Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct

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Hoffert JD, Nielsen J, Yu M-J, Pisitkun T, Schleicher SM, Nielsen S, Knepper MA. Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. Am J Physiol Renal Physiol 292: F691–F700, 2007. First published September 19, 2006; doi:10.1152/ajprenal.00284.2006.—We recently identified a novel phosphorylation site, serine-261 (pS261), in the COOH-terminus of the vasopressin-regulated water channel, aquaporin-2 (AQP2). To address whether phosphorylation at this site is regulated by vasopressin, a rabbit polyclonal phospho-specific antibody was generated. Dot blot and immunoblot analysis demonstrated that this antibody specifically recognizes AQP2 phosphorylated at pS261, and that phosphorylation of S256 (pS256), a site already known to be regulated by vasopressin, does not interfere with antibody recognition. Immunohistochemical analysis revealed intense pS261 labeling of inner medullary collecting duct (IMCD) from wild-type mice, while sections from AQP2 knockout animals showed a general absence of labeling. AQP2 pS261 was present in principal cells of all mouse and rat distal tubule segments from the connecting tubule to the terminal IMCD. Co-immunolabeling of collecting duct with phospho-specific and total AQP2 antibodies revealed that pS261 and pS256 have distinct subcellular distributions. Levels of pS256 increased, while the amount of pS261 significantly decreased in freshly isolated rat IMCD samples incubated with 1 nM [deamino-Cys1, D-Arg8]vasopressin for 30 min. Similarly, based on immunohistochemical labeling, the amount of pS261 was reduced in all collecting duct segments of Brattleboro rats treated with [deamino-Cys1, D-Arg8]vasopressin for 2 h. This study reveals a reciprocal change in S256 and S261 phosphorylation in response to short-term vasopressin exposure, suggesting that these residues may serve distinct roles in regulation of AQP2 subcellular distribution and collecting duct water permeability.

IMCD: water transport; serine-256; channel; inner medullary

THE MAMMALIAN COLLECTING DUCT regulates renal water excretion in response to the antidiuretic hormone arginine vasopressin. Vasopressin increases collecting duct water permeability (14), thereby permitting water reabsorption from the tubule lumen, leading to production of concentrated urine. A critical step in this response involves trafficking of vesicles containing the vasopressin-sensitive water channel aquaporin-2 (AQP2) to the apical plasma membrane of collecting duct principal cells (15). There is mounting evidence, including studies in cell culture (6, 12) and animal models (3, 13), that phosphorylation of residue serine-256 (pS256) in the COOH-terminus of AQP2 plays a critical role in its apical trafficking.

As part of a large-scale phosphoproteomics screen in native rat inner medullary collecting duct (IMCD) cells, we recently identified additional residues in the COOH-terminus of AQP2 that are phosphorylated, namely S261, S264, and S269 (9). In that study, mass spectrometry-based quantification of AQP2 phosphopeptides suggested that the relative abundance of phosphorylated S261 (pS261) decreases in response to short-term vasopressin treatment, although the exact cause of this decrease could not be definitively addressed using this method. Residue S261 is conserved among all identified mammalian AQP2 isoforms.

To further explore S261 phosphorylation in the context of vasopressin signaling, we have developed a new phospho-specific antibody to this site. The present study addresses initial antibody characterization as well as cellular and subcellular distributions of pS261 in various distal tubule segments from rat and mouse kidney. Finally, potential changes in S261 phosphorylation with short-term [deamino-Cys1,D-Arg8]vasopressin (dDAVP) exposure were addressed using both in vitro and in vivo methods.

METHODS

Animals

Protocol 1: isolated IMCD experiment. Pathogen-free male Sprague-Dawley rats (Taconic Farm, Germantown, NY) were maintained on an autoclaved pelleted rodent chow (413110–75-56, Zeigler Bros., Gardners, PA) and ad libitum drinking water. All experiments were conducted in accord with an animal protocol approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (ACUC protocol number H-0110).

Protocol 2: normal Wistar rats and CD-specific AQP2 knockout mice. The following animal protocols have been approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice. Wistar rats were obtained from Møllegaard Breeding Center (Ejby, Denmark). CD-specific knockout mice were from an in-house stock and were originally generated by Rojek et al. (19). Rats were maintained on a standard rodent diet (Altromin no. 1320, Chr. Petersen A/S, Ringsted) and had free access to water.

Protocol 3: short-term dDAVP infusion of Brattleboro rats. Homozygous Brattleboro rats were obtained from Harlan Nederland (Horst, The Netherlands). Before the experiment, rats had free access to standard rat chow (Altromin no. 1320, Chr. Petersen A/S). Six Brattleboro rats were treated with subcutaneous injections of 50 ng dDAVP (Sigma-Aldrich, catalog no. V1005) in 500 μl saline/animal, and six vehicle-injected Brattleboro rats served as controls. After 2 h, the rats were anesthetized by halothane and were killed during perfusion fixation of the kidneys. Between injection of dDAVP and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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fixation of the kidney, rats had free access to water but not food. This is the same protocol as previously described by Christensen et al. (3).

**Materials**

Synthetic, NH₂-terminally biotinylated peptides corresponding to amino acids 241–271 of the COOH-terminus of rat AQP2 (.EPDT-DWEEREVRRQSVELHSPQSLPRGSKAQ) were generated by Anaspec (San Jose, CA). For three of these peptides, various residues were modified by phosphorylation, including S256, S261, and doubly-phosphorylated S256 and S261. An affinity-purified rabbit polyclonal antibody (anti-pS261) was generated against a proprietary sequence from the COOH-terminus of rat AQP2 that included pS261 (PhosphoSolutions, Aurora, CA). The affinity-purified antibody anti-pS256, which recognizes AQP2 phosphorylated at Ser-256, has been described previously (17). Total AQP2 antibodies L127 (16) and chicken anti-AQP2 (1) were used for immunoblotting and immunofluorescence, respectively. Goat polyclonal antibodies to glucose-regulated protein 78 (GRP78) and cathepsin D were from Santa Cruz Biotechnology (Santa Cruz, CA). The Golgi matrix protein 130 (GM130) mouse monoclonal antibody was from BD Transduction Laboratories (San Jose, CA). FITC-conjugated Dolichos biflorus agglutinin (DBA) was from Sigma.

**IMCD Isolation and Treatment with dDAVP**

IMCD fractions were freshly prepared from the inner medulla of rat kidney using the method of Stokes et al. (20) with some modifications (10, 18). IMCD samples were incubated in bicarbonate buffer in the presence or absence of 1 mM dDAVP for 1, 2, 5, 15, or 30 min in a pH/temperature-controlled chamber with gentle mixing under an atmosphere of 95% air–5% CO₂ at 37°C.

**Immunoblotting and Dot Blotting**

Enriched IMCD pellets were solubilized in 1.5% SDS/Tris, pH 6.8. Total protein concentrations were determined by the bichinchoninic acid assay (Pierce) using BSA as the standard. Protein samples (10 μg) were subjected to SDS-PAGE and immunoblotting as described (4), with reagent and protocol modifications based on the Odyssey Infrared Imaging System (LiCor, Lincoln, NE). Affinity-purified anti-pS256 and anti-pS256 were used at 1:2,000 and 1:500, respectively, for both dot blot and immunoblot analysis. A fluorescently labeled secondary antibody IRDye 680 goat anti-rabbit IgG (LiCor) was used at 1:5,000 for detection of all primary antibodies. Band densities for both pS256 and pS261 were initially normalized to the total AQP2 signal, followed by calculation of the normalized abundance ratio (dDAVP vs. control) for each individual trial for each time point. These ratios were then converted to log₂ values followed by calculation of the mean ± SE (n = 3) for each time point. Paired t-tests were performed as appropriate.

**Immunohistochemistry**

Kidneys were perfusion fixed through the abdominal aorta. Blood was flushed from the kidneys with cold 0.01 M PBS (pH 7.4) for 15 s, before switching to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for an additional 1 h, followed by 3 × 10-min washes with 0.1 M cacodylate buffer, pH 7.4. The tissue was dehydrated in graded ethanol and left overnight in xylene. After tissue embedding in paraffin, 2-μm sections were cut on a rotary microtome (Leica Microsystems A/S, Herlev, Denmark).

For immunolabeling, the sections were dewaxed with xylene and rehydrated with graded alcohol before endogenous peroxidase activity was blocked by 0.5% H₂O₂ in absolute methanol for 10 min. Sections were microwave boiled in a target retrieval solution (1 mM Tris, pH 9.0, with 0.5 mM EGTA) for 10 min, before nonspecific binding was blocked with 50 mM NH₄Cl in PBS for 30 min, and 3 × 10 min blocking with PBS blocking buffer (1% BSA, 0.05% saponin, and 0.2% gelatin). The sections were incubated with primary antibody (diluted in PBS with 0.1% BSA and 0.3% Triton X-100) overnight at 4°C. The sections were washed 3 × 10 min with PBS wash buffer containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO P448, DAKO A/S, Glostrup, Denmark) for 1 h at room temperature. After 3 × 10-min rinses with...
PBS wash buffer, the sites of antibody-antigen reaction were visualized with a brown chromogen produced within 10 min by incubation with 0.05% 3,3′-diaminobenzidine tetrachloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H₂O₂. Mayer’s hematoxylin was used for counterstaining, and, after dehydration, cover slips were mounted with hydrophilic medium (Entokit, O. Kindler, Freiburg, Germany). For sections prepared for immunofluorescence, a secondary fluorescent antibody was used (goat anti-rabbit IgG, Alexa Fluor 488, and goat anti-chicken IgG Alexa Fluor, 546 A11040, Molecular Probes, Eugene, OR). After a 1-h incubation at room temperature, cover slips were mounted with a hydrophilic mounting media containing anti-fading reagent (n-propyl-gallact, P-3101, Sigma Chemical, St. Louis, MO). Light microscopy was carried out on a Leica DMRE (Leica Microsystems A/S, Herlev, Denmark). Laser confocal microscopy was carried out on a Leica TCS-SP2 laser confocal microscope (Heidelberg, Germany).

Assessment of Colocalization

To quantify the degree of colocalization between phosphorylated and total forms of AQP2, images were obtained with a Zeiss 510 confocal system equipped with UV-Vis lasers (Carl Zeiss, Jena, Germany). Images were acquired sequentially using a 364-nm laser line and emission between 385 and 470 nm for 4,6-diamidino-2-phenylindole, a 488-nm laser line and emission between 505 and 550 nm for Alexa 488, and a 561-nm laser line and emission over 585 nm for Alexa 568. Z-series of high-resolution (100 nm/pixel) images were obtained throughout the cells with a numerical aperture of 1.2. ZAP was performed on a Leica 510 laser scanning confocal system equipped with UV-Vis lasers (Carl Zeiss, Jena, Germany). Laser confocal microscopy was carried out on a Leica TCS-SP2 laser confocal microscope (Heidelberg, Germany).

RESULTS

Initial Characterization of the Anti-pS261 Antibody by Dot Blot Analysis

Three synthetic COOH-terminal AQP2 phosphopeptides (pS256, pS261, and pS256/261), as well as an unphosphorylated AQP2 peptide (see METHODS for sequence information), were spotted onto a nitrocellulose membrane and probed with anti-pS256, anti-pS261, and anti-total AQP2 antibodies (Fig. 1A). Anti-pS256 recognized AQP2 peptide phosphorylated at S256, but did not cross-react with either peptide phosphorylated at S261 or with unphosphorylated peptide. Anti-pS261 recognized AQP2 phosphorylated at S261, but did not cross-react with either peptide phosphorylated at S256 or unphosphorylated peptide. Both phospho-specific antibodies recognized the doubly phosphorylated AQP2 peptide. As expected, total AQP2 antibody recognized unphosphorylated as well as all phosphorylated peptides.

Residues S256 and S261 being in such close proximity to one another raised the possibility that phosphorylation of both sites simultaneously could affect phospho-specific antibody binding. To address this issue, both doubly phosphorylated (pS256/261) and singly phosphorylated (pS256, pS261) biotinylated AQP2 peptides were serially diluted and probed by dot blot using either anti-pS256 (Fig. 1B, left) or anti-pS261 (Fig. 1B, right) antibodies. Blots were reprobed with fluorescently labeled streptavidin to detect biotin, which reflected the total peptide signal. Raw spot intensities were normalized to the streptavidin signal to correct for potential differences in peptide loading. The normalized spot intensities were then plotted against fold peptide dilution. For both antibodies, plots of doubly and singly phosphorylated peptide dilutions were similar, demonstrating that simultaneous phosphorylation of both S256 and S261 does not affect phosphopeptide recognition by either phospho-specific antibody.

Immunolabeling of AQP2 pS261 in Mouse and Rat

To further characterize the specificity of the anti-pS261 antibody, immunoperoxidase labeling of kidney tissue sections from CD-specific AQP2 knockout mice (19) was performed. Strong immunolabeling was present in the IMCD of wild-type control mice (Fig. 2A), while a general absence of labeling was observed in the IMCD of knockout mice (Fig. 2B, note that a few cells were positive for pS261 in the knockout mice due to incomplete expression of the Cre-recombinase).

Fig. 2. Immunolabeling of pS261 in inner medulla from a wild-type mouse (A) and a collecting duct (CD)-specific AQP2 gene-knockout mouse (B). The expression of pS261 is strong and dispersed in the inner medulla of wild-type mice, while labeling is totally absent (except for a few cells indicated by arrow, see text for explanation) in the knockout mice. IMCD, inner medullary collecting duct. Scale bar: 25 μm.
Fig. 3. Immunolabeling of pS261 in normal rat kidney. Low-magnification images are shown of cortex (A), outer medulla (E), and inner medulla (I). High-magnification images are shown of labeling in connecting tubule (CNT; B) and cortical CD (CCD; C). The labeling of pS261 in the apical cell domain of some CNT and CCD principal cells had a distinct vesicular appearance (arrow). Labeling of pS261 in the basolateral cell domain was observed in the CNT cells (arrowhead) but not in CCD principal cells. D: double labeling of pS261 (green) and the CNT cell marker protein calbindin (red). Labeling in outer medullary collecting duct (OMCD) in the outer stripe (F) and inner stripe (G) of the outer medulla was dispersed and apical. H: double labeling of pS261 (green) and H^+^-ATPase (red), which is only expressed in intercalated cells, shows specific labeling of principal cells. Labeling of pS261 in the initial IMCD (J) was dispersed and vesicular, while more evenly dispersed and basolateral near the tip of the IMCD (K). L: immunofluorescence labeling of pS261 is shown in the middle IMCD. Scale bar: A, E, I, 100 μm; other images, 20 μm.
Fig. 4. Double immunofluorescence labeling of pS256 AQP2 and pS261 AQP2 with total AQP2 in CCD and IMCD. Overlay is shown of pS261 and total AQP2 labeling in the CCD (A) and initial part of the IMCD (C). Many cells in both segments showed a narrow apical area predominantly labeled with AQP2 (red) and a subapical area with labeling of both pS261 and AQP2 (yellow). Further away from the apical plasma membrane, punctate intracellular labeling of pS261 was seen (green), which did not colocalize with total AQP2. B and D: in the tissue sections labeled with pS256 and AQP2, there was colocalization at the apical plasma membrane and subapical area seen as yellow. Scale bar: overlay images, 20 μm; single-labeling images, 10 μm. Confocal immunofluorescence micrographs are shown of rat middle IMCD cells stained with antibodies against pS261 (E) or pS256 (F) in green and total AQP2 in red. Nuclear staining appears blue.
In tissue sections from Wistar rats, labeling of pS261 in the inner medulla (Fig. 3I) was much stronger than in the outer medulla (Fig. 3E), which was marginally stronger than in the cortex (Fig. 3A). The immunolabeling was totally ablated when the anti-pS261 antibody was preincubated with pS261 peptide (Fig. 3L, inset). In the cortical collecting duct (CCD) principal cells, labeling was predominantly localized in the apical or subapical domain (Fig. 3C). The apical labeling in some connecting tubule (CNT) cells and CCD principal cells had a distinct vesicular appearance. In the initial CCD (not shown) and CNT cells (Fig. 3B), labeling was observed in both the apical and the basolateral plasma membrane domain, as previously described for AQP2 (2). The expression of pS261 in the CCT was confirmed by double immunofluorescence labeling of tissue sections with antibodies against pS261 and the CNT cell marker calbindin (Fig. 3D). The labeling pattern in the outer medullary collecting duct (OMCD) of the outer stripe of the outer medulla (Fig. 3F) and inner stripe of the outer medulla (Fig. 3G) was dispersed with some labeling in the apical plasma membrane domain, similar to what was observed in the CCD. Double immunofluorescence labeling of tissue sections with antibodies against pS261 of AQP2 and H^+\text{ATPase}, which is expressed only in intercalated cells, confirmed that AQP2 pS261 is only present in principal cells (Fig. 3H). In initial and middle IMCD, labeling was dispersed with a tendency to be stronger and vesicular in the apical half of the IMCD cells (Fig. 3, J and L). Closer to the tip of the inner medulla, the labeling was more evenly distributed throughout the cells and also showed more distinct labeling of the basolateral domains (Fig. 3K). The immunolabeling pattern of pS261 was also examined in mice, but was not markedly different from the distribution in rats (not shown).

Next we compared the distribution of pS261 and pS256 by co-immunofluorescence labeling with total AQP2. We found that pS256 labeling colocalized (seen as yellow) with labeling of total AQP2 in CCD (Fig. 4B), OMCD (not shown), and initial IMCD (Fig. 4D). The degree of colocalization between pS256 and total AQP2 in the OMCD was relatively high (R_{coloc.} = 0.73 \pm 0.02, see methods) (Fig. 5, A and C). The colabeling was observed mainly in the apical plasma membrane and subapical domains in the CCD, OMCD, and initial IMCD. In contrast to pS256, immunolabeling of pS261 and total AQP2 only colocalized in the subapical domain of cells in CNT (not shown), CCD (Fig. 4A), OMCD (not shown), and initial IMCD (Fig. 4C). The pool of pS261 not colocalizing with AQP2 was dispersed intracellularly (Fig. 4, A and C, green). Labeling of AQP2 not colocalizing with pS261 was present in the apical plasma membrane domain (Fig. 4, A and C, red). The degree of colocalization between pS261 and total AQP2 in the OMCD (R_{coloc.} = 0.32 \pm 0.07) (Fig. 5, B and C) was significantly lower than for pS256 vs. total AQP2. In the middle portion of the inner medulla (Fig. 4, E and F), the labeling of both pS261 and pS256 was similar to total AQP2, being predominantly intracellular with relatively sparse apical staining.

Analysis of Subcellular Distribution of AQP2-pS261

To better define the subcellular distribution of AQP2-pS261 in rat collecting duct, double labeling was performed for AQP2 phosphorylated at pS261 and for various subcellular markers:

DBA for plasma membrane, GRP78 for endoplasmic reticulum (ER), GM130 for Golgi, and cathepsin D for lysosomes (Fig. 6). In all instances, results were similar for both IMCD and OMCD. DBA (Fig. 6A) exhibited strong apical labeling, which did not coincide with pS261, which was predominantly intracellular. GM130 (Fig. 6C) staining was punctate but also did not colocalize with pS261. Both GRP78 (Fig. 6B) and cathepsin D (Fig. 6D) labeling were extensively cytoplasmic; however, there was minimal colocalization with pS261. These results suggest that the majority of intracellular pS261 resides in a location other than the ER, Golgi, and lysosomes.

Analysis of S261 Phosphorylation During Short-term dDAVP Treatment in Isolated Rat IMCD

To determine the effects of short-term vasopressin on S261 phosphorylation, rat IMCD suspensions were enriched by differential centrifugation from freshly isolated whole inner medullas (according to methods) and incubated in the presence or absence of 1 nM dDAVP for 30 min. Proteins were immediately solubilized and subjected to immunoblotting using antibodies to pS256, pS261, and total AQP2 (Fig. 7A). All antibodies, including anti-pS261, recognized a discrete band at
~29 kDa, consistent with the size of the unglycosylated AQP2 monomer, as well as a broad smear centered at ~37 kDa, corresponding to various glycosylated forms of AQP2. S256 phosphorylation increased in samples treated with dDAVP for 30 min (Fig. 7A, left), as previously demonstrated (9, 21). In contrast, levels of both glycosylated and unglycosylated pS261 decreased in response to short-term dDAVP (Fig. 7A, middle). Neither of these effects was due to a change in overall AQP2 protein abundance (Fig. 7A, right). To further explore these changes in phosphorylation and to obtain quantitative data, a time course study was performed. Isolated rat IMCD samples were incubated with dDAVP as described above, and proteins were harvested at five different time points (1, 2, 5, 15, and 30 min) for immunoblot analysis. To provide a clearer visual representation, these ratios were converted to log2 values. After only 1 min of dDAVP treatment, pS256 increased approximately twofold (log2 = 1.17 ± 0.4) and remained elevated out to 30 min (Fig. 7B). The level of pS261 began to decrease by 15 min and was significantly reduced ~2.7-fold (log2 = −1.57 ± 0.77) by 30 min.

Analysis of S261 Phosphorylation During Short-term dDAVP Treatment in Brattleboro Rats

To investigate whether the decrease in pS261 expression after short-term dDAVP treatment could be recapitulated in vivo, we carried out immunoperoxidase labeling of pS261 in kidney tissue sections from Brattleboro rats treated with dDAVP for 2 h. Immunolabeling of pS261 AQP2 was markedly decreased in all kidney regions. In the initial part of the inner medulla, the labeling was virtually absent compared with untreated Brattleboro rats, which showed mainly dispersed
intracellular labeling (Fig. 8, A and B). In the middle of the inner medulla, the reduction in pS261 abundance with dDAVP treatment was detectable but less prominent than in the initial part of the inner medulla (Fig. 8, C and D), and a faint labeling in the apical and subapical domain was observed after 2 h of dDAVP (Fig. 8D, bottom inset). Some IMCD cells in the middle of the inner medulla also showed large granules in the subnuclear region (Fig. 8D, top inset). Initial IMCD cells from the same animals labeled with an anti-pS256 antibody showed an increase in labeling of the apical plasma membrane domain and less intracellular labeling compared with the untreated Brattleboro rats (Fig. 8, E and F).

**DISCUSSION**

This study presents an initial characterization of AQP2 phosphorylation at residue S261 using a newly developed phospho-specific antibody. This antibody successfully recognized a synthetic AQP2 peptide phosphorylated at S261 and did not cross-react with either unphosphorylated or S256-phosphorylated peptides. Importantly, phosphorylation at S256 did not interfere with antibody recognition of pS261. In addition, anti-pS261 strongly labeled collecting ducts from wild-type mice, but did not react with collecting duct cells from CD-specific AQP2 knockout mice. Although successfully used in both immunoblotting and immunohistochemistry applications, this antibody has not worked well for immunogold labeling and analysis by electron microscopy (personal observation).

Immunohistochemical analysis revealed that AQP2 phosphorylated at S261 was present in cells of the CNT as well as in principal cells of all collecting duct segments from CCD to terminal IMCD. In normal rats with free access to water, the subcellular distribution of pS261-AQP2 varied, depending on the tubule region, being apical and basolateral in CNT; mostly subapical in CCD, OMCD, and the initial part of the IMCD; and broadly distributed in cytoplasm in middle IMCD. In CCD, OMCD, and initial IMCD, pS261 appeared to have a distinct distribution compared with pS256. While pS261 staining was mostly subapical and punctate in appearance, pS256 staining was strongly apical with relatively little cytoplasmic labeling. Quantification of colocalization demonstrated that the pS256 signal overlapped significantly more than pS261 with total AQP2. The difference in distribution of pS261 and pS256 indicates the presence of distinct subcellular pools of AQP2 and suggests that phosphorylation at S261 may be involved in regulation of the subcellular localization of AQP2. An analysis of the subcellular distribution of AQP2-pS261 with various markers demonstrated that the majority of cytoplasmic pS261 is not located in the ER, Golgi, or lysosomes. Other candidate structures in which pS261 could reside might include early, late, or recycling endosomes or secretory vesicles.

The hypothesis that pS256 and pS261 play divergent roles in regulation of AQP2 is supported by evidence obtained in a previous study using protein mass spectrometry (9). In that study, we utilized capillary electrophoresis mass spectrometry to analyze changes in phosphoprotein abundance with short-term vasopressin exposure and found that the abundance of AQP2 monophosphorylated at S256 increased 7.2-fold, while the abundance of AQP2 monophosphorylated at S261 decreased 2.5-fold in the presence of 1 nM dDAVP for 10 min. However, the prior study could not clarify whether the decrease in monophosphorylated AQP2 at S261 was due to phosphorylation at S256 to produce the diphosphorylated form or due to an absolute reduction of AQP2 phosphorylated at S261. In the present study, immunoblotting with the pS261 antibody showed that pS261 was significantly decreased only after 30 min of dDAVP treatment, much later than the increase in pS256 (2 min), demonstrating an absolute reduction in AQP2 phosphorylated at S261. In the present study, immunoblotting with the pS261 antibody showed that pS261 was significantly decreased only after 30 min of dDAVP exposure, much later than the increase in pS256 at S261. The decrease in pS261 detected within 10 min of dDAVP exposure in the previous study was likely due to formation of diphosphorylated AQP2, which effectively decreased the mono-pS261 peptide signal during analysis by mass spectrometry. Thus both mechanisms appear to be in play.

In the present study, we were also able to detect a decrease in overall pS261 abundance in tissue sections from Brattleboro rats treated with dDAVP for 2 h. While there was nearly a complete absence of pS261 labeling in the initial part of the IMCD, there appeared to be a small amount of apical labeling of pS261 in the middle IMCD, suggesting that pS261 is not always located intracellularly. These sections also contained a number of larger intracellular structures that stained positive...
Fig. 8. Labeling of pS261 and pS256 in inner medulla of Brattleboro (BB) rats treated with dDAVP for 2 h. Immunoperoxidase labeling of pS261 in untreated control Brattleboro rats was dispersed in the initial part of inner medulla (IM1) (A) and virtually absent in the dDAVP-treated BB rats (B). There was also a decrease in the overall pS261 labeling in the middle part of the inner medulla (IM2) (C vs. D). The dDAVP-treated BB rats also showed a weak labeling in the apical or subapical area (D and bottom inset), and some cells showed large, strongly staining punctate structures (D, top inset, arrows). Tissue sections labeled with antibody directed against pS256 showed dispersed labeling in the initial IMCD of control BB rats (E), while the dDAVP-treated BB rats showed a marked increase in apical labeling and decreased intracellular labeling (F). Scale bar: 20 μm; higher magnification insets, 10 μm.
for pS261 (Fig. 8D, inset arrows). The identity of these structures remains to be determined. To be consistent with this localization, it can be speculated that phosphorylation at S261 either inhibits exocytosis or stimulates endocytosis.

While it is evident that there is a decrease in overall pS261 abundance with short-term vasopressin exposure, the exact cause of this decrease (e.g., increased phosphatase activity, decreased kinase activity, or protein degradation) is as yet unidentified. If the mechanism involves dephosphorylation of S261, a candidate phosphatase would be protein phosphatase 2B (calcineurin), which has been found in endosomes containing AQP2 and can dephosphorylate AQP2 in vitro (11). In addition, calcineurin Aα null mice were shown to have diminished apical expression of AQP2 in the presence of dDAVP (7). Although the authors suggested that this phenotype was due to a combination of defective ER to Golgi transport and decreased AQP2 S256 phosphorylation, it may be informative to explore phosphorylation of S261 in the context of short-term vasopressin in these animals. If regulation of a specific kinase is involved, we can speculate that it is a member of the so-called “proline-directed” serine/threonine kinase subfamily because of the presence of proline at position 262. This proline is mutated in a subset of patients with autosomal recessive nephrogenic diabetes insipidus (5). Members of the proline-directed protein kinase family include mitogen-activated protein kinases (ERK1, p38), as well as cyclin-dependent kinases (cdc2, Cdk5). Upon sequence comparison, there is no similarity between the proline-directed S261 consensus and the basophilic PKA consensus of S256 (.RQXS.), further evidence that phosphorylation at these two residues may be regulated by distinct signaling pathways. There is also the possibility that the decrease in pS261 abundance results from enhanced protein degradation by lysosomal and/or proteasomal pathways. Evidence suggests that both of these pathways regulate the transient decrease in AQP2 abundance following increased vasopressin exposure in a cortical collecting duct cell line (8).

In conclusion, this study presents the first direct evidence demonstrating regulation of AQP2 phosphorylation at S261 by vasopressin. It also shows that the intracellular distribution of pS261-AQP2 differs from that of total AQP2 as well as pS256-AQP2, suggesting a unique role in AQP2 trafficking or function. Although the signaling pathways regulating AQP2 phosphorylation at S261 remain unknown, the findings in this paper are tantalizing clues to guide future studies of regulation of water transport in the renal collecting duct.

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