Atrial natriuretic peptide and nitric oxide signaling antagonizes vasopressin-mediated water permeability in inner medullary collecting duct cells

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Short title: Regulation of water permeability in IMCD cells
Abstract:

AVP and atrial natriuretic peptide (ANP) have opposite effects in the kidney. AVP induces antidiuresis by insertion of aquaporin-2 (AQP2) water channels into the plasma membrane of collecting duct principal cells. ANP acts as a diuretic factor. An ANP- and nitric oxide (NO)/soluble guanylate cyclase (sGC) induced insertion of AQP2 into the plasma membrane is reported from different models. However, functional data on the insertion of AQP2 is missing. We used primary cultured inner medullary collecting duct (IMCD) cells and digital holographic microscopy, calcein quenching measurements and immunofluorescence and western blotting to analyze the effects of ANP and NO donors on AQP2 phosphorylation, membrane expression and water permeability. While AVP led to acceleration in osmotically induced swelling, ANP had no effect. However, in AVP pretreated cells ANP decreased significantly the kinetic of cell swelling. This effect was mimicked by 8-bromo-cGMP (8-Br-cGMP) and blunted by protein kinase G (PKG) inhibition. Stimulation of the NO/sGC pathway or direct activation of sGC with BAY 58-2667 had similar effects like ANP. In cells treated with AVP AQP2 was predominantly localized in the plasma membrane, after additional incubation with atrial natriuretic peptide AQP2 was mostly localized in the cytosol indicating an increased retrieval of AQP2 from the plasma membrane by ANP. Western blot analysis showed that ANP was able to reduce AVP-induced phosphorylation of AQP2 at position S256.

In conclusion, we show that the diuretic action of ANP or NO in the IMCD involves a decreased localization of AQP2 in the plasma membrane which is mediated by cGMP and PKG.

Key words: AQP2, cell volume kinetics, cGMP generating signaling pathways; natriuretic peptides; water homeostasis; uroguanylin, phosphodiesterase-5
Introduction:

Atrial natriuretic peptide (ANP) is a cyclic peptide secreted from atrial myocytes and has besides the regulation of renal function and water homeostasis broad physiologic properties including vasodilatation, anti-fibrosis, anti-hypertrophy, cytoprotection, anti-inflammation and neurohumoral modulation (39). ANP binds to the natriuretic peptide receptor A (NPR-A), which is linked to particulate guanylyl cyclase (GC), elevating intracellular cGMP levels in many tissues including the kidney (39). It inhibits the renin-angiotensin-aldosterone system and increases glomerular filtration rate (GFR) and urine flow (23; 39). In the kidney, ANP induces excretion of Na⁺, K⁺ and Cl⁻, vasodilation in afferent glomerular vessels, but post-glomerular vasoconstriction leading to an increase in GFR (39). ANP leads to increasing levels of cGMP and activation of protein kinase G (PKG) in inner medullary collecting duct cells (25).

Water reabsorption along the nephron is facilitated by aquaporin (AQP) water channels and the fine tuning of volume homeostasis occurs in the collecting duct. Within this segment water reabsorption across principal cells is regulated by AVP which binds to the basolaterally located vasopressin-2 receptor (V₂R), a G protein-coupled receptor which activates adenylyl cyclase, and stimulates PKA. Phosphorylation of AQP2 at S256 leads to translocation of AQP2 from intracellular vesicles to the luminal membrane, increasing water permeability (49). Defects in this pathway lead to diabetes insipidus (DI) with severe water loss via the kidney (33). While the central form of DI is associated with a defect in AVP production, common causes of nephrogenic DI are hereditary (NDI) or acquired like chronic lithium ingestion, hypokalemia and hypercalcemia (33).

The molecular effects of ANP on distal tubular Na⁺ and water reabsorption are still not completely understood. It was suggested that ANP diminishes Na⁺ and AVP-mediated water reabsorption in the collecting duct leading to natriuresis and diuresis.
Such effects of ANP on Na\(^+\) and AVP-mediated water reabsorption were, however, not observed by others (36; 38). Some authors showed an ANP/cGMP-mediated translocation of AQP2 to the plasma membrane in LLC-PK1 cells and in isolated kidney slices, mainly in the inner stripe of outer medullary collecting duct (OMCD) (3). Wang et al. described a biphasic effect of ANP injected in rats (50). After an initial diuretic phase of 30 min the rat kidneys switched to antidiuresis with apical targeting of AQP2 mainly in IMCD, but not the cortical collecting duct (CCD) (50). Natriuresis sustained over the entire period of 90 min (50).

The proteins of the NO/sGC pathway are expressed in IMCD (1; 47) and thus forms a second natriuretic system beside ANP/NPR-A in the collecting duct. The impact of NO on AVP-dependent water permeability is still controversially discussed. Some authors describe an inhibition of water permeability elevated by AVP (45), others saw no change of water permeability in the CCD or IMCD (15; 51). Bouley et al. reported a trafficking of AQP2 to the apical plasma membrane in OMCD upon NO donors (3). Moreover, the selective phosphodiesterase-5 inhibitor sildenafil induced a translocation of AQP2 to the plasma membrane in principal cells of the collecting duct in the inner stripe of kidney slices and in rats in vivo (4).

In this study we attempted to further clarify the functional and molecular mechanisms of cGMP-elevating substances acting via NPR-A or sGC on AQP2 trafficking and water permeability in primary cultured rat IMCD cells.
**Materials and methods:**

**Cell culture:**

Experiments were approved by a governmental-committee on animal welfare (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany) and were performed in accordance with national animal protection guidelines (A 60/1993 and A 67/09).

IMCD cells were isolated and cultivated as described previously (28; 35). In short, the inner medulla with papilla of sacrificed female Wistar rats was separated, minced and digested in phosphate buffered saline (PBS, Biochrom, Berlin, Germany) containing 0.2% hyaluronidase (Sigma, Deisenhofen, Germany) and 0.2% collagenase type CLS-II (Sigma) at 37°C for 90 min. Cells were centrifuged at 450 g, resuspended in PBS and centrifuged for a second time. Cells were seeded at a density of ~ 10^5 cells/cm² on glass cover slips or in flow chambers with six channels (µ-slide IV, IBIDI, Munich, Germany) both coated with collagen type IV (Becton-Dickinson, Heidelberg, Germany). Cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, Biochrom) containing penicillin 100 IU/ml, streptomycin 100 µg/ml, 1% non essential amino acids (Biochrom), 1% ultroser (Pall corporation, East Hills, USA) and 10 µM dbcAMP and the osmolality of culture media was elevated to ~ 600 mOsm/kg by addition of 100 mM NaCl and 100 mM urea in an 8% CO₂ atmosphere. The dbcAMP removed 16 h before the experiments. Cells were used for experiments 5 to 10 days after seeding.

**Experimental solutions:**

Osmolality was checked using an osmometer (Knauer, Berlin, Germany) and the appropriate calibration solution of 400 mOsm/kg (Braun, Berlin, Germany). Before the
measurements the medium was changed from DMEM to MEM (Biochrom) modified as described above to 600 mOsm/kg and without bicarbonate and serum but with 10 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid (HEPES) at pH 7.45. The hypoosmolar medium (200 mOsm/kg) was obtained by adding distilled water.

**Measurements of cell volume changes with digital holographic microscopy:**

Digital holographic microscopy (DHM) provides contactless, marker-free, quantitative phase-contrast imaging (20; 26; 29; 34) that is suitable for modular integration into commercial microscopes (19). The evaluation of the obtained quantitative phase contrast images provides data for thickness monitoring (20; 29) and cell tracking (24). For DHM phase contrast measurements on cell thickness/volume kinetics an iMIC microscope (TILL-Photonics, Graefelfing, Germany) was applied that had been modified for DHM (Center of Biomedical Optics and Photonics, Muenster, Germany).

Figure 1 shows the scheme of the setup and illustrates the principle of DHM (7; 20). The coherent light source was a frequency-doubled Nd:YAG laser (Coherent Compass 315M, Coherent, Luebeck, Germany, $\lambda = 532$ nm). A flow chamber enabled exchange of perfusion medium ($\mu$-slide IV, IBIDI, Martinsried, Germany). The sample was illuminated with laser light (object wave) and imaged by a microscope lens (Zeiss LD Plan-Neofluar 63x/0.75Korr, Carl Zeiss, Jena, Germany) and a tube lens a charge-coupled device camera (DMK 41BF02, The Imaging Source, Bremen, Germany). The object wave was superimposed with the slightly tilted reference wave (Fig. 1) for the generation of off-axis holograms which were recorded by the camera sensor and transferred to an image processing system (hologram acquisition software: FireView, Center for Biomedical Optics and Photonics, Muenster, Germany). The numerical reconstruction of the digitally recorded holograms was carried out by spatial-phase-shifting-based reconstruction
For cells in perfusion medium with a refractive index $n_{\text{medium}}$ and the assumption of a known homogeneously distributed integral cellular refractive index $n_{\text{cell}}$, the cell thickness $d(x,y)$ can be determined from the measured optical phase retardation $\Delta \varphi_{\text{cell}}$ of the cells to the surrounding medium (20):

$$d(x,y) = \frac{\lambda \Delta \varphi_{\text{cell}}(x,y)}{2\pi} \times \frac{1}{n_{\text{cell}} - n_{\text{medium}}}$$

(1)

with the light wavelength $\lambda$ (software: PhaseIllustrator, Center for Biomedical Optics and Photonics, Muenster, Germany). For each series 15 digital holograms were recorded before the initiation of the swelling process by adding the hypoosmolar medium followed by additional acquisition of 135 holograms at a frequency of 0.2-0.5 Hz. From the obtained maximum phase values $\Delta \varphi_{\text{cell,max}}(x,y)$ the maximum cell thickness $d(x,y)$ was obtained by equation (1) with $n_{\text{cell}} = 1.370 \pm 0.008$ (see section Determination of absolute cell volume and integral cellular refractive index of suspended IMCD cells) and $n_{\text{medium}} = 1.337 \pm 0.001$ (obtained by an Abbe refractometer).

Equation 2 was used to analyze the time dependence of the obtained cell thickness changes $d(t)$:

$$d(t) = d_{\min} + (d_{\max} - d_{\min})(1 - \exp(-t/\tau))$$

(2)

with the time $t$. The parameter $d_{\min}$ denotes the initial cell thickness and $d_{\max}$ is the cell thickness after the swelling process. The constant $\tau$ is reciprocally associated with the osmotic water permeability coefficient $P_f$ as described by Farinas et al. (10). The time constant $\tau$ was determined by fitting equation 2 to the measured relative cell
thickness change (Graph Pad Software, San Diego, USA). Assuming that the cells in
the confluent monolayer mainly swell in the vertical direction (27), changes in cell
thickness directly correlate with cell swelling. Changes of water permeability in per
cent can be calculated by ratios of different time constants $\tau$. During the cell swelling
process $n_{cell}$ is decreased by water uptake (34), which results in an underestimation
by using equation 2 for the determination of the absolute maximum cell thickness
d$(x,y)$. As the refractive index change is temporally correlated with the cell swelling
process, the effect of the refractive index change on the time constant $\tau$ is expected
to be small. This estimation is supported by the correlation of the results for $\tau$ for both
DHM and fluorescence self-quenching experiments (see Fig. 3 and Fig. 7). In
confluent IMCD monolayers 80-90% were AQP2-positive cells and showed typical
epithelial cobblestone pattern (see immunofluorescence Fig. 3A and D) (22). One
measurement per channel was performed. All cells of the captured image frame
containing 5-10 cells were analyzed.

Determination of the osmotic water permeability coefficient, absolute cell
volume and integral cellular refractive index of suspended IMCD cells

The osmotic water permeability coefficient $P_f$ was calculated as described in (10):

$$P_f = (\tau (A/V)_0 V_w \Phi_0)^{-1}$$

(3)

Here $\tau$ is the time constant, the term $(A/V)_0$ determines the ratio of the cell surface
area A to the cell volume V before the swelling process, $V_w$ is the molar volume of
water and $\Phi_0$ determines the medium osmolality before swelling experiments. For the
determination of A and V, IMCD cells were detached with trypsin-EDTA in 2 fold PBS
(Biochrom), centrifuged at 150 g and resuspended in hyperosmolar MEM
(600 mOsm/kg) buffered with 10 mM HEPES. Cells had a spherical shape with
radius \( r \). Thus, the absolute cell volume and cell surface area could be estimated to

\[ A = 4\pi r^2 \quad \text{and} \quad V = \frac{4}{3}\pi r^3. \]

The average radius of the suspended cells was determined to \( r = 8.6 \pm 0.1 \mu m \) (\( n = 148 \), data not shown) whereas the integral refractive index \( n_{\text{cell}} = 1.370 \pm 0.008 \) was obtained by DHM with the method described in (21). The result for \( n_{\text{cell}} \) was used for cell thickness determination with equation 1.

**Immunofluorescence studies:**

AQP2 was detected with a polyclonal antibody kindly provided by Dr. E. Klussmann (14). IMCD cells were cultured on glass cover slides. The cells were washed twice with PBS and fixed with PBS containing 4% paraformaldehyde (Sigma), pH 7.4, for 20 min and washed three times for 10 min each in PBS. The cell membranes were permeabilized in PBS containing 0.1% Triton X-100 (Sigma) for 5 min and washed three times with PBS. Blocking procedure was performed with 0.3% fish skin gelatin (Sigma) in PBS. Cells were incubated in a humid chamber at 37°C with an anti-AQP2-antibody or anti-NPR-A-antibody (Immundiagnostik AG, Bensheim, Germany) for 90 min. The cells were washed three times in PBS for 10 min and then incubated with a secondary goat-anti-rabbit antibody conjugated with Alexa488 (Invitrogen, Karlsruhe, Germany) for 60 min. Immunofluorescence images were captured with an epifluorescence Axiovert 100 microscope and a digital camera AxioCamMR (Carl Zeiss, Oberkochen, Germany) or using a laser scanning microscope (Axiovert 200M LSM 510 META and AxioCamHR, Zeiss).

The effect of AVP (0.5 µM, 15 min, Bachem, Weil am Rhein, Germany) or ANP (1 µM, 15 min, Peptide Institute, Osaka, Japan) on AQP2 localization was quantified using confocal images with the same settings for laser intensity, pinhole aperture, photomultiplier gain, and offset. ratio of cytosolic to cell membrane associated signal
served as an indicator for AQP2 membrane localization (14) and intensity was calculated using Metamorph 7.0 software (Visitron, Munich, Germany).

**cGMP measurement:**

Intracellular cGMP-levels were measured using a commercial ELISA kit (R&D Systems, Wiesbaden, Germany). Cells were grown to confluence in a 24 well micro titer plate and incubated in the presence of IBMX (1 mM) for 10 min with ANP, guanylin (GN), uroguanylin (UGN) (all 0.5 µM) (all Peptide Institute) or left untreated. The cGMP-concentration was measured in duplicates regarding the manufacturer’s instructions. Cells were lysed using 0.1 N HCl. The optical density was measured using a microplate reader (Tecan Spectra, Crailsheim, Germany). The cGMP concentrations were calculated using the WinFitting Software (Tecan).

**Measurements of cell volume changes by fluorescence self-quenching:**

All fluorescence self-quenching experiments were performed in the microplate reader (Infinite® 200, Tecan) at room temperature. Cells were cultivated in a 96 well plate (Nunc, Thermo Fischer Scientific, Schwerte, Germany) to confluence and were loaded for 60 min in hyperosmolar DMEM (600 mOsm/kg) under cell culture conditions with 5 µM calcein-acetoxymethylester (calcein-AM) (Invitrogen). Pluronic (Calbiochem Gibbstown, USA) was added to the loading medium 1:500 from a 10% stock solution. After the loading procedure cells were washed twice with MEM (600 mOsm/kg) without bicarbonate but with 10 mM HEPES-buffer. Stimulation with the peptide hormones was achieved under cell culture conditions in 50 µl MEM (600 mOsm/kg). AVP-prestimulated cells (15 min) were washed twice with medium and then subsequently incubated with UGN, ANP (both 1 µM), 8-Br-cGMP (500 µM) (Sigma), sodium nitroprussid (SNP, 10 µM), BAY 58-2667 (1 µM(44) alone or
KT5823 (1 µM, Calbiochem) combined with ANP for 15 min and the same protocol was carried out in DHM-measurements. Cells were excited at 494 nm and fluorescence emission was measured at 522 nm. The hypotonic conditions were performed by dispensing 100 µl distilled water. To calculate relative changes in fluorescence intensity during the swelling process, the first value maintained after the hypotonic change was set to 100%. The time course of calcein fluorescence was recorded for 300 s. Non-linear fittings were carried out with graph-pad-software as described above and the time constant $\tau$ was calculated.

Western Blot analysis

IMCD cells were cultured in 24 well dishes to confluency and were treated as indicated. The cells were lysed over night in sample buffer (4% SDS, 10% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl). Proteins were separated by SDS-polyacrylamide (4-20%) electrophoresis and transferred to a PVDF. The membrane was incubated with 5% BSA and then incubated with an antibody raised against phospho S256 AQP2 (17). After incubation with the secondary antibody membranes were covered with SuperSignal (Pierce, Bonn, Germany) before exposure (Kodak, Stuttgart, Germany). Signal intensities were calculated using BioDoc analysis software (Biometra). The antibodies were removed and the signal intensities for GAPDH were calculated as described above and the AQP2 S256 signals were normalized to GAPDH.

**Statistical analysis:**

Values are depicted as mean ± SEM. Data were compared with two-tailed unpaired $t$-test and one-way-ANOVA and Tukey’s multiple comparison tests where
appropriate. P < 0.05 were accepted as significant. The statistical analysis was done with GraphPad Prism4 software.

**Results:**

**Cell thickness changes as parameter for cell swelling analyzed by digital-holographic microscopy**

To monitor changes in water permeability of IMCD cells the kinetics of changes in cell thickness after a hypoosmolar challenge from 600 to 200 mOsm/kg were monitored using digital-holographic microscopy (DHM). This method allows marker free and dynamic analysis of cell geometries with high time and space resolution (20; 21; 24).

Figure 1 represents the schematic setup for the DHM. An example of the swelling of an IMCD cell monolayer after hypoosmolar medium change is shown in (Fig. 2).

IMCD cells in isoosmolar medium (600 mOsm/kg) are imaged in quantitative phase contrast in (Fig. 2A) and the changes of phase contrast 300 s after the medium change to 200 mOsm/kg is shown in (Fig 2B). Subsequent calculation of the cell thickness in an exemplary cell (dashed lines in Figs. 2A and 2B) by application of equation 1 (see methods) gave a cell swelling of ≈ 50% (Fig. 2C). We used these calculated cell thickness changes in cell swelling experiments as indicator for water permeability.

**Calculation of water permeability coefficient (Pf)**

The time constant $\tau$ for osmotically induced cell swelling (from 600 to 200 mOsm/kg) of control cells (93 ± 4 s, n = 36), the radius (8.6 ± 0.1 µm) and the ratio of cell surface to cell volume 3.500 ± 700 cm$^{-1}$ of suspended IMCD cells was inserted into equation 3 to calculate the basal $P_f$ of IMCD cells. The $P_f$ calculated from these values was 4.4 ± 0.5 µm/s.
Effects of AVP on AQP2 localization and water permeability

Primary cultured IMCD cells stained with an AQP2 antibody showed an intracellular localization of AQP2 when cells were left untreated (Fig. 3A). After incubation with AVP (0.5 µM, 15 min) staining with the AQP2-antibody indicates the expected translocation of AQP2 to the plasma membrane (Figs. 3D, 5C, and 6B). Functional insertion of AQP2 into the plasma membrane was indicated by the accelerated cell swelling upon hypoosmotic challenge (600 to 200 mOsmol/kg). The time constant $\tau$, indicating the time when the cells reach their half maximal thickness during the swelling process, was calculated by sigmoidal regression analysis to the data obtained by DHM (27). A representative plot is shown for an untreated cell (Fig 3B) and for an AVP-stimulated cell (Fig. 3E). As summarized in (Fig. 3C) the stimulation of cells with AVP led to a significant decrease of $\tau$ from 93 ± 4 s ($n = 36$) to 41 ± 3 s ($n = 22$). This indicates a 2.3 fold increase of water permeability of the plasma membrane upon AVP.

To quantify the changes in membrane expression of AQP2 modified by AVP or ANP we used immunofluorescence and confocal microscopy to calculate the ratio of the membrane/cytosol fluorescence signal, as similarly described by Henn et al. (14).

AVP stimulation led to a marked increase in this ratio from 1.08 ± 0.04 ($n = 30$) in unstimulated cells to 1.88 ± 0.09 ($n = 30$) in AVP-treated cells. Thus, the increase in water permeability upon AVP stimulation correlates with an increased accumulation of AQP2 in the plasma membrane.

Effects of ANP on AQP2 localization and water permeability

The functional expression of NPR-A in these IMCD cells was proven by immunofluorescence using an antibody directed against NPR-A (Fig. 4A). The
staining with the antibody indicates that NPR-A is present both at the plasma membrane and in the cytosol. The incubation of IMCD cells with ANP (0.5 µM for 15 min) significantly increased the cGMP level in IMCD cells from 1.3 ± 0.3 pM/well (n = 4) to 107.1 ± 5.6 pM/well (n = 5) (Fig. 4B), indicating a functional expression of the NPR-A. In contrast to ANP, the natriuretic peptides guanylin (GN) and uroguanylin (UGN) (each 0.5 µM, 15 min, n = 5 each), which activate guanylate cyclase C e.g. in intestinal and renal cells, had no significant effect on intracellular cGMP-levels in IMCD cells (Fig. 4B), suggesting the absence of this signaling pathway in IMCD cells.

Next we examined the influence of ANP on AQP2 shuttling to the plasma membrane and on the time constant $\tau$ after osmotically induced cell swelling as parameter of water permeability. ANP stimulation of IMCD cells did not lead to a change in AQP2 localization compared to untreated cells (Figs. 5A and 5B). Consequently, ANP had also no significant effect on the time constant $\tau$ (80 ± 4 s, n = 25). Figure 5D summarizes these effects on the kinetics of the swelling process for untreated cells and ANP stimulated cells. In line with this absence of ANP effects in unstimulated IMCD cells the ratio of the signal intensities of membrane bound to intracellular labeled AQP2 in ANP-stimulated cells (1.06 ±0.04, n = 30) was not different from control cells (1.08 ± 0.04, n = 30), as shown in (Fig. 5E). These data indicate that ANP did not alter plasma membrane localization of AQP2 and water permeability in unstimulated IMCD cells, despite the marked increase in cellular cGMP under these conditions.

**ANP decreases AVP-mediated changes in water permeability**

To analyze whether the diuretic action of ANP observed *in vivo* can be demonstrated as an influence on AVP-mediated increase in water permeability in IMCD cells, cells
were initially incubated with AVP (0.5 µM) for 15 min and then subsequently with ANP (1 µM) for 15 min. Indeed, in these AVP-prestimulated cells ANP led to a decrease in AQP2 membrane localization compared to AVP stimulation alone (Figs. 6A and 6B).

The time constant $\tau$ obtained during the osmotic challenge was $\sim 1.7$ fold higher compared to cells stimulated only by AVP, but still significantly lower than in unstimulated cells: $69 \pm 7$ s ($n = 25$) for ANP and AVP; $71 \pm 11$ s ($n = 12$) for 8-Br-cGMP and AVP, $41 \pm 3$ s ($n = 22$) for AVP alone, and $93 \pm 4$ s ($n = 36$) in unstimulated cells (Fig. 6C). AVP washout after 15 min stimulation and a subsequent period of 15 min without stimulation led to no significant increase in the time constant $\tau$ ($49 \pm 6$ s, $n = 8$). The ratio of fluorescence of membrane bound AQP2 to intracellular AQP2 showed a significant decrease in cells pretreated with AVP and subsequent ANP stimulation from $1.88 \pm 0.09$ ($n = 30$) in AVP-stimulated cells to $1.41 \pm 0.06$ ($n = 30$) after additional ANP stimulation (Fig. 6D). This indicates that ANP led to an increased retrieval of AQP2 from the plasma membrane and in consequence to a decrease in water permeability when cells were AVP stimulated.

**Calcein measurements and action of UGN on AVP-mediated water permeability**

Although we have shown that DHM is a suitable method to analyze changes in water permeability, it is associated with high effort for data analysis and the variance of measured values in cell thickness (Fig. 3) was not optimal at this time point. For these reasons a calcein fluorescence quenching method was used as a second approach to verify results from DHM experiments (6). These measurements indicate a $1.8 \pm 0.2$ fold increase of water permeability after stimulation with AVP (Fig. 7A), comparable to the results obtained by DHM (see above). ANP alone again had no effect on the water permeability of IMCD cells determined by this method.
(τ = 57 ± 8 s, n = 15) compared to unstimulated cells (58 ± 4 s, n = 4). In cells first stimulated with AVP for 15 min and a subsequent incubation with ANP led to an increase of the time constant from 32 ± 1 s, n = 190 under AVP alone to 66 ± 5 s, n = 26 after AVP and subsequent ANP. This ANP effect was dose-dependent, as shown in (Fig. 7B). AVP washout and a period of 15 min without any further stimulation did again not change the water permeability significantly compared to AVP-stimulated cells (36 ± 4 s, n = 21). In line with the absence of an effect on cellular cGMP UGN (1 µM, 15 min) had no effect on AVP-elevated water permeability (τ = 32 ± 3 s, n = 19).

The specific PKG-Inhibitor KT5823 (1 µM, 15 min) was used to verify, if the ANP effect was mediated by PKG. KT5823 completely blocked the effect of ANP (τ = 37 ± 3 s, n = 29) on AVP increased water permeability. The solvent for KT5823, dimethyl sulfoxide, alone had no significant effect (data not shown).

Next we examined whether activation of the NO/sGC pathway by SNP (10 µM) or BAY 58-2667 (1 µM, (44), had a similar influence on AVP-mediated water permeability. The NO donor SNP increased the time constant significantly to (τ = 60 ± 6 s, n = 24) and the NO-independent sGC activator BAY 58-2667 also increased τ (τ = 57 ± 5 s, n = 19) after AVP. Exemplary kinetics of these changes of calcine quenching are shown in (Fig. 7C) for AVP and in (Fig. 7D) for untreated cells.

**ANP diminishes AVP-induced phosphorylation of AQP2 at S256**

Phosphorylation status of AQP2 at S256 was examined in unstimulated cells, cells treated with AVP (500 nM, 15 min) or cells prestimulated with AVP and then incubated with ANP (1 µM, 15 min). Western blot analysis of whole cell lysates was performed with a specific antibody against phosphorylation at S256 and a representative blot is shown in (Fig. 8A) (17). AVP led to increasing amounts of S256
phosphorylated AQP2 (334 ± 46% of control, n = 5). Subsequent incubation with ANP diminished significantly AVP-elevated phosphorylation of S256 to (170 ± 22% of control levels, n = 5).

Discussion

Effects of ANP on Na\(^+\) and water transport have been reported \textit{in vivo} and \textit{in vitro} using various methodological approaches and cellular or animal models (9; 15; 27; 31; 32; 36-38). However, the cellular and molecular mechanisms responsible for these actions of ANP on collecting duct water permeability are still not completely understood.

We used primary cultured IMCD cells of the rat, which express endogenously important components of collecting duct water transport regulation: AQP2, V2R, NPR-A, and ENaC (5; 28) and can be easily studied with a number of functional or molecular methods. In this study we applied various technical approaches to investigate effects of ANP, NO, and the diuretic peptides guanylin and uroguanylin on basal and AVP dependent trafficking of AQP2. By using digital holographic microscopy, volumetric calcein fluorescence analysis and immunohistochemistry we show that ANP/cGMP reduces AVP-dependent AQP2 accumulation in the plasma membrane via a cGMP and PKG-dependent mechanism. Activation of the NO/sGC pathway by SNP or BAY 58-2667 had the same effect on AQP2 regulation as ANP. In contrast, UGN which also has been described as a natriuretic and diuretic peptide had no effect on recruitment of AQP2 inserted into the plasma membrane, suggesting that the diuretic effects of UGN are not mediated by AQP2 membrane retrieval in IMCD.

DHM as suitable method and validation by calcein quenching:
DHM has been established as a marker free method to monitor changes in cell geometry with high time and space resolution (7; 20; 26; 29). In previous studies Farinas et al. used total internal reflection and an interference microscope with an integrated Mach-Zehnder interferometer (10; 11) to analyze changes in water permeability. Using spatial phase shifting digital holographic reconstruction (7) in combination with subsequent convolution based propagation (20) in DHM we demonstrate a 2.3 ± 0.2 fold increase of water permeability upon AVP stimulation compared to resting IMCD cells. Using calcein fluorescence as volume marker a similar increase in water permeability induced by AVP was observed (1.8 ± 0.2 fold). These values are in line with earlier published data on AVP dependent swelling in cell lines and primary cultured IMCD cells mediated via AQP2 (8; 27; 46; 48). Therefore, both methods are suitable for the analysis of cell volume kinetics in these primary cultured IMCD cells which reflect physiological AQP2 regulation.

Calculation of water permeability coefficient (Pf)

Water fluxes across membranes usually are quantified using the water permeability coefficient Pf. The calculated Pf was 4.4 ± 0.5 µm/s based on cell thickness changes obtained by DHM and 7 ± 2.2 µm/s in cell volume kinetics by calcein fluorescence measurements. DHM is based on direct one dimensional cell thickness measurements, calcein fluorescence as volume marker indicates indirectly cell volume changes due to self quenching of the molecule. This could explain the longer time constant τ obtained in DHM compared to calcein measurements. Using laser scanning microscopy of IMCD cells Maric et al. published a basal water permeability coefficient of approximately 13 µm/s in trypzinated cells (27). In a later study they determined a basal Pf of untrypsinated IMCD monolayers of approximately 5 µm/s (30). Thus, the Pf values obtained in our study are within the range of those reported
before for primary cultured IMCD cells. These coefficients are also in line with further
published basal $P_f$ values in cell lines. Deen et al. reported a basal $P_f$ of
approximately 7 µm/s in MDCK cells transfected with AQP2 (8) and in a human
collecting duct cell line (CD8) basal $P_f$ was about 10 µm/s (48).

**Effects of natriuretic peptides on water permeability:**

Water homeostasis is controlled by several mechanisms like the sympathetic nervous
system, the renin-angiotensin-aldosterone system and the natriuretic NO and ANP
systems. Regulation of water reabsorption occurs in the kidney predominantly in the
collecting duct. Bouley et al. proposed that ANP acting through cGMP and other
cGMP-elevating agents like SNP and L-arginine have an AVP-like effect on cellular
localization of AQP2 based on studies in proximal tubular LLC-PK1 cells transfected
with AQP2 and *in vitro* in kidney slices in OMCD (3). In a further study they observed
trafficking of AQP2 upon injection of a PDE5 inhibitor predominantly in the rat OMCD
(4). Wang et al. showed a trafficking of AQP2 90 min after the beginning of a
continuous injection of ANP in rats in the IMCD (50). In that study it remained unclear
whether this was a cGMP effect, the initiation of blunted ANP signaling or a counter
regulatory process. Within this study we can exclude such systemic counter
regulatory effects acting on AQP2 regulation.

Immunohistochemical examination of AQP2 membrane localisation and DHM
analysis of cell swelling presented here shows that ANP does not lead to a
translocation of AQP2 into the plasma membrane or change in osmotic water
permeability in resting IMCD cells (Fig. 5A and B). ANP, however, enhances the
retrieval of AQP2 from the plasma membrane after AVP-mediated stimulation of
water permeability and thereby reduces this AVP-dependent water permeability.
Consistent with an enhanced retrieval of AQP2 from the plasma membrane after AVP
stimulation, ANP stimulation resulted in a significant increase of the time constant $\tau$ for osmotically induced cell thickness changes to almost control levels. This indicates a decrease in osmotic water permeability by 60% compared to AVP treated cells (Figs. 6 and 7A). The action of ANP on established AVP effects decreased in a dose-dependent manner (Fig. 7B). Similar results on $\tau$ were obtained in AVP-prestimulated cells with 8-Br-cGMP-treatment (Fig. 6). Our results confirm earlier studies of ANP actions on water permeability in isolated, AVP-prestimulated CCD and IMCD segments of rabbit and rat kidney. In rat and rabbit cortical collecting ducts ANP-mediated a decrease of the water permeability coefficient ($P_f$) by 20 and 50-90%, respectively (9; 31). In AVP-prestimulated IMCD segments of the rat ANP led to 18-48% lower values of $P_f$, dependent on ANP concentrations (32). In those earlier studies the underlying mechanisms remained unclear.

In line with increased AQP2 retrieval from the plasma membrane upon ANP treatment in AVP-prestimulated IMCD cells we were able to measure decelerated kinetics of cell swelling. The effect of ANP was not observed in the absence of AVP. ANP seems to interfere with the AVP-dependent trafficking of AQP2, for example by decreasing the amount of S256 phosphorylation of AQP2 (Fig. 8). ANP is therefore apparently not involved in the control of basal water permeability in the inner medullary collecting duct. We were able to show that the classical ANP-signaling pathway including cGMP and PKG is involved in the inhibition of AVP-dependent AQP2 trafficking by ANP. Our data indicate that ANP via NPR-A, cGMP, and PKG lead to decreased membrane localization for AQP2 and diminished S256 phosphorylation, necessary for AQP2 shuttling to the plasma membrane (18). This is followed by reduced water permeability and explains the observed changes in water permeability in the above mentioned studies using isolated CCD and IMCD segments. A similar diminished surface expression by a cGMP-mediated mechanism
was reported also for the sodium-potassium-chloride cotransporter (NKCC2) in the thick ascending limb (2).

**Effects of agonists of the NO/sGC signaling pathway on AQP2 regulation:**

The action of activators of the NO/sGC signaling pathway on collecting duct water reabsorption is controversial. Inhibition of AVP-mediated water permeability was described (13), however others were not able to validate these data (15; 51). Similar to the effects of ANP in our study we saw a reverse of AVP-elevated water permeability to control levels (Fig. 7A) also upon SNP and the NO-independent sGC activator BAY-582667 (44) in IMCD cells, suggesting a common final pathway for these two signaling systems.

**No effects of guanylin peptides on water permeability:**

Besides ANP, GN and UGN have been proposed as saluretic and diuretic peptides acting also in the kidney (41). Furuya et al. described in rats treated with intravenous GN a decrease of cell thickness in the IMCD segment. They suggested an effect of GN on ion channel transport properties which indirectly would modify cell volume (12). We recently described such effects in the proximal tubule as well as in the CCD on K⁺ conductances for these peptides (40-42). In the present study UGN and GN had no effect on intracellular cGMP-levels in IMCD cells (Fig. 4) and UGN did not alter the effect of AVP on AQP2 (Fig. 7A), suggesting that these peptides do not activate their classical GC-C and cGMP dependent signaling in these IMCD cells.

An alternative signaling for UGN has been described in human proximal tubular IHKE cells via a pertussis toxin-sensitive G protein (16; 43). In mouse and human CCD segments we also demonstrated a cGMP-independent signaling pathway coupled to
phospholipase A$_2$ for these peptides, leading to changes in K$^+$-conductances (40; 42). However, our results indicate, that these peptides, compared to ANP, had no
direct influence on the regulation of AQP2.

In summary, our study demonstrates that short term ANP stimulation of resting IMCD
cells has no influence on AQP2-dependent water permeability. ANP, however
counteracts AVP-dependent increases in water permeability. This inhibitory effect of
ANP on the established antidiuretic AVP answer is mediated via NPR-A, cGMP, and
PKG. This effect of ANP is due to an ANP-mediated decrease of membrane AQP2-
expression and a diminished amount of S256 phosphorylated AQP2. On the other
hand, the described diuretic effects of guanylin peptides \textit{in vivo} cannot be explained
by this mechanism.

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\section*{Disclosures:}

None

Reference List


**Figure 1**

**Experimental set-up of DHM.** Configuration of laser (off-axis), microscope (inverse) and PC in digital-holographic microscopy. The digital-holographic microscopy is a quantitative phase contrast and interferometric method. A laser beam is divided by a beam splitter in an object wave, illuminating the specimen through the condensor and the microscope lens. Reference wave and object wave interfere in an off-axis geometry. Morphologic changes of the biological specimen lead to a changed pattern of interferogram. The computer simultaneously reconstructs numerical intensity and the phase distribution of the holograms.

**Figure 2**

**Representive DHM measurement.** (A) Holographic phase contrast image of IMCD cells in 600 mOsmol/kg at t = 0 before the reduction of osmolality. (B) The same IMCD cells at t = 300 s after a decrease in osmolality to 200 mOsmol/kg. (C) Cross-sections through the quantitative phase images (dotted white lines in A and B) as well as the corresponding calculated cell thickness d using refraction indices $n_{cell} = 1.375$ and $n_{medium} = 1.337$. The cell swelling ($\approx$ 50 percent) is clearly detected. The analysis of the holographic phase contrast images for the change in relative thickness is performed by a PV-wave-based software (Phaselillustrator, Center for Biomedical Optics and Photonics, University Hospital, Muenster, Germany), scale bar = 20 µm.

**Figure 3**

**Membrane expression of AQP2 correlates with accelerated cell swelling.** In control cells AQP2 is mainly located intracellularly (A), detected by immunofluorescence. Upon AVP stimulation AQP2 translocates from intracellular vesicles to the plasma membrane (D). Holograms were captured with a rate of 0.2-
0.5 Hz. Two examples of representative curve fittings of the time course of the cell thickness changes are shown in (B) and (E). The results of the kinetic measurements are presented in (C). Quantification of immunofluorescence (F) indicates enhanced plasma membrane expression of AQP2 labeled by a specific antibody under AVP (0.5 µM, 15 min) stimulation. The dashed line represents the time point of the change from 600 to 200 mOsm/kg. The scale bar = 20 µm, *= statistically significant in unpaired student t-test, values are means ± SEM, n = cells of at least three independent cell cultures and measurements.

**Figure 4**

NPR-A is expressed in IMCD cells, ANP leads to intracellular cGMP-elevation. Expression of NPR-A in IMCD cells using an anti NPR-A antibody (A). The addition of 0.5 µM ANP for 15 min to the cell culture medium led to increased levels of intracellular cGMP (B), indicating that ANP acts in IMCD cells through NPR-A and particulate guanylyl cyclase. In contrast to ANP the natriuretic and diuretic peptides GN and UGN (0.5 µM, 15 min) did not lead to increased intracellular cGMP-levels (B). Values are means ± SEM, scale bar = 20 µm, * = statistically significant in one-way-ANOVA with Tukey’s multiple comparison test as post test.

**Figure 5**

Effect of ANP stimulation on AQP2-distribution and water permeability. Cellular distribution of AQP2 in resting IMCD cells (A) and after AVP stimulation (0.5 µM; 15 min) (C). Unlike AVP stimulation, ANP-treatment of IMCD cells (1 µM, 15 min) did not lead to a translocation of AQP2 to the plasma membrane, analyzed with epifluorescence microscopy (B). ANP alone had no influence on time constant τ (D). Similar results were obtained by quantification of the ratio of membranous to cytosolic fluorescence of AQP2 (E). n = cells of at least three independent cell cultures and measurements.

**Figure 6**

ANP retrieval of AQP2 in AVP-pretreated cells, effects of ANP and 8Br-cGMP on time constant τ. In AVP-pretreated (0.5 µM, 15 min) cells, ANP (1 µM, 15 min) led to a withdrawal of AQP2 from the plasma membrane (A) compared to only AVP-stimulated cells (B). The kinetics of the changes in cell thickness upon an osmotic challenge (600 to 200 mOsm/kg) are summarized in (C). In AVP-stimulated cells ANP and 8-Br-cGMP (500 µM, 15 min) induced an increase in τ from antidiuretic to almost control levels. The quantitative results of the immunofluorescence indicate a diminished membrane expression of AQP2 in cells pretreated with AVP and following ANP stimulation (D). * = significantly different to AVP, n = cells of at least three independent cell cultures and measurements.

**Figure 7**

Summary of time constants and exemplary kinetics of calcein fluorescence quenching. The mean time constants τ of different treatments gained by calcein measurements are shown in A. The effect of ANP on AVP-mediated elevated water permeability can be blocked in a dose-dependent manner by ANP (B). Exemplary
kinetics after hypoosmolar medium exchange are shown for control cells (C) and for AVP stimulated cells (D). For the wash out experiments, cells were prestimulated with AVP for 15 min (0.5 µM), washed twice with medium and then stimulated for 15 min with ANP (1 µM, 100 nM, 10 nM, 1 nM), BAY 58-2667 (1 µM), KT5823 (1 µM), SNP (10 µM) or UGN (1 µM) or left without peptide stimulation. * = significantly different from AVP-treatment in ANOVA with Tukey’s multiple comparison test, n = measurements of at least three independent cell cultures

Figure 8
ANP leads to a decrease in AVP-induced S256 phosphorylation of AQP2. A representative western blot analysis of whole cell lysates with specific antibody directed against phosphorylated S256 is shown here. The cells were left untreated (lane 1 and 2) or stimulated with AVP alone (500 nM, 15 min, lane 3 and 4) or ANP (1 µM, 15 min, lane 5 and 6) after washout of AVP (A). Quantification of the immunoblots is shown in (B). Signal intensities were normalized to the control lane. * = significantly different to AVP in student-t test, n = experiments of at least three independent cell cultures
Figure 1

object illumination wave

condensor

IMCD cells in perfusion chamber

microscope objective

beam splitter

reference wave

tube lens

CCD

monitor
Figure 2

(A) $t = 0$

(B) $t = 300 \text{ s}$

(C) $\Delta \Phi_{\text{cell}}$ vs $x$ (in $\mu m$) for $t = 0 \text{ s}$ and $t = 300 \text{ s}$.
Figure 3

Figure 3 A

Figure 3 B

Figure 3 C

Figure 3 D

Figure 3 E

Figure 3 F
Figure 4

A

B

Control  ANP  GN  UGN

cGMP [pM/well]

n = 4  n = 5  n = 5

*
Figure 5

[Images of cellular structures showing differences in control and ANP treated samples.]

**D**

Control ANP

- $\tau$ [s]
- $n = 36$ for Control, $n = 25$ for ANP

**E**

Ratio: membrane/intracellular

- $n = 30$ for Control, $n = 30$ for ANP
Figure 6

A B

AVP wash out AVP, ANP

0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00

n = 30

C

Control AVP ANP AVP then ANP AVP then Br-cGMP

$\tau$ [s]

n = 36 n = 22 n = 25 n = 16 n = 12

D

Ratio: membrane/intracellular

AVP wash out AVP, ANP

$\text{n} = 30$

* * *
Figure 7

A

Control AVP ANP
washout AVP/ANP
washout AVP+nitro stimulation
washout AVP/ANP and KT925
washout AVP/SNP
washout AVP/BAY 58-2667

B

n = 30
n = 112
n = 15
n = 26
n = 24
n = 19
n = 19

C

D

C

Control

\[ \tau = 56 \pm 1 \text{ s} \]

D

AVP

\[ \tau = 28 \pm 1 \text{ s} \]
Figure 8

A

kDa

55

36

28

Control  AVP  AVP/ANP

AQP2

GAPDH

B

Expression of AQP2 arbitrary units % of Control

n = 5

n = 5*

AVP  AVP/ANP

*