was supported by a Lundbeck Junior Group Leader Fellowship to LNN, additional funding towards a PhD stipend from Aarhus University Graduate School of Science and Technology to SM, and support by the Lundbeck Foundation and The Danish Council for Independent Research - Medical Sciences to BMC. We also thank the Danish Molecular Bioimaging Center at University of Southern Denmark for access to spinning disk microscopy.


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**Figure legends**
Figure 1. Sub-cellular localization and the effect of forskolin stimulation on the diffusion of AQP3-EGFP.
A: Representative epi-fluorescence micrograph showing plasma membrane localization of AQP3-EGFP in MDCK cells. B: Representative micrograph of a cell stably expressing AQP3-EGFP captured by spinning disk confocal microscopy with focus at the basal membrane. The boxed area shows a representative example of a crop selected for kICS analysis. A and B: Fluorescence is shown in inverted contrast. Scale bars are 20 µm. C: Graph showing average diffusion coefficients of AQP3-EGFP upon DMSO (control) and 50 µM forskolin stimulation for 30 min. The diffusion was monitored using spinning disk microscopy. D: Diffusion plot for AQP3-EGFP (spinning disk) showing –Dt, the diffusion coefficient D and time t versus the time in seconds. The grey line represents DMSO treatment, the black line indicates forskolin stimulation. The plot was generated by averaging over all data for each time point giving an average diffusion plot. In this type of plot, parallel lines result in equal diffusion coefficients and non-parallel lines result in different diffusion coefficients as the slope of the line is the diffusion coefficient. * indicates p<0.05.

Figure 2. Sub-cellular localization and the effect of forskolin stimulation on the diffusion of AQP3-myc-EGFP. A: Representative epi-fluorescence image of MDCK cells stably expressing AQP3-myc-GFP in the membrane. Fluorescence is shown in inverted contrast. Scale bar is 20 µm. B: Graph showing average diffusion coefficients of AQP3-myc-EGFP upon DMSO (control) and 50 µM forskolin stimulation for 30 min. The diffusion was monitored using spinning disk microscopy. C: Diffusion plot for AQP3-myc-EGFP (spinning disk) showing –Dt, the diffusion coefficient D and time t versus the time in seconds. The grey line represents DMSO treatment, the black line is forskolin stimulation. * indicates p<0.05.

Figure 3. QD labeling and the effect of forskolin stimulation on the diffusion of AQP3-myc-EGFP. A and B: MDCK cells stably expressing AQP3-myc-EGFP on the membranes were labeled with anti-c-myc and secondary antibody conjugated-QD655 and imaged live using epi-fluorescence microscope. EGFP (A) and QD655 (B) (acquired at 100 ms exposure time). Fluorescence is shown in inverted contrast. Scale bars are 20 µm. C: Graph showing average diffusion coefficients of AQP3-myc-EGFP-QD upon DMSO (control) and 50 µM forskolin stimulation for 30 min (pre-labeling treatment). D: Diffusion plot for AQP3-myc-EGFP-QD (pre-labeling treatment) showing –Dt, the diffusion coefficient D and time t versus the time in seconds. The grey line represents DMSO treatment, the black line indicates forskolin stimulation. E: Graph showing average diffusion coefficients of AQP3-myc-EGFP-QD upon DMSO (control) and 50 µM forskolin stimulation for 15 min (post-labeling treatment). F: Diffusion plot for AQP3-myc-EGFP-QD (post-labeling treatment) showing –Dt, the diffusion coefficient D and time t versus the time in seconds.
The grey line represents DMSO treatment, whereas the black line indicates forskolin stimulation. * indicates p<0.05. G and I. Representative epi-fluorescence micrographs of MDCK cells with superimposed trajectories. Trajectories of AQP3-myc-EGFP-QD were constructed from the stack of 250 frames acquired at 20 ms integration time. The trajectory in the box is enlarged and shown in H (from G) and J (from I).

Figure 4. Schematic representation of average diffusion coefficients of AQP3-EGFP after chemical alteration of cytoskeleton. Every stimulation experiment was carried out in parallel to DMSO control.

Figure 5. Immunoelectron microscopical images of an AQP3-EGFP transfected MDCK cell treated with DMSO. A: Abundant AQP3-EGFP labeling of the lateral plasma membrane is seen (insert D, arrows), whereas sparse labeling of intracellular vesicles (insert B, arrowhead) and the apical plasma membrane (insert C, open arrowhead) are observed. Magnification: A, x12.000; B and C, x75.000; D, x51.000.

Figure 6. Immunoelectron microscopical images of an AQP3-EGFP transfected MDCK cell treated with forskolin. A: Similar to the control, abundant AQP3-EGFP labeling of the lateral plasma membrane is seen (insert B and C, arrows), whereas sparse labeling of intracellular vesicles (insert B, arrowheads) and the apical plasma membrane (insert B, open arrowhead) are observed. Magnification: A, x7.000; B, x70.000 and C, x56.000.

Figure 7. Immunoelectron microscopical images of a non-transfected MDCK cell. A: No labeling of the lateral plasma membrane is observed (insert B). Magnification: A, x12.500 and B, x51.000.
<table>
<thead>
<tr>
<th></th>
<th>Diffusion coefficient (μm²/sec)</th>
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<tbody>
<tr>
<td></td>
<td>Actin Disruption</td>
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<tr>
<td>DMSO</td>
<td></td>
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<tr>
<td>Cytochalasin D</td>
<td>0.0179 ± 0.0055</td>
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<tr>
<td>DMSO</td>
<td>0.0169 ± 0.0052</td>
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<tr>
<td>AQP3-EGFP (Spinning disk)</td>
<td>0.0179 ± 0.0055</td>
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