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

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Klokkers J, Langehanenberg P, Kemper B, Kosmeier S, v Bally G, Riethmüller C, Wunder F, Sindic A, Pavenstädt H, Schlatter E, Edemir B. Atrial natriuretic peptide and nitric oxide signaling antagonizes vasopressin-mediated water permeability in inner medullary collecting duct cells. Am J Physiol Renal Physiol 297: F693–F703, 2009. First published July 1, 2009; doi:10.1152/ajprenal.00136.2009.—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, calcine-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 significantly decreased the kinetics of cell swelling. This effect was mimicked by 8-bromo-cGMP and blunted by PKG inhibition. Stimulation of the NO/sGC pathway or direct activation of sGC with BAY S8-2667 had similar effects to ANP. In cells treated with AVP, AQP2 was predominantly localized in the plasma membrane, and after additional incubation with ANP 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.

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

Atrial natriuretic peptide (ANP) is a cyclic peptide secreted from atrial myocytes and has, besides the regulation of renal function and water homeostasis, broad physiological properties, including vasodilatation, antifibrosis, antihypertrophy, 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 the 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 postglomerular vasoconstriction, leading to an increase in GFR (39). ANP leads to increasing levels of cGMP and activation of PKG in inner medullary collecting duct (IMCD) 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 adenyl 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 (NDI) are hereditary 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 (9, 31, 32, 37). 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. (50) described a biphasic effect of ANP injected into rats. After an initial diuretic phase of 30 min, the rat kidneys switched to anti-diuresis with apical targeting of AQP2 mainly in the IMCD, but not the cortical collecting duct (CCD) (50). Natriuresis was sustained over the entire period of 90 min (50).

The proteins of the nitric oxide/sGC pathway are expressed in the IMCD (1, 47) and thus form a second natriuretic system beside ANP/NPR-A in the collecting duct. The impact of nitric oxide (NO) on AVP-dependent water permeability is still controversially discussed. Some authors describe an inhibition of water permeability elevated by AVP (45), and others saw no change in water permeability in the CCD or IMCD (15, 51). Bouley et al. (3) reported a trafficking of AQP2 to the apical plasma membrane in OMCD upon NO donors. 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 killed female Wistar rats was separated, minced, and digested in 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 ~106 cells/cm² on glass coverslips 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 DMEM (Biochrom) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids (Biochrom), 1% ultralow (Pall, East Hills, NY), and 10 µM dibutyryl (db)-cAMP, and the osmolality of the culture media was elevated to ~600 mosmol/kgH2O by addition of 100 mM NaCl and 100 mM urea in an 8% CO2 atmosphere. The db-cAMP was removed 16 h before the experiments. Cells were used for experiments 5–10 days after seeding.

Experimental solutions. Osmolality was checked using an osmometer (Knauer, Berlin, Germany) and the appropriate calibration solution of 400 mosmol/kgH2O (Braun, Berlin, Germany). Before the measurements, the medium was changed from DMEM to MEM (Biochrom) modified as described above to 600 mosmol/kgH2O and without bicarbonate and serum but with 10 mM HEPES at pH 7.45. The osmolosmolar medium (200 mosmol/kgH2O) 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 (λ = 532 nm, Coherent Compass 315M, Coherent, Lubeck, Germany). A flow chamber enabled exchange of perfusion medium (µ-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 of 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). The numerical reconstruction of the digitally recorded holograms was carried out by spatial-phase-shifting-based reconstruction (7, 20) (deHolo software, Center for Biomedical Optics and Photonics based on PV-Wave, Visual Numerics, Stuttgart, Germany).

For cells in the perfusion medium with a refractive index nmedium and the assumption of a known homogeneously distributed integral cellular refractive index ncell, the cell thickness d(x,y) can be determined from the measured optical phase retardation Δφcell of the cells to the surrounding medium (20)

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

with the light wavelength λ (Phasellustrator software, Center for Biomedical Optics and Photonics). For each series, 15 digital holograms were recorded before the initiation of the swelling process by adding the hyposmolar medium followed by additional acquisition of 135 holograms at a frequency of 0.2–0.5 Hz. From the obtained maximum phase values Δφcell,max (x,y), the maximum cell thickness d(x,y) was obtained by Eq. 1 with ncell = 1.370 ± 0.008 (see Determination of osmotic water permeability coefficient, absolute cell volume, and integral cellular refractive index of suspended IMCD cells) and nmedium = 1.337 ± 0.001 (obtained by an Abbe refractometer).

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

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

with time t. The parameter d_{\text{min}} denotes the initial cell thickness, and d_{\text{max}} is the cell thickness after the swelling process. The constant τ is reciprocally associated with the osmotic water permeability coefficient Pwater as described by Farinas et al. (10). The time constant τ was determined by fitting Eq. 2 to the measured relative cell thickness change (Graph Pad Software, San Diego, CA). 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 in water permeability (percent) can be calculated by ratios of different time constants τ. During the cell swelling process, n_{\text{cell}} is decreased by water uptake (34), which results in an underestimation by using Eq. 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 Figs. 3 and 7). In confluent IMCD monolayers, 80–90% were AQ22-positive cells and showed a typical epithelial cobblestone pattern (see immunofluorescence in Fig. 3, A and D) (22). One measurement per channel was performed. All cells of the captured image frame containing 5–10 cells were analyzed.

**Determination of 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 Ref. 10

$$P_{f} = \left(\frac{\sigma(AV)}{V_{m} \Phi_{v}}\right)^{-1}$$

Here, $\sigma$ is the time constant, the term $(AV)/V_{m}$ determines the ratio of the cell surface area $A$ to the cell volume $V$ before the swelling process, $V_{m}$ is the volume of the cell, and $\Phi_{v}$ determines the medium osmolality before swelling experiments. For the determination of $A$ and $V$, IMCD cells were detached with trypsin-EDTA in twofold PBS (Biochrom), centrifuged at 150 g, and resuspended in hyperosmolar MEM (600 mosmol/kgH$_{2}$O) 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 be $A = 4\pi r^{2}$ and $V = 4/3\pi r^{3}$. The mean radius of the suspended cells was determined to be $r = 8.6 \pm 0.1 \mu$m ($n = 148$, data not shown) whereas the integral refractive index $n_{ref}$ was $1.370 \pm 0.008$ was obtained by DHM with the method described in Ref. 21. The result for $n_{ref}$ was used for cell thickness determination with Eq. 1.

**Immunofluorescence studies.** AQ22 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. A 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, 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 Alexa 488 (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 (Axiolab 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 AQ22 localization was quantified using confocal images with the same settings for laser intensity, pinhole aperture, photomultiplier gain, and offset. The ratio of cytosolic to cell membrane-associated signal was used as an indicator for AQ22 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 microplate and incubated in the presence of IBMX (1 mM) for 10 min with ANP, guanylin (GN), uroguanylin (UGN; all 0.5 μM, Peptide Institute) or left untreated. The cGMP concentration was measured in duplicate according to 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 WinFitting Software (Tecan).

**Measurements of cell volume changes by fluorescence self-quenching.** All fluorescence self-quenching experiments were performed with the microplate reader (Infinite 200, Tecan) at room temperature. Cells were cultivated in a 96-well plate (Nunc, Thermo Fisher Scientific, Schwerte, Germany) to confluence and were loaded for 60 min in hyperosmolar DMEM (600 mosmol/kgH$_{2}$O) under cell culture conditions with 5 μM calcine-AM (Invitrogen). Pluronic (Calbiochem, Gibbstown, NJ) was added to the loading medium (1:500) from a 10% stock solution. After the loading procedure, cells were washed twice with MEM (600 mosmol/kgH$_{2}$O) without bicarbonate but with 10 mM HEPES buffer. Stimulation with the peptide hormones was achieved under cell culture conditions in 50 μM MEM (600 mosmol/kgH$_{2}$O). 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 nitroprusside (SNP; 10 μM), BAY 58-2667 (1 mM) (44) alone, or KT5823 (1 μM, Calbiochem) combined with ANP for 15 min, and the same protocol was carried out for 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 calcine fluorescence was recorded for 300 s. Nonlinear fittings were carried out with GraphPad software as described above, and the time constant $\tau$ was calculated.

**Western blot analysis.** IMCD cells were cultured in 24-well dishes to confluence and were treated as indicated. The cells were lysed overnight in sample buffer (4% SDS, 10% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl). Proteins were separated by SDS-PAGE (4–20%) and transferred to a polyvinylidene difluoride membrane. 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 means ± SE. Data were compared with a two-tailed unpaired t-test and one-way ANOVA and Tukey’s multiple comparison tests where appropriate. $P < 0.05$ was accepted as significant. The statistical analysis was done with GraphPad Prism4 software.

**RESULTS**

**Cell thickness changes as parameter for cell swelling analyzed by DHM.** To monitor changes in water permeability of IMCD cells, the kinetics of changes in cell thickness after a hyposmolar challenge from 600 to 200 mosmol/kgH$_{2}$O were monitored using 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 DHM. An example of the swelling of an IMCD cell monolayer after hyposmolar medium change is shown in Fig. 2. IMCD cells in isosmolar medium (600 mosmol/kgH$_{2}$O) are imaged in quantitative phase contrast in Fig. 2A, and the changes in phase contrast 300 s after the medium change to 200 mosmol/kgH$_{2}$O are shown in Fig. 2B. Subsequent calculation of the cell thickness in an exemplary cell (dashed lines in Fig. 2, A and B) by application of Eq. 1 (see METHODS) gave a cell swelling of ~50% (Fig. 2C). We used these calculated cell thickness changes in cell swelling experiments as an indicator of water permeability.

**Calculation of $P_{f}$**. The time constant $\tau$ for osmotically induced cell swelling (from 600 to 200 mosmol/kgH$_{2}$O) 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$^{-2}$) of suspended IMCD cells was inserted into Eq. 3 to calculate the
basal $P_f$ of IMCD cells. The $P_f$ calculated from these values was $4.4 \pm 0.5 \mu m/s$.

**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 hyposmotic challenge (600 – 200 mosmol/kgH₂O). 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 in $\tau$ from $93 \pm 4$ (n = 36) to $41 \pm 3$ s (n = 22). This indicates a 2.3-fold increase in 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 to the cytosolic fluorescence signal, as similarly described by

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**Fig. 2.** Representative DHM measurement. A: holographic phase-contrast image of inner medullary collecting duct (IMCD) cells in 600 mosmol/kgH₂O 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/kgH₂O. 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 $n_{\text{cell}} = 1.375$ and $n_{\text{medium}} = 1.337$. The cell swelling ($\sim50\%$) 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 (PhaseIllustrator, Center for Biomedical Optics and Photonics, University Hospital, Muenster, Germany). Scale bar = 20 μm.

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**Fig. 3.** Membrane expression of aquaporin-2 (AQP2) correlates with accelerated cell swelling. In control cells, AQP2 is mainly located intracellularly (A), detected by immunofluorescence. Upon arginine vasopressin (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 mosmol/kgH₂O. Scale bar = 20 μm. Values are means ± SE; n = cells from at least 3 independent cell cultures and measurements. *Statistically significant in unpaired Student’s $t$-test.
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 the 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 (n = 4) to 107.1 ± 5.6 pM/well (n = 5) (Fig. 4B), indicating a functional expression of NPR-A. In contrast to ANP, the natriuretic peptides GN and UGN (each 0.5 μM, 15 min, n = 5 each), which activate GC-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 τ after osmotically induced cell swelling as a parameter of water permeability. ANP stimulation of IMCD cells did not lead to a change in AQP2 localization compared with untreated cells (Fig. 5, A and B). Consequently, ANP had also no significant effect on the time constant τ (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 the 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 with AVP stimulation alone (Fig. 6, A and B).

The time constant τ obtained during the osmotic challenge was ~1.7-fold higher compared with cells stimulated only by AVP but still significantly lower than in unstimulated cells: 69 ± 7 s (n = 25) for ANP and AVP; 71 ± 11 s (n = 12) for 8-Br-cGMP and AVP, 41 ± 3 s (n = 22) for AVP alone, and 93 ± 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 a significant increase in the time constant τ (49 ± 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 ± 0.09 (n = 30) in AVP-stimulated cells to 1.41 ± 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.

Calcine measurements and action of UGN on AVP-mediated water permeability. Although we have shown that DHM is a suitable method for analyzing 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 calcine fluorescence-quenching method was used as a second approach to verify results from DHM experiments (6). These measurements indicate a 1.8 ± 0.2-fold increase in 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 with unstimulated cells (58 ± 4 s, n = 30). In cells first stimulated with AVP for 15 min and a subsequent incubation with ANP, these led to an
increase in the time constant from $32 \pm 1$ s, $n = 190$ under AVP alone to $66 \pm 5$ 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 again did not change the water permeability significantly compared with AVP-stimulated cells ($36 \pm 4$ 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 ($32 \pm 3$ s, $n = 29$).

The specific PKG inhibitor KT5823 (1 μM, 15 min) was used to verify whether the ANP effect was mediated by PKG. KT5823 completely blocked the effect of ANP ($37 \pm 3$ s, $n = 29$) on AVP-increased water permeability. The solvent for KT5823, dimethyl sulphoxide, 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 ($60 \pm 6$ s, $n = 24$), and the NO-independent sGC activator BAY 58-2667 also increased $\tau$ ($57 \pm 5$ s, $n = 19$) after AVP. Exemplary kinetics of these changes of calcein quenching are shown in Fig. 7C for AVP and in Fig. 7D for untreated cells.

ANP diminishes AVP-induced phosphorylation of AQP2 at S256. The 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. AVP led to increasing amounts of S256-phosphorylated AQP2 (334 ± 46% of control, $n = 5$). Subsequent incubation with ANP significantly diminished AVP-elevated phosphorylation of S256 to $170 \pm 22\%$ of control levels ($n = 5$).

**DISCUSSION**

Effects of ANP on Na$^+$ and water transport have been reported in vivo and in vitro using various methodological approaches and cellular or animal models (9, 15, 27, 31, 32,
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 the epithelial Na\(^+\)/H\(^+\) channel, 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 GN and UGN on basal and AVP-dependent trafficking of AQP2. By using DHM, 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 has been established as a marker-free method to monitor changes in cell geometry with high time and space resolution. In previous studies, Farinas et al. used total internal reflection and an interference microscope with an integrated Mach-Zehnder interferometer to analyze changes in water permeability. Using spatial phase-shifting digital holographic reconstruction in combination with subsequent convolution-based propagation in DHM, we demonstrate a 2.3 ± 0.2-fold increase in water permeability upon AVP stimulation compared with resting IMCD cells. Using calcein fluorescence as a 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). 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 \(P_f\). Water fluxes across membranes usually are quantified using the water permeability coefficient \(P_f\). The calculated \(P_f\) was 4.4 ± 0.5 \(\mu\)m/s based on cell thickness changes obtained by DHM and 7 ± 2.2 \(\mu\)m/s in cell volume kinetics by calcein fluorescence measurements. DHM is based on direct one-dimensional cell thickness measurements, while calcein fluorescence as a volume marker indicates indirectly cell volume changes due to self-quenching of the molecule. This could explain the longer time constant \(\tau\) obtained in DHM compared with calcein measurements. Using laser-scanning microscopy of IMCD cells, Maric et al. published a basal \(P_f\) of 13 \(\mu\)m/s in trypsinized cells. In a later study, they determined a basal \(P_f\) of untrypsinated IMCD monolayers of 5 \(\mu\)m/s. Thus the \(P_f\) 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 studies.
published basal $P_f$ values in cell lines. Deen et al. (8) reported a basal $P_f$ of $\sim 7 \mu m/s$ in MDCK cells transfected with AQP2, and in a human collecting duct cell line (CD8) basal $P_f$ was $\sim 10 \mu m/s$ (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. (3) 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 the OMCD. In a further study, they observed trafficking of AQP2 upon injection of a PDE5 inhibitor predominantly in the rat OMCD (4). Wang et al. (50) showed a trafficking of AQP2 90 min after the beginning of a continuous injection of ANP into rats in the IMCD. In that study, it remained unclear whether this was a cGMP effect, the initiation of blunted ANP signaling, or a counterregulatory process. Within this study, we can exclude such systemic counterregulatory effects acting on AQP2 regulation.

Immunohistochemical examination of AQP2 membrane localization and DHM analysis of cell swelling presented here show that ANP does not lead to a translocation of AQP2 into

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**Fig. 7. Summary of time constants and exemplary kinetics of calcein fluorescence quenching.**

**A:** mean time constants $\tau$ of different treatments gained by calcein measurements. $B$: effect of ANP on AVP-mediated elevated water permeability can be blocked in a dose-dependent manner by ANP. $C$ and $D$: exemplary kinetics after hyposmolar medium exchange for control cells (C) and for AVP-stimulated cells (D). For washout experiments, cells were prestimulated with AVP for 15 min (0.5 $\mu M$), washed twice with medium, and then stimulated for 15 min with ANP (1 $\mu M$, 100 nM, 10 nM, 1 nM), BAY 58-2667 (1 $\mu M$), KT5823 (1 $\mu M$), SNP (10 $\mu M$), or UGN (1 $\mu M$) or left without peptide stimulation. *Significantly different from AVP treatment in ANOVA with Tukey’s multiple comparison test, $n$ = measurements of at least 3 independent cell cultures.
the plasma membrane or change in osmotic water permeability in resting IMCD cells (Fig. 5, A 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 in 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 with 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 for \( \tau \) 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 CCDs, ANP mediated a decrease in \( P_f \) 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 IMCD. We were able to show that the classic 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 leads 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. (12) described in rats treated with intravenous GN a decrease in 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 classic 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 with 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 is mediated via NPR-A, cGMP, and PKG. This effect of ANP is due to an ANP-mediated decrease in membrane AQP2 expression and a diminished amount of S256-phosphorylated AQP2. On the other hand, the described diuretic effects of guanylin peptides in vivo cannot be explained by this mechanism.
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