Extracellular pH affects phosphorylation and intracellular trafficking of AQP2 in inner medullary collecting duct cells

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1Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; and 2BK21 Plus KNU Biomedical Convergence Program, Department of Biomedical Science, Kyungpook National University, Taegu, Korea

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Choi HJ, Jung HJ, Kwon TH. Extracellular pH affects phosphorylation and intracellular trafficking of AQP2 in inner medullary collecting duct cells. Am J Physiol Renal Physiol 308: F737–F748, 2015. First published January 28, 2015; doi:10.1152/ajprenal.00376.2014.—Kidney collecting duct cells are continuously exposed to changes of extracellular pH (pHe). We aimed to study the effects of altered pHr on desmopressin (dDAVP)-induced phosphorylation (Ser256, Ser261, Ser264, and Ser269) and apical targeting of aquaporin-2 (AQP2) in rat kidney inner medullary collecting duct (IMCD) cells. When freshly prepared IMCD tubule suspensions exposed to HEPES buffer with pH 5.4, 6.4, 7.4, or 8.4 for 1 h were stimulated with dDAVP (10−10 M, 3 min), AQP2 phosphorylation at Ser256, Ser264, and Ser269 was significantly attenuated under acidic conditions. Next, IMCD cells primary cultured in transwell chambers were exposed to a transepithelial pH gradient for 1 h (apical pH 6.4, 7.4, or 8.4 vs. basolateral pH 7.4 and vice versa). Immunocytochemistry and cell surface biotinylation assay revealed that exposure to either apical pH 6.4 or basolateral pH 6.4 for 1 h was associated with decreased dDAVP (10−9 M, 15 min, basolateral)-induced apical targeting of AQP2 and surface expression of AQP2. Fluorescence resonance energy transfer analysis revealed that the dDAVP (10−9 M)-induced increase of PKA activity was significantly attenuated when LLC-PK1 cells were exposed to pH 6.4 compared with pH 7.4 and 8.4. In contrast, forskolin (10−7 M)-induced PKA activation and dDAVP (10−9 M)-induced increases of intracellular Ca2+ were not affected. Taken together, dDAVP-induced phosphorylation and apical targeting of AQP2 are attenuated in IMCD cells under acidic pHr, likely via an inhibition of vasopressin V2 receptor-G protein-cAMP-PKA actions.

Acidosis; collecting duct; fluorescence resonance energy transfer; vasopressin; vasopressin V2 receptor

RENAL TUBULAR EPITHELIAL CELLS, including kidney collecting duct cells, are continuously exposed to changes of the microenvironment, e.g., luminal fluid shear stress, transepithelial osmotic gradients, and changes of luminal or interstitial pH. We previously demonstrated the effects of altered luminal fluid shear stress on the translocation of aquaporin-2 (AQP2) and reorganization of the actin cytoskeleton (F-actin) in kidney inner medullary collecting duct (IMCD) cells (14). Immunocytochemistry demonstrated that exposure to luminal fluid shear stress per se in the microfluidic channel induced polymerization of F-actin associated with increased apical targeting of AQP2, even in the absence of vasopressin stimulation. This finding suggests that changes of the microenvironment, including extracellular pH (pHe), could be importantly involved in the apical targeting of AQP2.

AQP2s are a family of membrane proteins that transport water molecules through biomembranes (18, 21, 31). AQP2 is the water-selective channel protein that plays a key role in arginine vasopressin (AVP)-mediated water reabsorption in collecting ducts (2, 20, 21, 27, 30, 31, 41). Vasopressin V2 receptor (V2R)-mediated cAMP/PKA signaling has been shown to be a principal signaling pathway for both AQP2 trafficking and protein expression via activation of Gsα-mediated adenyl cyclase activity (7, 16, 30, 41, 42).

However, despite the well-known effects of AVP stimulation, the concurrent effects of changes in the luminal or interstitial microenvironment in vivo, e.g., pH, on the phosphorylation and apical targeting of AQP2 in collecting duct cells are not well understood. Urine pH can vary between 4.6 and 8.0, depending on the amount of urinary net acid excretion. A recent clinical study (3) demonstrated that almost 60% of patients with stones had a urine pH of <6, a value consistent with that found in the general population. It has been demonstrated that the affinity of vasopressin for V2R was significantly lower under the condition of acidic pH compared with that at neutral pH in LLC-FLAG-V2R cells (44). Moreover, rats with metabolic acidosis had significantly decreased urinary excretion of AQP2, whereas renal AQP2 mRNA and protein expressions were increased (28). In addition, it has been demonstrated that urine alkalinization promoted the excretion of urinary exosomal AQP2 independent of vasopressin stimulation in rats (9).

Therefore, we hypothesized that changes in pHr could affect intracellular pH (pHi) of collecting duct cells, resulting in an altered response of AQP2 phosphorylation (Ser256, Ser261, Ser264, and Ser269) and apical targeting of AQP2 to vasopressin stimulation. First, desmopressin (dDAVP)-induced AQP2 phosphorylation (Ser256) was examined in IMCD tubule suspensions exposed to pH 5.4, 6.4, 7.4 and 8.4, respectively. To study the selective effects of apical or basolateral pH on dDAVP-induced intracellular trafficking of AQP2 in collecting duct cells, IMCD cells from rat kidneys were primary cultured in a transwell chamber system. The effects of acidic (pH 6.4), neutral (pH 7.4), or alkaline (pH 8.4) conditions in the apical or basolateral sides of cells were examined on dDAVP-induced intracellular trafficking and cell surface expression of AQP2.

Moreover, to further elucidate the underlying signaling mechanisms, fluorescence resonance energy transfer (FRET) analysis was exploited, and time-lapse changes of AVP- or forskolin-induced PKA activity and intracellular Ca2+ dynamics were monitored in LLC-PK1 cells exposed to acidic (pH 6.4), neutral (pH 7.4), or alkaline (pH 8.4) microenvironments.
**MATERIALS AND METHODS**

*Primary culture of IMCD cells from rat kidneys.* The animal protocols were approved by the Animal Care and Use Committee of Kyungpook National University (KNU 2012-10). Primary cultures enriched in IMCD cells were prepared from pathogen-free male Sprague-Dawley rats (200–250 g, Charles River, Seongnam, Korea), as previously described in detail (6, 23, 24). Briefly, rats were anesthetized under enflurane inhalation, and kidneys were rapidly removed. After the IMCD cell suspension had been isolated, cells were seeded on either collagen-coated 35-mm glass-base dishes (Asahi Techno Glass, Tokyo, Japan) for pH measurements or semi-permeable filters of the transwell system (0.4-μm pore size, Transwell Permeable Supports, catalog no. 3450, Corning) for cell surface biotinylation assays. IMCD cells were fed every 48 h and grown in hypertonic culture medium (640 mosmol/kg H2O) supplemented with 10% FBS at 37°C in a 5% CO2-95% air atmosphere for 3 days and then in PBS-free culture medium for an additional 1 day before the experiment on day 5. The culture medium was DMEM-F-12 without phenol red, containing 80 mM urea, 130 mM NaCl, 10 mM HEPES, 2 mM l-glutamine, 10,000 U/ml penicillin-streptomycin, 50 nM hydrocortisone, 5 μM 3,3,5-triiodothyronine, and 10% FBS (pH 7.4, 640 mosmol/kg H2O).

**IMCD tubule suspensions.** Fresh IMCD tubules were prepared from rat kidneys as previously described (5, 35). Rats were anesthetized under enflurane inhalation. The kidney inner medulla was dissected, minced, and digested in IMCD suspension buffer (250 mM sucrose and 10 mM triethanolamine, pH 7.4) containing collagenase B (3 mg/ml) and hyaluronidase (2 mg/ml). The IMCD suspension was then continuously agitated in a warm water bath at 37°C for 90 min. Thereafter, the IMCD tubule suspension was centrifuged at 600 × g for 30 s to separate the IMCD-enriched fraction (pellet) and non-IMCD fraction (supernatant). The IMCD-enriched fraction was collected and washed twice with ice-cold IMCD suspension buffer. For the experiments, IMCD tubule suspensions were exposed to HEPES-buffered saline solution (162.5 mM NaCl, 2.5 mM Na2HPO4, 4 mM KCl, 25 mM HEPES, 2 mM CaCl2, 1.2 mM MgSO4, and 5.5 mM glucose) with different pH (pH 5.4, 6.4, 7.4, or 8.4) for 1 h, which was established by adding HCl (for pH 5.4) or NaOH (for pH 6.4, 7.4, and 8.4) (43). dDAVP stimulation (10−10 M) was done during the last 3 min (Fig. 1A).

**Semi-quantitative immunoblot analysis.** SDS-PAGE was performed on 12% polyacrylamide gels, as previously described (34). Primary antibodies used were anti-AQP2 (H7661AP, 1:2,000) (15, 23), anti-phosphorylated (p)-AQP2 at Ser356 (K0307AP, 1:3,000) (34), anti-p-AQP2 at Ser256, Ser264, and Ser269 (Phosphosolutions, 1:1,000), and anti-β-actin (Sigma, 1:100,000).

**Immunofluorescence microscopy of primary cultured IMCD cells.** IMCD cells were grown to confluence in each transwell chamber (0.4-μm pore size, Transwell Permeable Supports, catalog no. 3460, Corning) for 4 days. On day 5, IMCD cells were subjected to a transepithelial pH gradient for 1 h (pH 6.4, 7.4, or 8.4 at the apical side vs. pH 7.4 at the basolateral side and vice versa) at 37°C. dDAVP stimulation (10−9 M, 15 min, basolateral side only) was done during the last 15 min, and cells were then fixed with 3% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. After fixation, cells were washed twice in PBS and permeabilized with 0.3% Triton X-100 in PBS at room temperature for 15 min. Cells were washed and then labeled with anti-AQP2 (H7661AP, 1:200) antibody, and nuclei were stained with 4’,6-diamidino-2-phenylindole. AQP2 localization was determined using laser confocal microscopy (Zeiss LSM 5 EXCITER, Jena, Germany), and a ×63 (numerical aperture: 1.4) objective lens (Zeiss) was used. Digital images were collected and analyzed using the Zeiss Aim Image Examiner program.

**Cell surface biotinylation assays.** IMCD cells were primary cultured for 4 days on semipermeable filters of the transwell system (0.4-μm pore size, Transwell Permeable Supports, catalog no. 3450, Corning). On day 5, they were exposed to a transepithelial pH gradient for 1 h (apical pH 6.4, 7.4, or 8.4 vs. basolateral pH 7.4 and vice versa) at 37°C. Cells were then washed three times with ice-cold 10 mM PBS containing 1 mM CaCl2 and 0.1 mM MgCl2 (PBS-CM; pH 7.5) and incubated at 4°C for 45 min in ice-cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, and 125 mM NaCl, pH 8.9) containing 1 mg/ml sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin, Thermo Scientific, Rockford, IL) on the apical side. Cells were washed once with quenching buffer (50 mM Tris-HCl in PBS-CM, pH 8.0) followed by two washes with PBS-CM. Lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1.15 mM PMSF, 0.4 μg/ml leupeptin, 0.1 μg/ml Pefabloc, 0.01 μM okadaic acid, 1 mM Na3VO4, and 25 mM NaF) was added, and lysates were sonicated with 2 × 6 pulses at 20% of amplitude. Lysates were centrifuged at 10,000 g for 5 min at 4°C. The supernatant was transferred to columns where Neutavidin agarose resin (200 μl) had been previously loaded (Thermo Scientific) and incubated for 60 min at room temperature.
with end-over-end mixing. After being washed four times with PBS containing protease inhibitors, 1× sample buffer containing DTT was added to the column, and samples were incubated for 60 min at room temperature. Samples were heated at 65°C for 10 min.

**pH measurements.** pH was measured using the pH-sensitive fluorescent dye BCECF-AM (Molecular Probes). BCECF-AM is converted to intracellular esterases. Primary cultured IMCD cells from rat kidneys were seeded and cultured on collagen-coated 35-mm glass-base dishes (Asahi Techno Glass) in hypertonic culture media (pH 7.4 and 640 mosmol/kgH2O) for 4 days. On day 5, cells were incubated with nonserum IMCD culture media (DMEM-F-12 without phenol red, 80 mM urea, 130 mM NaCl, and 10,000 U/ml penicillin-streptomycin) containing 2 μM BCECF-AM at 37°C in 5% CO2-95% air for 30 min. Cells were washed twice in PBS and changed to nonserum IMCD culture media (pH 7.4) for 10 min in a 37°C incubator before experiments. IMCD cells were mounted in the chamber on the temperature-controlled stage of Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan). Cells were continuously perfused with Na+-free solution with different pH values (pH 6.4, 7.4, and 8.4, see below) for 60 min by a six-channel system (VC-2, Warner Instruments, Hamden, CT), and the perfusate was collected by the peristaltic pump (EYELA MP3, Tokyo, Japan).

pH was measured using the dual-excitation fluorescence ratio method. The fluorescence was monitored at 530 nm with excitation wavelengths of 440 nm (a wavelength at which the fluorescence is relatively pH insensitive) and 490 nm (a wavelength at which the fluorescence is very pH sensitive). pH was time lapse obtained by the Nikon Ti-E inverted microscope equipped with a Cascade 512B EMCCD camera (Roper Scientific, Trenton, NJ) and excitation and emission filter wheels (MAC5000, Luddl Electronic Products, Hathorne, NY). All systems were controlled by MetaMorph software (Universal Imaging, Downingtown, PA). Fluorescent images were acquired sequentially through cyan fluorescent protein (CFP) filter channels. Images were acquired using the 4 × 4 binning mode and 200-ms exposure time. A ×60 (numerical aperture: 1.4) objective lens (Nikon) was used for measurements. The 490-to-440-nm ratio was created by MetaMorph software. Data were obtained from five independent experiments in both groups, respectively.

**pH** was calculated by the nigericin-calibration technique (3). IMCD cells were exposed to Na+-free solution (0 mM Na+, 105 mM K+, 0 mM NH4+, 49 mM N-methyl-d-glucamine−, 5 mM Mg2+, 0 mM Ca2+, 63 mM CI−, 106.4 mM aspartate−, 0.4 mM H2PO4−, 1.6 mM HPO42−, 17.8 mM HEPES−, 0 mM HCO3−, 5 mM SO42−, 10 mM EGTA2−, 5.5 mM glucose, 5 mM alanine, and 14.4 mM HEPES) with different pH values (6.4, 7.4, and 8.4) by adding KOH containing 10 μM of the K+/H+ exchanger nigericin. When the K+ concentration was equal between intracellular and extracellular compartments, pH and pHe should be the same with nigericin (490-to-440-nm ratio: 1.0, pH 7.0) (3). Therefore, data were normalized to make the ratio at pH 7.0 equal to unity. Raw data of intensity at each excitation wavelength were corrected for background-corrected fluorescence emissions as pH values as pH 7.0 equal to unity. Raw data of intensity at each excitation wavelength were corrected for background-corrected fluorescence emissions as pH values as pH 7.0 equal to unity. Raw data of intensity at each excitation wavelength were corrected for background-corrected fluorescence emissions as pH values as pH 7.0 equal to unity. Raw data of intensity at each excitation wavelength were corrected for background-corrected fluorescence emissions as pH values as pH 7.0 equal to unity.

**FRET imaging for PKA activity.** LLC-PK1 cells stably transfected with AKAR3EV (PKA FRET-based indicator) (29), LLC-PK1 cells in DMEM (phenol red free) were subjected to time-lapse imaging. LLC-PK1 cells transiently transfected with YC3.60 were cultured on collagen-coated 35-mm glass-base dishes (Asahi Techno Glass). The plasmid for YC3.60 was also provided by Prof. M. Matsuda (Kyoto University). YC3.60 was composed of calmodulin (CaM), enhanced YFP, a glycylglycine linker, the CaM-binding peptide of myosin light chain kinase (M13), and enhanced CFP (29). Ca2+ binding to the CaM moiety initiates an intramolecular interaction between CaM and M13 domains, which causes the chimeric protein to shift from a spread form to a more compact form, therefore increasing the efficiency of FRET from CFP to YFP (29). With this Y36.60 indicator, changes of intracellular Ca2+ levels were time lapse obtained by the Nikon Ti-E inverted microscope. Images were acquired every 15 s using the 4 × 4 binning mode and 200-ms exposure time. The FRET-to-CFP ratio was created with MetaMorph software, and the value was normalized to 1 at the starting time point in all groups. Data were obtained from five independent experiments in both groups, respectively.

**Statistical analyses.** Values are presented as means ± SE. Data were analyzed by one-way ANOVA followed by Bonferroni’s multiple-comparison test. Multiple-comparisons tests were only applied when a significant difference was determined by ANOVA. P values of <0.05 were considered as significant.

**RESULTS**

Effects of pHe on dDAVP-induced AQP2 phosphorylation in IMCD tubule suspensions. To examine whether changes of pHe affect dDAVP-induced AQP2 phosphorylation in IMCD cells, freshly prepared IMCD tubule suspensions were incubated for 1 h in HEPES-buffered saline solution with different pH (pH 5.4, 6.4, 7.4, or 8.4). First, AQP2 phosphorylation was examined with specific antibodies against p-AQP2 at Ser256 (PKA phosphorylation consensus site), and the p-AQP2-to-AQP2 ratio was examined in IMCD tubule suspensions treated with either vehicle or dDAVP (10−10 M, 3 min; Fig. 1, A and B). Semiquantitative immunoblot analysis revealed that dDAVP-induced AQP2 phosphorylation (Ser256) was significantly decreased when tubule suspensions were exposed to pH 5.4 and 6.4 compared with pH 7.4 and 8.4 (Fig. 1B). Moreover, the decrease of AQP2 phosphorylation was more prominent when tubule suspensions were exposed to more acidic conditions (pH 5.4) compared with pH 6.4 (Fig. 1B), suggesting that acidic pHe decreases dDAVP-induced phosphorylation of AQP2 in IMCD cells and that lowering pH values have even more prominent effects. Based on this finding, three pH levels, i.e., pH 6.4, 7.4, and 8.4, were chosen for further experiments, as urine pH can vary between 4.6 and 8.0 in humans.
Next, the effects of dDAVP at pH 6.4, 7.4, and 8.4 were compared with vehicle-treated control at pH 6.4, 7.4, and 8.4, respectively, in IMCD tube suspensions (Fig. 2). Changes of dDAVP-induced AQP2 phosphorylation at Ser256, Ser261, Ser264, and Ser269 in IMCD tube suspensions were examined by semiquantitative immunoblot analysis. At pH 7.4, AQP2 was significantly phosphorylated at Ser256, Ser264, and Ser269 after short-term dDAVP stimulation (10^{-10} M, 3 min), whereas AQP2 phosphorylation at Ser261 was markedly decreased (Fig. 2, A and D–G). AQP2 phosphorylation at Ser256 was increased after dDAVP stimulation (10^{-10} M, 3 min) under the alkaline condition (pH 8.4), whereas Ser261 phosphorylation was decreased (Fig. 2, C–E). In contrast, Ser264 and Ser269 were not phosphorylated after short-term dDAVP stimulation (10^{-10} M, 3 min) under pH 8.4 (Fig. 2, C, F, and G), possibly due to slower phosphorylation than the corresponding increase in Ser256 phosphorylation, which has been demonstrated in a previous study (10). In contrast, at pH 6.4, Ser256 and Ser269 of AQP2 were not significantly phosphorylated, and phosphorylation at Ser264 of AQP2 was even decreased after dDAVP stimulation (10^{-10} M, 3 min; Fig. 2, B, D, F, and G).

Moreover, the effects of dDAVP stimulation (10^{-10} M, 3 min) on AQP2 phosphorylation were compared among IMCD tube suspensions exposed to pH 6.4, 7.4, or 8.4 for 1 h (Fig. 3, A–F). When compared with dDAVP-induced AQP2 phosphorylation at pH 7.4, AQP2 phosphorylation at Ser256 (76 ± 3%, P < 0.05), Ser264 (44 ± 2%, P < 0.05), and Ser269 (26 ± 4%, P < 0.05) was significantly decreased under the acidic condition (pH 6.4; Fig. 3, B, C, E, and F).

![Figure 2: Semiquantitative immunoblot analysis of AQP2 and p-AQP2 (Ser256, Ser261, Ser264, and Ser269) in IMCD tube suspensions. A–C: IMCD tube suspensions were incubated for 1 h in HEPES-buffered saline solution with different pH [pH 6.4 (B), 7.4 (A), or 8.4 (C)]. IMCD tube suspensions were stimulated with either vehicle or dDAVP (10^{-10} M) for the last 3 min. Immunoblots of IMCD tube suspensions reacted with antibodies against AQP2 and p-AQP2 (deglycosylated ~29 kDa and glycosylated ~35–50 kDa). D–G: densitometric analysis. *P < 0.05 compared with vehicle-treated IMCD tube suspensions. n is the number of IMCD tube suspension preparations from independent experiments.](http://ajprenal.physiology.org/).
and F), whereas Ser\(^{261}\) was more phosphorylated (122 ± 6%, \(P < 0.05\); Fig. 3, B and D). Under the alkaline condition (pH 8.4), however, Ser\(^{256}\) and Ser\(^{264}\) of AQP2 were phosphorylated similar to the condition of pH 7.4 (Fig. 3, B, C, and E). In contrast, increased phosphorylation of Ser\(^{261}\) (133 ± 3%, \(P < 0.05\); Fig. 3, B and D) and decreased phosphorylation of Ser\(^{269}\) (50 ± 3%, \(P < 0.05\), Fig. 3, B and F) were observed.

**Changes of pH in primary cultured IMCD cells from rat kidneys.** Changes of pH of IMCD cells were monitored when cells were exposed to Na\(^{+}\)/H\(^{-}\)-free solution containing nigericin with different pH values (pH 6.4, 7.4, or 8.4) for 60 min, respectively. Changes of pH were measured in IMCD cells where BCECF-AM was applied. As shown in Fig. 4A, changes of the 490-to-440-nm ratio were time lapse monitored when IMCD cells were exposed to Na\(^{+}\)/H\(^{-}\)-free solution of pH 6.4, 7.4, and 8.4 containing 10 M nigericin and high K\(^{+}\) (105 mM). The background-corrected fluorescence intensity was then normalized to 1 when pH\(_{i}\) was 7.0 (Fig. 4B). Within 3 min of exposure to pH\(_{e}\) 6.4 or 8.4, pH\(_{i}\) was rapidly changed (Fig. 4C). At 60 min, pH\(_{i}\) became 6.1, 7.4, or 8.2 when cells were exposed to pH\(_{e}\) 6.4, 7.4, or 8.4, respectively (\(n = 5\) cells/group; Fig. 4C).

**Decreased dDAVP-induced apical translocation of AQP2 in IMCD cells when cells were exposed to acidic pH.** It is conceivable that cellular pH is more sensitive to basolateral pH in IMCD cells as the apical membrane is tight. To examine whether pH changes at the apical side (i.e., apical pH) or basolateral side (i.e., basolateral pH) of IMCD cells affect dDAVP (basolateral)-induced AQP2 expression in the apical plasma membrane, cell surface biotinylation assays were performed in IMCD cells cultured in transwell chambers. IMCD cells were exposed to a transepithelial pH gradient for 1 h (pH 6.4, 7.4, or 8.4 at the apical side vs. pH 7.4 at the basolateral side and vice versa). Changes of actual pH values in culture media in both compartments were directly measured after 1 h by a pH meter (Mettler-Toledo) and are shown in Table 1.

In the presence of dDAVP stimulation (10\(^{-9}\) M, 15 min, basolateral side only), cell surface biotinylation assays demonstrated that the biotinylated AQP2-to-total AQP2 ratio was significantly decreased when IMCD cells were exposed to
Apical compartment pH 6.4 for 1 h (60 ± 6% of dDAVP-treated IMCD cells exposed to apical pH 7.4, P < 0.05; Fig. 5). Moreover, the biotinylated AQP2-to-total AQP2 ratio was more decreased when IMCD cells were exposed to basolateral pH 6.4 for 1 h (48 ± 5% of dDAVP-treated IMCD cells exposed to basolateral pH 7.4, P < 0.05; Fig. 6). In contrast, under the alkaline condition (pH 8.4) in either the apical or basolateral side of cells, the biotinylated AQP2-to-total AQP2 ratio after dDAVP stimulation (10^{-9} M, 15 min, basolateral side only) was similar to that observed under pH 7.4 (Figs. 5 and 6).

Laser scanning confocal microscopic examination of AQP2 labeling was done in IMCD cells cultured in transwell chambers.

**Table 1. Changes of pH values in culture media after a 1-h transepithelial pH gradient on inner medullary collecting duct cells in transwell chambers**

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<th>Treatment</th>
<th>pH Values</th>
<th>pH Changes</th>
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<td>Apical pH 6.4, 7.4, or 8.4 Versus Basolateral pH 7.4</td>
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<td>Apical compartment</td>
<td>Initial pH (n = 6)</td>
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<td></td>
<td>pH values at 1 h (n = 6)</td>
<td>7.1 ± 0.02</td>
<td>7.5 ± 0.05</td>
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<tr>
<td>Basolateral compartment</td>
<td>Initial pH (n = 6)</td>
<td>7.4</td>
<td>7.4</td>
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<tr>
<td></td>
<td>pH values at 1 h (n = 6)</td>
<td>7.5 ± 0.03</td>
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<th>Apical pH 7.4 Versus Basolateral pH 6.4, 7.4, or 8.4</th>
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<td></td>
<td>pH values at 1 h (n = 6)</td>
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<td>pH values at 1 h (n = 6)</td>
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Consistent with the findings of cell surface biotinylation assays, dDAVP stimulation (10^{-9} M, 15 min, basolateral side only) induced less AQP2 labeling in the apical plasma membrane when cells were exposed to apical or basolateral compartment pH 6.4 for 1 h (Fig. 7, B and E) compared with exposure to apical compartment pH 7.4 and 8.4 for 1 h (Fig. 7, A and C) or basolateral compartment pH 7.4 and 8.4 for 1 h (Fig. 7, D and F). When cells were exposed to apical or basolateral compartment pH 6.4 for 1 h, intracellular AQP2 labeling was more prominent (Fig. 7, B and E).

**FRET analysis for PKA activity.** The cAMP/PKA signaling pathway is a principal signaling pathway for both AQP2 trafficking and protein expression. We used a PKA FRET-based indicator (AKAR3EV) (17) to investigate the effects of pH on dDAVP-induced PKA activity. Since IMCD cells were resistant to transfection of the PKA FRET-based indicator, LLC-PK1 cells, which are the proximal tubular origin from pig kidneys and express V2R (36), were used for FRET analysis. With this probe, time-lapse imaging was taken to study the changes in PKA activity in stably transfected LLC-PK1 cells expressing AKAR3EV during exposure to different pHs in the presence of 10^{-9} M dDAVP stimulation. Abundant expression of AKAR3EV and a dDAVP-induced increase in FRET levels were observed in cells (Fig. 8A). When cells were stimulated with a high concentration of dDAVP (10^{-8} M), FRET levels were increased irrespective of pH changes (Fig. 8, A and B). However, when cells were treated with a lower concentration of dDAVP (10^{-9} M), increased FRET levels, i.e., increased PKA activity, were only seen in cells exposed to pH 7.4 and 8.4 (Fig. 8, A and C) but not during exposure to pH 6.4. In contrast, increased FRET levels were seen in cells stimulated with forskolin (both 10^{-6} and 10^{-7} M), independent of pH.

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**Fig. 4. Changes of intracellular pH (pHi) in primary cultured IMCD cells where BCECF-AM was applied. A: time course of changes in the fluorescence excitation 490-to-440-nm ratio during exposure to Na^+ -free solution of pH 6.4, 7.4, and 8.4 containing 10 M nigericin and high K^+ (105 mM). B: dependence of the normalized fluorescence excitation ratio on pH. C: changes of pHi in IMCD cells exposed to extracellular pH (pHe) 6.4, 7.4, or 8.4 for 60 min.**
changes (Fig. 9, A and B), suggesting that acidic pH could attenuate dDAVP-induced PKA activity by affecting vasopressin binding to the V2R and/or Gs/H9251-mediated adenylyl cyclase activity.

Consistent with this, the effect of pH on forskolin-induced AQP2 phosphorylation (Ser256) was examined. In contrast to the observed changes in apical pH on AQP2 expression at the apical plasma membrane of IMCD cells, *P < 0.05 compared with dDAVP-treated IMCD cells exposed to apical pH 7.4. 

n is the number of preparations of IMCD cell lysates.
that the forskolin (10^{-6} M, 3 min)-induced p-AQP2-to-AQP2 ratio was similar irrespective of pH_e 6.4, 7.4, or 8.4 (Fig. 10).

**FRET analysis for intracellular Ca^{2+} levels.** To examine whether dDAVP-induced intracellular Ca^{2+} dynamics are affected by changes of pH_e, analysis of FRET levels using YC3.60 as an indicator of intracellular Ca^{2+} (29) was performed. LLC-PK1 cells transiently transfected with YC3.60 were analyzed for FRET responses after dDAVP stimulation with changes of pH_e (Fig. 11A). Intracellular Ca^{2+} levels were increased after dDAVP stimulation (10^{-8} or 10^{-9} M, 25 min), which were not affected by changes of pH_e (Fig. 11, B and C).

**DISCUSSION**

In the present study, we examined the effects of pH_e on dDAVP-induced phosphorylation (Ser^{256}, Ser^{261}, Ser^{264}, and Ser^{269}) and apical targeting of AQP2 in IMCD tubule suspensions and primary cultured IMCD cells from rat kidneys. AQP2 phosphorylation after short-term dDAVP stimulation was attenuated when tubule suspensions were exposed to acidic pH (pH 5.4 and 6.4) compared with that induced under conditions of neutral pH (pH 7.4) and alkaline pH (pH 8.4). Moreover, IMCD cells exposed to a transepithelial pH gradient in the transwell chamber demonstrated that exposure to either apical or basolateral pH 6.4 significantly decreased dDAVP (basolateral side)-induced apical expression of AQP2 compared with that under exposure to pH 7.4 and 8.4. Importantly, FRET analysis was done to study the effects of pH_e on dDAVP- and forskolin-induced intracellular PKA activity and Ca^{2+} levels. dDAVP-induced PKA activity was significantly attenuated when LLC-PK1 cells were exposed to acidic pH compared with neutral or alkaline pH, whereas forskolin-induced PKA activation was not affected. The dDAVP-induced increase of intracellular Ca^{2+} was not affected by changes of pH_e.
Previous studies demonstrated that pH regulates the hydroosmotic action of AVP in the toad urinary bladder (4, 33) and rabbit kidney collecting ducts (26). In the isolated perfused rabbit cortical collecting tubule, when bath pH values were decreased, vasopressin-induced changes in mean hydraulic conductance was decreased, whereas reduced luminal pH did not have an effect on vasopressin-induced hydraulic conductance (26). In particular, in cultured cells of rat kidney papillary collecting ducts, Ishikawa et al. (13) demonstrated that acidic pH reduced AVP- and forskolin-induced cAMP production and AVP-mediated Ca\(^{2+}\) mobilization. Acidic pH (pH 6.8) markedly suppressed the receptor binding of \[^3H\]AVP in papillary collecting duct cells, resulting in a reduction of AVP-induced activation of adenylate cyclase (13). This finding was further supported by a recent study (44) demonstrating that V2Rs displayed a significantly reduced affinity for vasopressin under acidic conditions in LLC-FLAG-V2R cells, whereas an increase of osmolality had no effect on V2R binding to vasopressin.

Fig. 8. Fluorescence resonance energy transfer (FRET) analysis for PKA activity in dDAVP-stimulated LLC-PK1 cells exposed to different pHs. A: time-lapse live cell FRET imaging analysis in LLC-PK1 cells expressing PKA FRET-based indicator (AKAR3EV). B and C: LLC-PK1 cells were exposed to different pH (pH 6.4, 7.4, or 8.4) for 1 h and stimulated by dDAVP (10\(^{-8}\) or 10\(^{-9}\) M) for the last 15 min. FRET intensity was analyzed every 15 s during 20 min. The values of FRET ratio were calculated from 5 (B) or 11 (C) independent experiments. Red line, exposure to pH 6.4; black line, exposure to pH 7.4; green line, exposure to pH 8.4. *P < 0.05 compared with dDAVP-treated LLC-PK1 cells exposed to pH 7.4 and 8.4.

Fig. 9. FRET analysis for PKA activity in forskolin-stimulated LLC-PK1 cells exposed to different pHs. A and B: time-lapse live cell FRET imaging analysis in LLC-PK1 cells expressing PKA FRET-based indicator (AKAR3EV). LLC-PK1 cells were exposed to different pH (pH 6.4, 7.4, or 8.4) for the last 15 min. FRET intensity was analyzed every 15 s during 20 min. Values of the FRET ratio were calculated from five independent experiments. Red line, exposure to pH 6.4; black line, exposure to pH 7.4; green line, exposure to pH 8.4.
Transcellular water reabsorption across renal tubular epithelial cells is dependent on the expression of AQPs (32). Vasopressin plays a key role in water reabsorption in the kidney connecting tubule and collecting ducts, where AQP2, the vasopressin-regulated water channel protein, is regulated by intracellular trafficking for short-term regulation and by altered protein abundance for long-term adaptation of water balance. The most important mediator of both trafficking and protein abundance is vasopressin for the regulation of renal water reabsorption and body water homeostasis. Vasopressin-induced AQP2 trafficking is mediated by the binding of vasopressin to V2Rs, resulting in Gs protein-mediated stimulation of adenylate cyclase, resulting in increased cAMP levels. The increased concentration of cAMP causes activation of PKA, which subsequently phosphorylates AQP2 and regulatory proteins and stimulates AQP2-expressing vesicles to be fused with the apical plasma membrane, increasing osmotic water permeability (20, 21, 31). It has been demonstrated that AQP2 is phosphorylated at four serine sites (Ser256, Ser261, Ser264, and Ser269) in the COOH-terminal tail of rat AQP2 (12). Ser256 is part of a consensus motif (RRQS) for phosphorylation by PKA (8). Previous studies (10, 11) have demonstrated that AQP2 phosphorylation at Ser256, Ser264, and Ser269 was increased in IMCD tubule suspensions after short-term dDAVP stimulation, whereas Ser261 showed a decrease in phosphorylation in response to vasopressin or cAMP. Interestingly, phosphorylation at Ser269 was dependent on the prior phosphorylation of Ser256 (10).

Therefore, our results revealing decreased phosphorylation of AQP2 (Ser256, Ser264, and Ser269) in IMCD tubule suspensions and apical translocation of AQP2 in IMCD cells when they were stimulated with dDAVP (10^{-8} M, 3 min) under conditions of acidic pH were consistent with previous findings demonstrating the decrease of AVP-induced cAMP production and receptor binding of [3H]AVP under conditions of acidic pH.
pH in papillary collecting duct cells (13). This finding was further supported by FRET analysis. dDAVP (10^{-9} M)-induced PKA activity was significantly attenuated when LLC-PK1 cells were exposed to acidic pH compared with neutral or alkaline pH, whereas forskolin-induced PKA activation was not affected. The results indicate that acidic pH decreases dDAVP-induced phosphorylation and apical targeting of AQP2 in IMCD cells via an inhibition of V2R-G protein-cAMP-PKA actions.

In contrast, one could reconsider the results in relation to normal urine pH values in human and rodents. Under normal conditions, urine pH in human is <6 in a majority of the general population (22, 39), and in different strains of rodents, urine pH stays at or just above 6.5 and mostly below 7.5 (37). Based on this, one can assume that urine alkalization by administration of NaHCO_3 might be, at least in part, effective in concentrating urine in the condition of nephrogenic diabetes insipidus. Moreover, since alkaline pH in urine may stimulate stone formation, which will be aggravated by concentrated urine with increased apical expression of AQP2, this might be pathophysiologically relevant.

In primary cultured IMCD cells, vasopressin stimulation induced an increase of intracellular Ca^{2+} concentration to 350–400 nM (25). Vasopressin causes an increase in intracellular Ca^{2+} mobilization from ryanodine-sensitive intracellular Ca^{2+} stores, which is thought to play an important role in CaM-mediated translocation of AQP2-expressing vesicles (6, 31). Consistent with this, FRET analysis in the present study demonstrated that dDAVP (10^{-8} and 10^{-9} M) induced an increase of intracellular Ca^{2+} levels in IMCD cells. Ca^{2+} oscillation was not detected, since the FRET images were taken every 15 s for 25 min. However, the dDAVP-induced increase of intracellular Ca^{2+} levels was not affected by changes of pH. This was in contrast to changes of dDAVP (10^{-5} M)-induced PKA activity, which need to be further elucidated.

In addition to the decreased affinity of the V2R to vasopressin in conditions of acidic pH, systemic metabolic acidosis is also known to be associated with decreased expression of V2R mRNA in collecting ducts from rat kidneys (38). This is consistent with the observed vasopressin resistance in chronic renal failure commonly complicated with metabolic acidosis, demonstrating significantly decreased V2R mRNA levels (40) and AQP2 protein abundance (19).

In summary, the present study revealed that dDAVP-induced phosphorylation (Ser^{256}, Ser^{264}, and Ser^{269}) and apical targeting of AQP2 were significantly attenuated in IMCD cells under acidic pH. This is likely via an inhibition of V2R-G protein-cAMP-PKA actions.

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EXTRACELLULAR pH AFFECTS dDAVP-INDUCED AQP2 TRAFFICKING


