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Phosphorylation and dephosphorylation of aquaporin-2 at serine 269 and its subcellular distribution during vasopressin-induced exocytosis and subsequent endocytosis in the rat kidney

Kinue Shimizu[1], Megumi Sano[1], Aoi Kita[1], Nobuhiko Sawai[1,2], Akiko Iizuka-Kogo[1], Hiroshi Kogo[1], Takeo Aoki[1,3], Kuniaki Takata[1,4], and Toshiyuki Matsuzaki[1]

[1]*Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan*
[2]*Present address: Department of Molecular Medicine and Anatomy, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan*
[3]*Present address: School of Radiological Technology, Gunma Prefectural College of Health Sciences, 323-1 Kamioki-Machi, Maebashi, Gunma 371-0052, Japan*
[4]*Present address: Gunma Prefectural College of Health Sciences, 323-1 Kamioki-Machi, Maebashi, Gunma 371-0052, Japan*

Summary. Aquaporin-2 (AQP2) is a water channel protein that is trafficked between intracellular vesicles and the plasma membrane of kidney collecting duct cells upon vasopressin stimulation. Vasopressin changes the phosphorylation states of the AQP2 C-terminal serines (Sers), Ser256, Ser261, Ser264, and Ser269, in rats and mice, which is thought to play a role in controlling trafficking. Here, we focused on Ser269. We developed a specific antibody to Ser269-phosphorylated AQP2. Using immunofluorescence microscopy, we examined its localization in the rat kidney following injection of vasopressin and a vasopressin type 2 receptor-specific antagonist (OPC-31260). Ser269-phosphorylated AQP2 was almost undetectable in the water-loaded rat kidney, but was detected intracellularly soon after vasopressin injection, and then highly accumulated on the apical membrane of connecting tubule and collecting duct principal cells. In addition to the apical membrane, Ser269-phosphorylated AQP2 was also detected on the basolateral membrane of connecting tubule cells and inner medullary collecting duct principal cells. OPC-31260 injection following vasopressin stimulation caused internalization of AQP2, a pool of which was phosphorylated at Ser269. These results suggest that 1) AQP2 is phosphorylated at Ser269 intracellularly upon vasopressin stimulation and is rapidly trafficked to the plasma membrane, and 2) AQP2 can be internalized from the plasma membrane even if it remains phosphorylated at Ser269.

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Address for correspondence: Toshiyuki Matsuzaki, M.D., Ph.D. Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan
Tel: +81-27-220-7900, Fax: +81-27-220-7906
E-mail: matoshi@gunma-u.ac.jp

Introduction

Aquaporins (AQPs) are membrane water channels that allow rapid and substantial water transfer across lipid bilayers (Tani

and Fujiyoshi, 2014). To date, 13 AQP isoforms have been identified in mammals (Ishibashi *et al.*, 2011). The mammalian kidney reabsorbs large amounts of water from the glomerular filtrate, which requires at least four AQP isoforms (Takata *et al.*, 2004): AQP1, AQP2, AQP3, and AQP4. AQP1 is present in both the apical and basolateral membranes of proximal tubule cells and is involved in near-isosmolar water reabsorption (Nielsen *et al.*, 1993). In the collecting duct system, there are three AQP isoforms (AQP2 on the luminal side, and AQP3 and AQP4 on the abluminal side) that enable urinary concentration upon vasopressin stimulation (Ikeda and Matsuzaki, 2015; Takata *et al.*, 2008). Among these, AQP2 is a fundamental and critical molecule in vasopressin-controlled urinary concentration (Fushimi *et al.*, 1993). AQP2 is mainly localized in the apical region of principal cells along connecting tubules and the collecting duct system (Nielsen *et al.*, 2002). It largely remains in intracellular vesicles in the absence of plasma vasopressin (or in the presence of low levels), and vesicular exocytosis is accelerated upon vasopressin stimulation, which causes AQP2 to accumulate in the apical membrane for water reabsorption to occur (Brown, 2003). A complete understanding of the AQP2 trafficking mechanism and its regulation remains a long-term goal.

One possibly important phenomenon that regulates AQP2 trafficking is its phosphorylation (Brown *et al.*, 2008; Ikeda and Matsuzaki, 2015). There are four serines (Sers) located near the carboxyl terminal of rat and mouse AQP2 that could be phosphorylated or dephosphorylated in response to vasopressin. Among these, Ser256 is phosphorylated by kinases such as protein kinase A upon vasopressin stimulation; therefore, Ser256 is thought to be the most critically important phosphorylation site (Fushimi *et al.*, 1997). Recently, however, phosphorylation of Ser264, Ser269, and Ser256 was found to increase following vasopressin stimulation, whereas Ser261 phosphorylation decreases (Hoffert *et al.*, 2008; Hoffert *et al.*, 2007), thereby suggesting that the intracellular trafficking of AQP2 is regulated by much more complex mechanisms than previously envisaged.

The current study focused on the relationship between phosphorylation of AQP2 at Ser269 and AQP2 trafficking. Moeller et al. (2009) previously reported that Ser269 is phosphorylated upon vasopressin stimulation and that the localization of Ser269-phosphorylated AQP2 is restricted to the apical membrane of collecting duct cells, thereby suggesting that phosphorylation occurs at the apical membrane and plays a role in the membrane retention of AQP2. However, there is a lack of information regarding the localization of Ser269-phosphorylated AQP2 soon after vasopressin treatment or during endocytotic processes after abolishing the effects of vasopressin. To clarify these processes, we prepared a Ser269-phosphorylated AQP2-specific antibody and performed detailed time course analysis of Ser269 phosphorylation and the intracellular localization of this protein upon treatment with vasopressin or a vasopressin type 2 receptor-specific antagonist.

Materials and Methods

Anti-Ser269-phosphorylated AQP2 antibody

A peptide corresponding to amino acids 265–271 of rat AQP2 (i.e., LPRGSKA) was chosen as the immunogen. Synthetic oligopeptides that were phosphorylated or not phosphorylated at Ser269 (named TM56 and TM57, respectively), and to which cysteine residues were added at their N-termini, were obtained from Operon Biotechnology (Tokyo, Japan). The rabbit polyclonal antibody to Ser269-phosphorylated AQP2 was developed using the TM56 oligopeptide that was conjugated to keyhole limpet hemocyanin using an Imject maleimide-activated mcKLH kit (77611, Thermo Scientific, Rockford, IL). To obtain the specific antibody, affinity purification was performed as follows. Serum was applied to a TM57-coupled agarose gel column (Sulfolink coupling resin; 20401, Thermo Scientific) in order to completely adsorb the antibody to the non-phosphorylated peptide, and the serum that passed through was collected. The adsorbed serum was then applied to the TM56-coupled agarose gel column, and the specific antibody was eluted and dialyzed using phosphate-buffered saline (PBS). This antibody was denoted pS269Ab.

Other antibodies

An antibody against phospho-Ser269 AQP2 was purchased from PhosphoSolutions (Catalog #p112-269, Lot #cs914b; Aurora, CO). AQP2 was detected using an affinity-purified rabbit anti-rat AQP2 antibody (designated Ra AQP2Ab in

this study; Tajika *et al.*, 2004) or a goat anti-human AQP2 antibody (designated Goat AQP2Ab in this study; sc-9882, Santa Cruz Biotechnology, Santa Cruz, CA), which also reacts with rat AQP2. A mouse anti-calbindin D28K monoclonal antibody (ab82812, Abcam, Cambridge, MA) was purchased. The secondary antibodies used were Rhodamine Red-X-conjugated donkey anti-rabbit IgG (711-295-152, Jackson Immunoresearch, West Grove, PA), DyLight 488-conjugated donkey anti-goat IgG (705-485-147, Jackson Immunoresearch), Alexa Fluor 488-conjugated donkey anti-goat IgG (A11055, Life Technologies, Grand Island, NY), Alexa Fluor 647-conjugated donkey anti-mouse IgG (A31571, Life Technologies), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (P0448, DAKO, Glostrup, Denmark), and HRP-conjugated mouse anti-goat IgG (31400, Thermo Scientific).

Vasopressin and the vasopressin type 2 receptor-specific antagonist

Arginine vasopressin (AVP; V9879, Sigma-Aldrich, St. Louis, MO) was diluted in water to a concentration of 1 mg/mL and used as a stock solution. The vasopressin type 2 receptor-specific antagonist, OPC-31260, was kindly provided by Otsuka Pharmaceutical (Tokushima, Japan) and diluted in physiological saline for each experiment. Physiological saline was purchased from Otsuka Pharmaceutical.

Animal treatments

All animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, approved by the Animal Care and Experimentation Committee of Gunma University (admission nos. 10-029, 12-020, and 14-013), and performed at the Bioresource Center of Gunma University Graduate School of Medicine. Male Sprague Dawley rats (200 g) were obtained from Japan SLC (Shizuoka, Japan) and maintained using normal rat chow and water. To reduce the endogenous vasopressin level, rats were water-loaded by providing free access to 5% dextrose prepared in water overnight, and then to 5% dextrose and 1% ethanol prepared in water overnight (Gimenez and Forbush, 2003). AVP was administered by intraperitoneal injection (1 μg in 1 mL of physiological saline) or via the femoral vein (100 ng in 0.2 mL of physiological saline). OPC-31260 was administered via the femoral vein (1 mg in 0.2 mL of physiological saline). All administrations via the femoral vein were performed under anesthesia with ketamine/xylazine. The kidneys were excised under anesthesia with ketamine/xylazine or after decapitation. Detailed protocols for the animal treatments performed during each experiment are described in the figure legends.

Immunoblotting

Two rats were water-loaded before starting the experiment. The kidneys were excised from one water-loaded rat. AVP was intraperitoneally administered to the other water-loaded rat, and the kidneys were excised after 30 minutes. Four kidney inner medulla homogenate samples were prepared for analysis: 1) water-loaded rat kidney, 2) AVP-administered rat kidney, 3) AVP-administered rat kidney treated with phosphatase, and 4) AVP-administered rat kidney treated with the reaction buffer used for phosphatase treatment. In detail, the water-loaded rat kidney and one of the AVP-administered rat kidneys were homogenized in PBS containing a protease inhibitor (Complete Mini; 11836153001, Roche Applied Science, Mannheim, Germany) and a phosphatase inhibitor (PhosSTOP; 04906837001, Roche Applied Science) on ice using a glass homogenizer. The other AVP-administered rat kidney was homogenized in the same buffer without the phosphatase inhibitor. These homogenates were centrifuged at 800 *g* for 5 minutes to remove the nuclei and tissue debris. The protein concentration was measured using a Pierce BCA Protein Assay Kit (23225, Thermo Scientific). An aliquot of tissue homogenate without the phosphatase inhibitor was treated with 15 units/μL lambda protein phosphatase (P0753, New England Biolabs, Beverly, MA), and another aliquot was treated with the reaction buffer alone for 40 minutes at 30°C. These four samples were denatured in an equal volume of buffer containing 50 mM Tris-HCl (pH 7.5), 4% SDS, 300 mM dithiothreitol, 50% glycerol, and 0.01% bromophenol blue at 37°C for 30 minutes. SDS-PAGE was performed using homemade 12.5% polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane (FluoroTrans W, Pall, Port Washington, NY). MagicMark XP Western Protein Standard (LC5602, Life Technologies) was used as the

molecular weight standard. After blocking with StartingBlock T20 (TBS) blocking buffer (37543, Thermo Scientific) for 15 minutes, the membrane was incubated with the primary antibody, pS269Ab, overnight at 4°C. After washing with Tris-based buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100), the membrane was incubated with HRP-conjugated goat anti-rabbit IgG at room temperature for 90 minutes. Visualization was performed using ECL Prime western blotting detection reagent (RPN2232, GE Healthcare, Buckinghamshire, UK), and images were captured by a CCD camera (ImageQuant LAS4000, GE Healthcare). To verify the presence of AQP2, the membrane was stripped of the antibodies by incubating it twice for 30 minutes each with stripping buffer (25 mM glycine and 1% SDS, pH 2.0) at room temperature. After blocking with StartingBlock T20 (TBS) blocking buffer (37543, Thermo Scientific) for 15 minutes at room temperature, the membrane was sequentially incubated with Ra AQP2Ab and a HRP-conjugated secondary antibody and visualized using the same method. Thereafter, the membrane was stripped of the antibodies again and labeled with Goat AQP2Ab and HRP-conjugated mouse anti-goat IgG using the same method.

Immunofluorescence microscopy and laser confocal microscopy

Indirect immunofluorescence microscopy was performed using our standard protocol (Matsuzaki *et al.*, 2009). Kidneys were sliced and immersed in fixative (4% paraformaldehyde prepared in 0.1 M phosphate buffer [pH 7.4]) overnight at 4°C. After washing with PBS, the fixed kidney slices were dehydrated using a graded ethanol series, cleared in xylene, infiltrated, and embedded in paraffin. Paraffin sections of the kidney specimens were cut and mounted on coated glass slides (Platinum; PLC-01, Matsunami, Osaka, Japan). After deparaffinization and rehydration, these sections were placed in 20 mM Tris-HCl (pH 9.0) and heated in a microwave (MI-77, Azumaya, Tokyo, Japan) to 97°C for 30 minutes for antigen retrieval. After blocking with PBS containing 5% normal donkey serum, the sections were sequentially incubated with primary and secondary antibodies. Double labeling for AQP2 and Ser269-phosphorylated AQP2 was performed using Goat AQP2Ab and pS269Ab. To verify the specificity of pS269Ab, the following controls were performed: (1) pS269Ab was applied to the tissue specimens after preincubation with either the TM56 or TM57 peptide (10 μg/ml), and (2) kidney sections subjected to antigen retrieval were incubated with 8 units/μL lambda protein phosphatase (P0753, New England Biolabs) or the reaction buffer alone for 2 hours at 30°C and then incubated with pS269Ab. Finally, the specimens were mounted with Vectashield mounting media (H-1000, Vector Laboratories, Burlingame, CA) and observed under a BX-62 microscope equipped with Nomarski differential interference-contrast and epifluorescence optics (Olympus, Tokyo, Japan). All images were captured using a CoolSNAP K4 CCD camera (Photometrics, Tucson, AZ) and MetaMorph software version 6.1 (Molecular Devices, Silicon Valley, CA), and then processed using Adobe Photoshop software (Adobe Systems, San Jose, CA). Confocal images were captured using an FV1000 laser confocal system (Olympus) and processed using Adobe Photoshop software.

Results

Specificity of pS269Ab

We generated pS269Ab and confirmed its specificity using both immunoblotting and immunohistochemistry. Immunoblotting with pS269Ab was performed using kidneys obtained from AVP-administered rats (Figure 1A, AVP), in which Ser269 of AQP2 should be highly phosphorylated (Hoffert *et al.*, 2008; Hoffert *et al.*, 2006), and using kidneys from water-loaded rats (Figure 1A, water-loaded). Specific bands for the unglycosylated form (approximately 25 kDa) and glycosylated form (approximately 32–38 kDa) of AQP2 were only detected in the AVP-administered kidneys. When the tissue homogenate was treated with lambda protein phosphatase (AVP PP (+)), both the unglycosylated and glycosylated forms almost disappeared. When the tissue homogenate was treated with reaction buffer without lambda protein phosphatase (AVP PP (−)), the intensities of these bands were also reduced, which is possibly caused by endogenous phosphatase activity. After stripping the membrane of pS269Ab, it was incubated with Ra AQP2Ab (Figure 1B), and again stripped and incubated with Goat AQP2Ab (Figure 1C) to confirm the presence of AQP2 in all homogenates. Both the unglycosylated

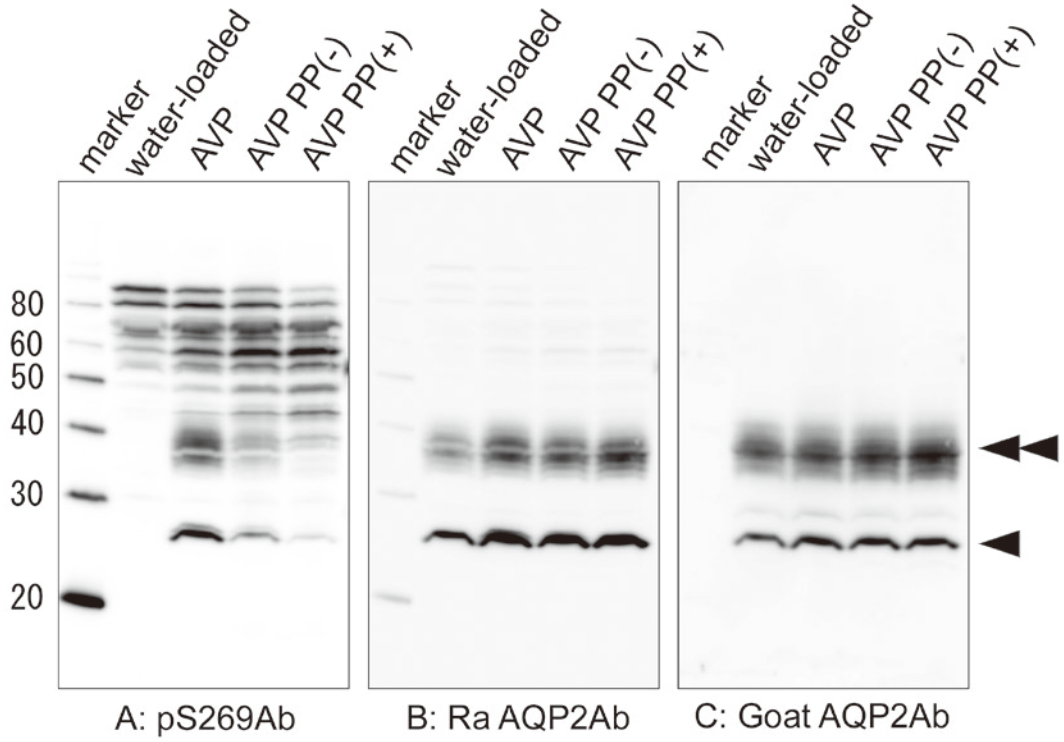

Fig. 1. Specificity of pS269Ab in immunoblotting. Tissue samples were prepared as described in the Materials and Methods. The denatured kidney lysate (20 μg) was subjected to SDS-PAGE and immunoblotting with pS269Ab (A). After stripping the membrane of the antibodies, AQP2 was detected using Ra AQP2Ab (B) and again using Goat AQP2Ab (C). The arrowhead and double-arrowhead indicate the unglycosylated (approximately 25 kDa) and glycosylated (approximately 32–38 kDa) forms of AQP2, respectively.

and glycosylated forms were similarly detected in all kidney homogenates.

Immunofluorescence microscopy with pS269Ab was performed on kidney sections obtained from a water-treated rat and an AVP-administered rat (Figure 2). Almost no labeling for Ser269-phosphorylated AQP2 was seen in the water-loaded rat kidney with pS269Ab (Figure 2B); however, intense labeling was seen along the collecting ducts of the AVP-administered rat (Figure 2D). When the section obtained from the AVP-administered rat was treated with lambda protein phosphatase, the labeling completely disappeared (Figure 2F), whereas intense labeling was still observed in the reaction buffer-treated section (data not shown). We further verified the specificity of pS269Ab by preadsorption with peptides. pS269Ab exhibited intense labeling along the collecting duct system in the kidney section obtained from the AVP-treated rat (Figure 2G). This labeling was abolished by addition of the Ser269-phosphorylated peptide (TM56) (Figure 2H), but not by addition of the non-phosphorylated peptide (TM57) (Figure 2I).

To compare our results obtained using pS269Ab with the findings of previous studies (Hoffert *et al.*, 2008; Moeller *et al.*, 2009), we tested the quality of a commercial anti-phospho-Ser269 AQP2 antibody (PhosphoSolutions; Catalog #p112-269, Lot #cs914b) used in these reports. This commercial antibody intracellularly labeled collecting duct cells of the water-loaded rat kidney in the presence or absence of lambda protein phosphatase treatment (arrows in Figure 3B and A, respectively). The antibody intensely labeled collecting duct cells of the AVP-injected rat (Figure 3C), and this labeling was still observed when the specimen was treated with lambda protein phosphatase and then incubated with the antibody (arrows in Figure 3D). These results suggest that the commercial antibody we obtained seems to

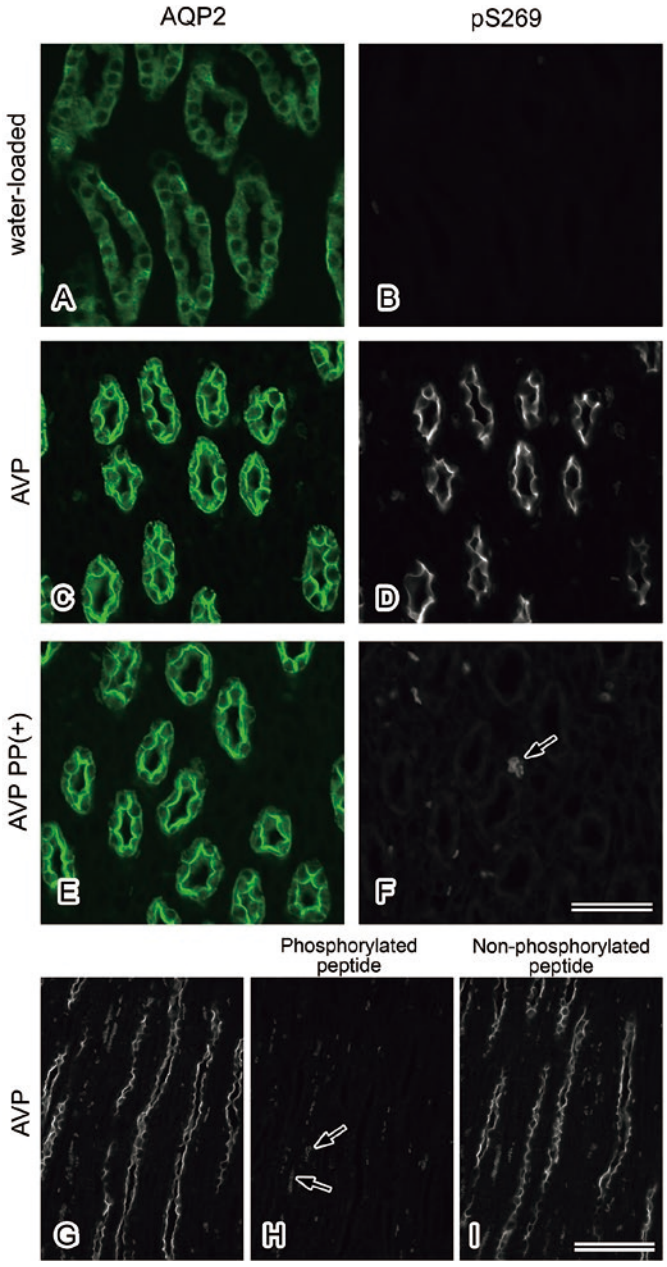

Fig. 2. Specificity of pS269Ab in immunohistochemistry. (A–F) Paraffin sections were processed for double immunofluorescence labeling with Goat AQP2Ab (green) and pS269Ab (white). (A, B) A kidney section from a water-loaded rat. (C, D) A kidney section from an AVP-administered rat. (E, F) A kidney section from an AVP-administered rat was treated with lambda protein phosphatase before immunolabeling to investigate the specificity of pS269Ab. The specimens were observed under a conventional fluorescence microscope. Images of inner medullae were taken under the same conditions for each primary antibody. An arrow indicates autofluorescence of erythrocytes. Bar, 100 μm. (G–I) Peptide preadsorption was performed on the kidney sections from an AVP-administered rat to investigate the specificity of pS269Ab. (G) A section was incubated with pS269Ab. (H) A section was incubated with pS269Ab in the presence of the Ser269-phosphorylated antigen peptide (TM56, 10 μg/ml). (I) A section was incubated with pS269Ab in the presence of the non-phosphorylated peptide (TM57, 10 μg/ml). Specimens were observed under a conventional fluorescence microscope. Each low-magnification photograph of the inner medullae was taken under the same conditions. Arrows indicate autofluorescence of erythrocytes. Bar, 50 μm.

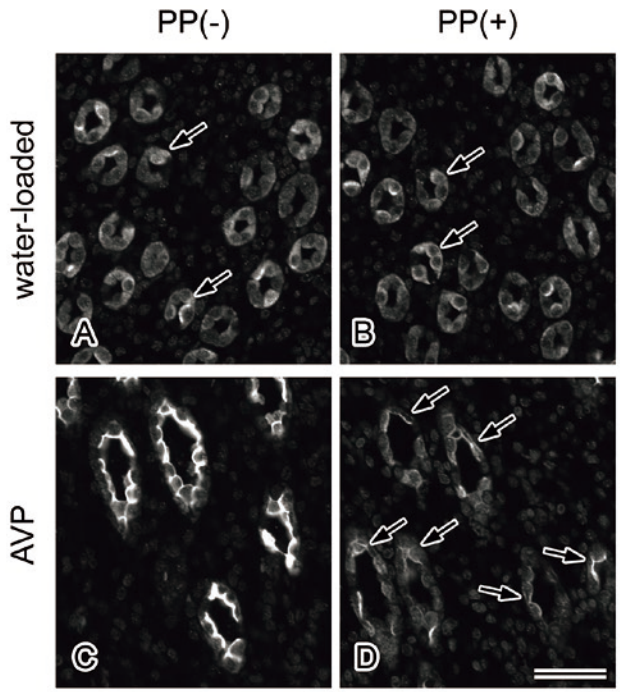

Fig. 3. Specificity of the commercial anti-phospho-Ser269 AQP2 antibody. Kidneys were excised from a water-loaded rat (A, B) and an AVP-administered rat (C, D). The same kidney specimens were used as in Figure 2A–F. Paraffin sections were processed for immunofluorescence labeling with a commercial anti-phospho-Ser269 AQP2 antibody (PhosphoSolutions, Catalog #p112-269, Lot #cs914b) diluted 1:1000, which is 10-fold more diluted than recommended by the manufacturer, under identical conditions. The section was treated with lambda protein phosphatase (PP (+); B, D) before immunolabeling according to the protocol used in Figure 2E and F. PP (−) indicates that the specimens were treated with the buffer without lambda protein phosphatase (A, C). The specimens were observed under a conventional fluorescence microscope. Each image of inner medullae were taken under the same conditions. Bar, 50 μm.

cross-react with non-phosphorylated AQP2. Therefore, we only used our homemade antibody pS269Ab in the following studies.

AQP2 Ser269 is highly phosphorylated upon AVP treatment and is subsequently dephosphorylated upon OPC-31260 injection

We examined changes in AQP2 Ser269 phosphorylation following treatment with vasopressin and the vasopressin type 2 receptor-specific antagonist OPC-31260 by performing immunofluorescence microscopy with pS269Ab. It is difficult to analyze AQP2 trafficking in detail, particularly the *in vivo* endocytotic processes that occur in the kidney, because it is impossible to wash out vasopressin, although it is possible to study cultured cells. To overcome this, we used OPC-31260 to abolish the effects of vasopressin. Water-loaded rats received an intraperitoneal AVP injection and were then administered OPC-31260. Kidneys were excised at several time points and analyzed. First, we obtained the kidneys from four animals: 1) the water-loaded control, 2) a rat treated with AVP for 30 minutes, 3) a rat treated with AVP for 30 minutes and subsequently with OPC-31260 for 30 minutes, and 4) a rat treated with AVP for 30 minutes and subsequently with OPC-31260 for 60 minutes. We performed double labeling for AQP2 with Goat AQP2Ab and for Ser269-phosphorylated AQP2 with pS269Ab in the kidney sections. Representative images of initial portions of inner medullae are shown in Figure 4. When we examined the distribution of AQP2 at a lower magnification, fluorescent labeling was present throughout the collecting duct system, including the connecting tubules, with similar intensities regardless of the different treatments. At a higher magnification, labeling for AQP2 was present intracellularly in the water-loaded rat kidney (Figure 4A), accumulated largely on the apical membrane of all regions and also on the basolateral membrane of connecting tubule and inner medullary collecting duct cells at 30 minutes after AVP injection (Figure 4B), and was again seen intracellularly at 30 minutes (Figure 4C) and 60 minutes (Figure 4D) after OPC-31260 injection. These changes verified the effects of water loading, AVP, and OPC-31260. In these tissue sections, we examined changes in Ser269 phosphorylation and the intracellular distribution of this protein using pS269Ab. Although fluorescent labeling for Ser269-phosphorylated AQP2 was almost undetected in the water-loaded kidney (Figure 4E), it was seen throughout the collecting duct system, including the connecting tubules, at 30 minutes after AVP injection (Figure 4F). At a higher magnification, Ser269-phosphorylated AQP2 highly accumulated on the apical membrane of AQP2-expressing cells at 30 minutes after AVP injection (Figure 4F). Then, we examined changes in Ser269 phosphorylation after OPC-31260 injection following AVP treatment and found that labeling for Ser269-phosphorylated AQP2 almost disappeared

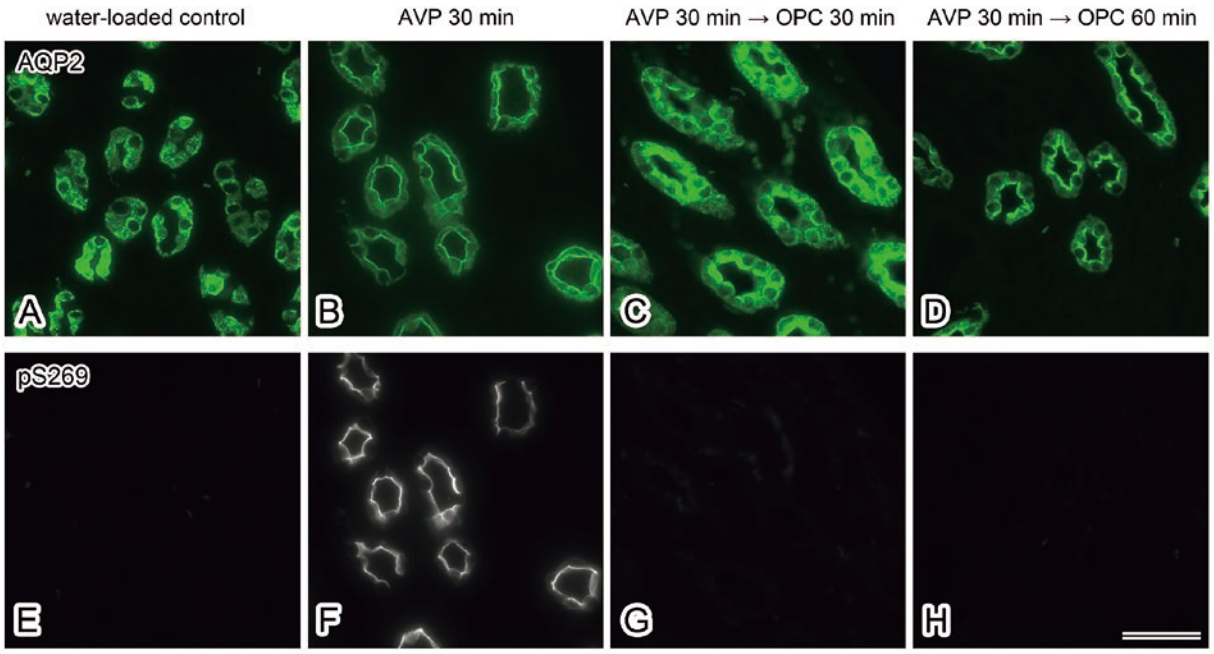

Fig. 4. Changes in AQP2 Ser269 phosphorylation following treatment with vasopressin and OPC-31260. Four rats were analyzed at the same time. All rats were water-loaded before starting the experiment. (A, E) The first rat: kidneys were excised as a water-loaded control. (B, F) The second rat: AVP was intraperitoneally administered to the water-loaded rat, and the kidneys were excised after 30 minutes. (C, G) The third rat: AVP was intraperitoneally administered to the water-loaded rat, OPC-31260 was intravenously administered after 30 minutes, and the kidneys were excised at 30 minutes after OPC-31260 injection. (D, H) The fourth rat: AVP was intraperitoneally administered to the water-loaded rat, OPC-31260 was intravenously administered after 30 minutes, and the kidneys were excised at 60 minutes after OPC-31260 injection. Paraffin sections were subjected to double labeling with Goat AQP2Ab and pS269Ab. The specimens were observed under a conventional fluorescence microscope. Images of the initial portion of the inner medulla from each animal were taken under the same conditions for each primary antibody. Images of AQP2 (green) and Ser269-phosphorylated AQP2 (white) from the same areas are shown in the upper and lower panels, respectively. Bar, 50 μm.

in the kidney at 30 minutes (Figure 4G) and 60 minutes (Figure 4H). However, in some animals and some collecting duct cells, labeling of Ser269-phosphorylated AQP2 was still seen at 30 minutes after OPC-31260 injection following AVP treatment.

Ser269-phosphorylated AQP2 is seen intracellularly soon after AVP treatment and soon after OPC-31260 injection following AVP treatment

We performed further experiments to determine if Ser269-phosphorylated AQP2 is restricted to the membrane, as previously described (Moeller *et al.*, 2009). We obtained the kidneys soon after AVP treatment. Figure 5A and B show the kidneys of a single animal obtained at 5 and 15 minutes after AVP injection, respectively. When we observed the kidney obtained at 5 minutes after AVP injection at a lower magnification, bright labeling for Ser269-phosphorylated AQP2 was seen in the outer and inner medullary collecting ducts. In addition, there was weak labeling in the connecting tubules and cortical collecting ducts. At a higher magnification, labeling for Ser269-phosphorylated AQP2 was largely seen intracellularly, especially in the inner medullary collecting duct principal cells (Figure 5A). In the outer medullary and cortical collecting ducts, labeling for Ser269-phosphorylated AQP2 was largely observed on the apical membrane, although a little was intracellular. Much more labeling for Ser269-phosphorylated AQP2 was observed on the apical membrane throughout the collecting duct system in the kidneys obtained at 15 minutes after AVP injection than in

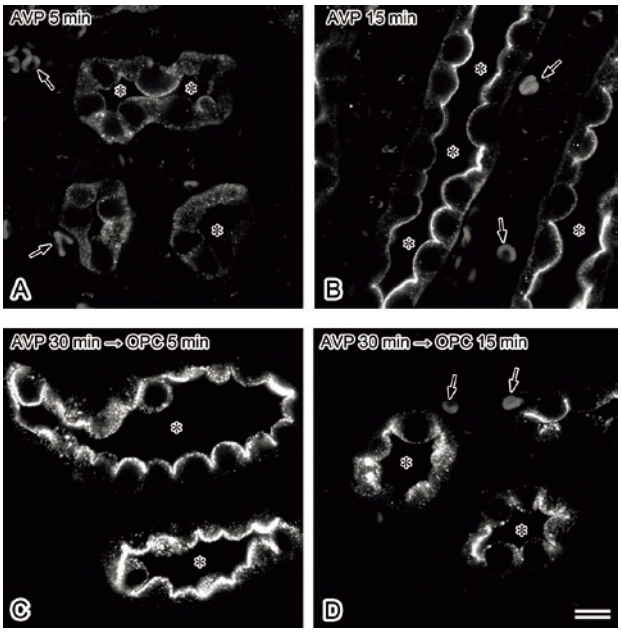

Fig. 5. Ser269-phosphorylated AQP2 is intracellularly detected soon after vasopressin treatment and after OPC-31260 injection following AVP treatment. (A, B) AVP was intraperitoneally administered to a water-loaded rat. The left and right kidneys were removed from the same animal after 5 minutes (A) and 15 minutes (B), respectively, under anesthesia. (C, D) Two water-loaded rats were intraperitoneally administered AVP, and the left kidneys from each animal were excised 30 minutes later. Thereafter, both rats were intravenously administered OPC-31260 and the right kidneys were excised under anesthesia after 5 minutes (C) or 15 minutes (D). Paraffin sections were labeled with pS269Ab. Specimens were observed under a laser confocal microscope. Representative images of Ser269-phosphorylated AQP2 in the initial portion of the inner medullary collecting ducts are shown. Asterisks indicate the lumen of collecting ducts. Arrows indicate autofluorescence of erythrocytes. Bar, 10 μm.

those obtained at 5 minutes after AVP injection (Figure 5B). To confirm that labeling for Ser269-phosphorylated AQP2 was observed intracellularly soon after AVP treatment, two more animals were examined. Labeling for Ser269-phosphorylated AQP2 was definitely observed intracellularly immediately after AVP treatment.

We further obtained the kidneys soon after OPC-31260 injection following AVP treatment. Figure 5C and D show specimens from different animals. Water-loaded rats received an intraperitoneal AVP injection, and the left kidneys were excised 30 minutes later to verify the effects of AVP. Rats then received an intravenous OPC-31260 injection, and the right kidneys were excised after 5 or 15 minutes. Ser269-phosphorylated AQP2 largely accumulated on the apical membrane after AVP treatment, similar to the findings presented in Figure 4F, which confirms the effect of AVP. In the kidneys obtained at 5 minutes after OPC-31260 injection, the labeling for Ser269-phosphorylated AQP2 was weaker, especially in cortical collecting ducts and outer medullary collecting ducts, in which some labeling was intracellular. In inner medullary collecting ducts, a large amount of labeling was seen in the subapical cytoplasmic region (Figure 5C). At 15 minutes after OPC-31260 injection, the labeling for Ser269-phosphorylated AQP2 was weaker than that observed at 5 minutes. However, some labeling was still cytoplasmic in inner medullary collecting duct cells (Figure 5D). To confirm that labeling for Ser269-phosphorylated AQP2 was seen intracellularly soon after OPC-31260 injection following AVP treatment, three more animals were examined. Labeling for Ser269-phosphorylated AQP2 was definitely seen intracellularly after OPC-31260 injection following AVP treatment.

The lack of labeling in sections treated with lambda protein phosphatase (data not shown) confirmed that the intracellular labeling observed soon after AVP treatment and soon after OPC-31260 injection following AVP treatment corresponded specifically to Ser269-phosphorylated AQP2.

Ser269-phosphorylated AQP2 is seen on the basolateral membrane as well as the apical membrane of connecting tubules and inner medullary collecting ducts

Basolateral trafficking of AQP2 occurs in the connecting tubule and inner medullary collecting duct cells (Christensen *et al.*, 2003), which was confirmed in the AVP-treated kidney by staining with Goat AQP2Ab in the current study (arrows in Figure 6A, D). When we observed the samples carefully, there was relatively strong labeling for Ser269-phosphorylated AQP2 on the basolateral membrane of the connecting tubule cells (arrows in Figure 6B), which were positive for calbindin D28K (Figure 6C), a connecting tubule marker (Christensen *et al.*, 2003). Weak labeling was also seen on the basolateral membrane of the inner medullary collecting duct cells (arrows in Figure 6E).

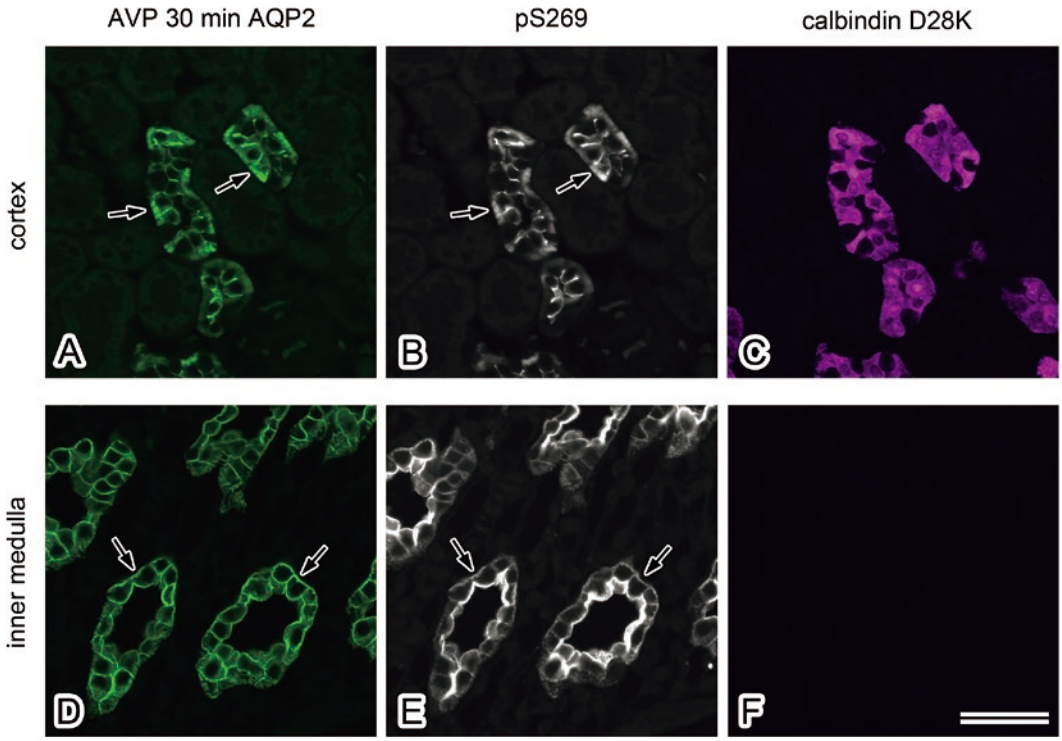

Fig. 6. Ser269-phosphorylated AQP2 is detected at the basolateral membrane, as well as the apical membrane. A kidney specimen obtained from an AVP-administered rat was triple-labeled with Goat AQP2Ab, pS269Ab, and a mouse anti-calbindin D28K antibody. Images of AQP2 (green), Ser269-phosphorylated AQP2 (white), and calbindin D28K (purple) in the same areas are shown in the left, middle, and right panels, respectively. (A–C) Cortex. Calbindin D28K-positive tubules are connecting tubules and distal convoluted tubules. (D–F) Middle portion of the inner medulla. Specimens were observed under a conventional fluorescence microscope. The apical labeling in (E) is overexposed to better show the basolateral staining (arrows). Bar, 50 μm.

Discussion

AQP2 Ser269 phosphorylation occurs intracellularly

Moeller et al. (2009) previously reported that Ser269-phosphorylated AQP2 is restricted to the apical membrane in vasopressin-treated rat kidneys based on the labeling of their antibody. In the current study, we developed pS269Ab and made several new findings. One of our new findings is that Ser269-phosphorylated AQP2 is intracellularly detected soon after vasopressin stimulation. At 30 minutes after vasopressin injection, Ser269-phosphorylated AQP2 was mostly restricted to the membrane, which is similar to the results of a previous study (Moeller *et al.*, 2009). Moeller et al. (2014) subsequently suggested that an apical membrane-associated kinase phosphorylates Ser269 of AQP2. Our current finding, however, suggests that AQP2 Ser269 is phosphorylated in the intracellular compartment upon vasopressin stimulation, and that AQP2 then very rapidly traffics to the plasma membrane. Phosphorylation of Ser256 is thought to be the key event for exocytotic insertion into the plasma membrane (Fushimi *et al.*, 1997; Lu *et al.*, 2004; Nejsum *et al.*, 2005; Noda *et al.*, 2008). Noda et al. (2008) showed that AQP2 phosphorylation at Ser256 reduces the binding of G-actin to AQP2 but induces the interaction between AQP2 and tropomyosin 5b, resulting in F-actin destabilization, which induces trafficking of AQP2 to the plasma membrane. Therefore, phosphorylation of Ser256 must be one of the requirements for AQP2 trafficking. On the other hand, a pool of Ser256-phosphorylated AQP2 can be observed intracellularly even under unstimulated conditions (Christensen *et al.*, 2000). Taking our data and previous findings together,

we speculate that Ser256 phosphorylation switches AQP2 into a "ready to go" state and that Ser269 phosphorylation may even promote the exocytotic insertion of this protein; therefore, phosphorylation of Ser269 might be a critical step for AQP2 trafficking to the plasma membrane.

Dephosphorylation of Ser269 is not necessarily required for AQP2 endocytosis

Previous analyses of AQP2 endocytosis were usually performed in cultured cells that express this protein because it is easy to turn off stimulation via a washout (Aoki *et al.*, 2012; Lu *et al.*, 2008; Tajika *et al.*, 2004). In the current study, we performed *in vivo* studies using a vasopressin type 2 receptor-specific antagonist, OPC-31260, to evaluate a living animal kidney (Yamamura *et al.*, 1992). Some studies report that injection of OPC-31260 into living animals causes AQP2 internalization (Christensen *et al.*, 1998; Saito *et al.*, 1997) and decreases Ser256 phosphorylation (Christensen *et al.*, 2000). These studies indicate the utility of OPC-31260 in *in vivo* studies. In the current analyses, to investigate the dephosphorylation of Ser269 and its relationship with AQP2 endocytosis, OPC-31260 was injected after exogenous AVP administration. We are unsure of the levels of the vasopressin type 2 receptor on the plasma membrane at 30 minutes after AVP administration and if OPC-31260 affects this because the vasopressin type 2 receptor, which binds to vasopressin, is internalized and degraded upon desensitization (Bouley *et al.*, 2005; Robben *et al.*, 2004; Yi *et al.*, 2007). A previous physiological study, however, showed that OPC-31260 administration inhibits the antidiuretic action of exogenous AVP (Yamamura *et al.*, 1992). We revealed that OPC-31260 injection after exogenous AVP administration caused rapid AQP2 internalization and decreased Ser269 phosphorylation, suggesting that this agent turns off the vasopressin signaling cascade. Using these protocols, we further found that Ser269-phosphorylated AQP2 can be detected intracellularly following OPC-31260 injection. This is another new finding of the current study. There are various possible explanations for this result because it is not possible to follow the dynamics of individual AQP2 molecules. However, Ser269-phosphorylated AQP2 detected intracellularly following OPC-31260 injection is not the equivalent of AQP2 that was intracellularly retained because almost all the labeling for Ser269-phosphorylated AQP2 accumulated on the surface membrane at 30 minutes after vasopressin treatment. Therefore, the current results suggest that AQP2 can be internalized from the plasma membrane even if it remains phosphorylated at Ser269. In contrast with our results, previous studies reported that Ser269 phosphorylation may be important for AQP2 retention on the plasma membrane (Hoffert *et al.*, 2008; Moeller *et al.*, 2010). Furthermore, Moeller et al. (2010) reported that the internalization of the AQP2-S269D mutant (which mimics the Ser269-phosphorylated state) from the surface membrane is significantly slower than that of wild-type AQP2 in transfected cultured cells, which is caused by decreased interactions between AQP2-S269D and the proteins required for AQP2 endocytosis. At first glance, our current finding seems to contradict these earlier studies; however, it does not if we consider that AQP2 forms homotetramers. Kamsteeg et al. (1999, 2000) showed that AQP2 forms homotetramers and that the subcellular localization of an AQP2 tetramer depends on the stoichiometry of Ser256-phosphorylated and –non-phosphorylated monomers. Specifically, three monomers, but not all monomers, per tetramer must be phosphorylated at Ser256 for its steady-state localization in the apical membrane. Similarly, our finding together with earlier studies (Hoffert *et al.*, 2008; Moeller *et al.*, 2010) raises the possibility that endocytosis of an AQP2 tetramer is regulated by the stoichiometry of Ser269-phosphorylated and –non-phosphorylated monomers. For example, Ser269 dephosphorylation in some, but not all, monomers per tetramer might be sufficient for the internalization of an AQP2 tetramer. This would explain why Ser269-phosphorylated AQP2 was detected intracellularly during endocytotic processes after OPC-31260 injection following AVP treatment. Taken together, we presume that Ser269 phosphorylation makes each AQP2 monomer remain at the plasma membrane but this phosphorylation does not preclude internalization.

Ser269-phosphorylated AQP2 is found on the basolateral membrane, as well as the apical membrane

AQP2 is localized on the basolateral membrane in addition to the apical membrane of the connecting tubule and inner medullary collecting duct cells (Christensen *et al.*, 2003), although its functional importance for transepithelial water

transfer remains unknown because the basolateral membrane of these cells bears other AQP isoforms such as AQP3 and/or AQP4 (Matsuzaki *et al.*, 2002; Takata *et al.*, 2005). We revealed that Ser269-phosphorylated AQP2 was also weakly detected on the basolateral membrane of these cells, in which its restricted accumulation on the apical membrane was reported by Moeller et al. (2009). Our current finding suggests that phosphorylation of Ser269 in the intracellular compartment, as described in our current work, does not affect the polarity of AQP2 trafficking.

AQP2 Ser269 phosphorylation is highly dependent on vasopressin stimulation

The data indicate that AQP2 Ser269 phosphorylation is highly dependent on vasopressin stimulation. In addition to the responsible kinase, which has not been identified, some phosphatase activity must be important because rapid dephosphorylation occurred following OPC-31260 treatment. This being the case, an important question is how is this phosphatase activated? We speculate that turning off the AVP signal might turn on the phosphatase activity. Further studies are required to elucidate this.

In clinical medicine, the amount of AQP2 excreted in urine is a useful indicator of vasopressin activity (Ishikawa, 2000; Verkman, 2012). Hence, AQP2 Ser269 phosphorylation in rodents, which occurs at threonine 269 in humans, could also be a useful biomarker of vasopressin action.

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