Quantitative analysis of aquaporin-2 phosphorylation

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RENAL WATER EXCRETION IS REGULATED in response to changes in the circulating level of the neurohypophysial hormone vasopressin as part of a feedback mechanism that maintains plasma osmolality within a tight range of 290–294 mosmol/kg H2O. To determine what percentage of AQP2 is present in the apical plasma membrane of collecting duct cells, the major observations that are the basis of the above conclusions are largely qualitative in nature. Application of quantitative approaches has the potential of refining and clarifying our understanding of the processes involved. Consequently, in this paper, we developed an immunoblotting-based approach to carry out relative quantification of phosphorylation at each of the known sites in the presence and absence of vasopressin. In addition, we performed immunogold electron microscopy (EM) of native inner medullary collecting duct cells from the same rat models and image quantification to determine what percentage of AQP2 is present in the apical plasma membrane in the absence and presence of vasopressin.

METHODS

Animal Models

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). 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) or 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.

Study 1. Four rats were treated with intravenous injection of 1 ng of dDAVP in 200 μl of saline/animal, and four saline-injected rats served as controls. After 60 min, the rats were anesthetized, and the kidneys were perfusion-fixed. Between injection of dDAVP and fixation of the kidney, animals had free access to water but not food. Tissue was processed for immunogold EM and immunoblotting (see pertinent subsection below).

Study 2. Three control rats were injected with 500 μl vehicle intramuscularly in the hind leg. Three experimental rats were injected with 2 nmol of dDAVP. After 60 min, animals were processed for tissue isolation (see pertinent subsection below).

Study 3. Rats had free access to 200 mM sucrose water for 16 h before experimentation. Three control rats were injected with saline...
solution intramuscularly in the hind leg, and three experimental rats were injected with 2 nmol of dDAVP. Following injection, the rats had free access to 200 mM sucrose water. After 60 min, animals were processed for tissue isolation (see pertinent subsection below).

Urine Osmolality Measurements

Urine samples from the control and experimental rats before injection and after injection were collected, and urine osmolality (mosmol/kgH2O) was measured using a vapor pressure osmometer (Wescor Vapro 5520, St. Michaels, MD).

Inner Medulla Isolation

Rats were euthanized, kidneys were removed, and the whole inner medulla (IM) was dissected. The whole IM was homogenized in ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine, pH 7.6, containing 1 mg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1× HALT phosphatase inhibitor cocktail, Pierce, Rockland, IL) using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) at maximum speed for three 15-s intervals. Total protein concentrations were measured (BCA kit; Pierce Chemical, Rockland, IL) using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) at maximum speed for three 15-s intervals. Total protein concentrations were measured (BCA kit; Pierce Chemical, Rockland, IL) using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) at maximum speed for three 15-s intervals.

Measurement of Percentage of AQP2 Phosphorylated at Specific Sites Using Phosphopeptide Standards

SDS-PAGE was performed on 12% polyacrylamide gels. In addition to the IM samples, each gel was loaded with phosphopeptide standards over a range of quantities (MW of peptide is 4,200 g/mol). The sequence of each synthetic peptide spanned amino acids 241–271 of the COOH-terminal tail of AQP2 and was phosphorylated at the appropriate residue according to the antibody being used (see below). Each standard was detectable by the appropriate phospho-specific antibody and by the total AQP2 antibody; thus the same standards were used to calibrate the blot with a given phospho-specific antibody and the blot with the total AQP2 antibody. The range of standards was optimized in each case such that the signal from the immunoblot for either total AQP2 or the specific phosphoform in the IM samples would fall within a standard curve generated using the phosphopeptides. For each phosphorylation site that needed to be quantified, two immunoblots were produced. These immunoblots were identical in format and produced on the same day. No stripping of immunoblots was performed. The first immunoblot was probed using a phospho-specific antibody (targeted against Ser256, Ser261, Ser264, or Ser269). The second immunoblot was probed for total AQP2 using an antibody recognizing total AQP2 independently of its phosphorylation status (LKEM Ab K007 targeted to amino acids 237–255 of rat AQP2) (5).

The method for determination of the percentage of AQP2 phosphorylated at a given site is illustrated in Fig. 1, showing quantification of pSer269-AQP2 in Sprague-Dawley rat IMs. Figure 1A shows samples from vehicle- and dDAVP-treated rats (60 min on the left). The combined band densities for the glycosylated and nonglycosylated bands are determined computationally as illustrated by the boxes surrounding the bands. The protein bands below the boxed-in areas are believed to represent a histone that is recognized by COOH-terminal AQP2 antibodies (8). The band densities of peptide standards,
containing the pSer269 phosphorylation, are determined in a similar manner (right). As shown in this representative example, pSer269-AQP2 was markedly increased by acute dDAVP treatment. Figure 1B shows the analogous immunoblot probed for total AQP2. Here, the peptide standards are the same as in Fig. 1A. Since the epitope for the total AQP2 antibody used for this blot is upstream from the phosphorylation sites (5), the presence of a phosphorylated serine at any site will not affect antibody binding. In preliminary studies (not shown), the peptide standard range was determined so that samples will lie within the bounds of the standard curve constructed. The percentage of AQP2 phosphorylated can be calculated by combining the information from the two blots, taking into account differences in the amounts of each sample loaded (see METHODS).

In the first study, we treated Sprague-Dawley rats with dDAVP or vehicle for 60 min and determined the percentage of AQP2 phosphorylated at Ser256, Ser264, and Ser269 (Table 1). (We had insufficient material for Ser261 immunoblotting in this sample set.) Ser269 phosphorylation underwent a large increase as seen previously (5), from under 3% to almost 26% in the presence of dDAVP. Ser264 phosphorylation trended toward an increase, but the percentage of phosphorylation at this site was far lower than that at Ser269 in the presence of dDAVP. Interestingly, despite the strong increase in pSer269, phosphorylation at Ser256 did not increase in response to dDAVP. Nevertheless, there was a high percentage of Ser256 phosphorylation in both the presence and absence of dDAVP.

These results offer an opportunity to test again the role of Ser269 vs. Ser256 phosphorylation in AQP2 trafficking, since Ser269 phosphorylation increased but Ser256 phosphorylation did not. To address this, we carried out immunogold labeling of the opposite kidney used for immunoblotting from each rat (same rats as Table 1) to quantify the percentage of AQP2 in the apical plasma membrane, using a semiautomated approach as described in METHODS. Figure 2 shows an example of an EM image and the counting procedure. Images of the principal cell were obtained at a magnification and resolution that allowed individual gold particles to be observed. If individual gold particles were not apparent, several higher magnification images were obtained and manually “sewn” together (Fig. 2A). The magnification and resolution of these images were sufficient to analyze a whole principal cell at once (Fig. 2B). High-magnification EM images exemplifying the immunogold counting technique using image enhancement are depicted in Fig. 2, C and D. Gold particles within three diameters of the apical surface (i.e., within 30 nm) were counted as in the apical plasma membrane, while gold particles anywhere else in the

Table 1. Percent phosphorylation of AQP2 at Ser256, Ser264, and Ser269: effect of acute dDAVP in inner medullas of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Phosphorylation Site</th>
<th>Vehicle (n = 4)</th>
<th>dDAVP (n = 4)</th>
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<tbody>
<tr>
<td>Ser256</td>
<td>49.1 ± 2.6</td>
<td>34.3 ± 1.0*</td>
</tr>
<tr>
<td>Ser264</td>
<td>1.9 ± 0.3</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>Ser269</td>
<td>2.6 ± 1.5</td>
<td>25.9 ± 3.8*</td>
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</tbody>
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Values are means ± SE. AQP2, aquaporin-2. *P < 0.05 compared with vehicle-treated group, 2-tailed test, unpaired t-test with Welch correction.
AQP2 underwent a significant decrease in phosphorylation. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased. As shown in our previous studies (6, 7), pSer261-AQP2, while Ser264 phosphorylation was moderately increased.
that employs synthetic phosphopeptide standards to determine the amounts of phosphorylated AQP2 relative to total AQP2, as illustrated in Fig. 1. The same standards can be used for both determinations because the peptide-directed total AQP2 antibody, which recognizes the AQP2 peptides upstream from the COOH-terminal phosphorylation sites, recognizes each of the four phosphopeptide standards at equal affinity, and phosphorylation does not affect the ability of the antibody to bind.

The original intent of the study was to quantify phosphorylation at the Ser269 site, which is the third from last amino acid (S*K*A) in AQP2. Our focus on this site was because of our recent finding that Ser269-phosphorylated AQP2 is localized exclusively in the apical plasma membrane, with no detectable pSer269-AQP2 anywhere else in collecting duct principal cells (5, 14). This observation and the associated mutational studies in Madin-Darby canine kidney cells (5) led us to hypothesize that phosphorylation at this site inhibits internalization of AQP2, and recent studies have confirmed that Ser269 phosphorylation partly modulates AQP2 endocytosis (13). Thus vasopressin-induced increases in Ser269 phosphorylation appear to be integrally involved in the well-known role of vasopressin to regulate the distribution of AQP2 between the plasma membrane and endosomal compartments. This idea is in line with previous views (1, 11, 19) that vasopressin regulates apical plasma membrane AQP2 abundance by controlling both AQP2 exocytosis and AQP2 endocytosis. Regulation of AQP2 exocytosis is believed to be dependent on Ser256 phosphorylation (9, 10). In contrast, both prostaglandin E2 and dopamine can induce AQP2 internalization independently of the phosphorylation state of Ser256-AQP2 (16).

In the current studies, the vasopressin analog dDAVP produced a consistent multifold increase in Ser269 phosphorylation in inner medullas from three independent sets of experiments in Sprague-Dawley rats as well as in cultured mpkCCD cells. In contrast, despite this large increase in Ser269 phosphorylation, no significant increase in pSer269-AQP2 was observed in study 1 (Table 1). We took advantage of this observation to address whether AQP2 redistribution can be observed in the renal inner medullary collecting duct without an increase in Ser269 phosphorylation. Immunogold labeling of fixed tissue from the opposite kidney showed that AQP2 was indeed redistributed to the apical plasma membrane in response to dDAVP. However, the increase in plasma membrane abundance was not as great as has been found in previous studies (17) or as great as the increase in water permeability normally seen in isolated, perfused collecting ducts (19). As previously emphasized (1, 18), the steady-state level of AQP2 in the apical plasma membrane depends on a balance of exocytosis and endocytosis, and the overall redistribution appears to involve regulation of both processes. Thus, if the usual regulation of exocytosis by vasopressin did not occur in the first set of experiments described above (as suggested by the lack of an increase in Ser256 phosphorylation), the overall steady-state redistribution of AQP2 would be attenuated as observed. In this study, acute vasopressin treatment may have resulted in a reduction in the intrinsic rate of AQP2 endocytosis related to the increase in Ser269 phosphorylation, but the increase in AQP2 redistribution may be attenuated owing to a minimal increase in Ser256 phosphorylation. These observations fit well with studies in Madin-Darby canine kidney cells that determined that mimicking phosphorylation of AQP2 at S269 (by a Ser-to-Asp mutation resulting in a fixed negative charge) resulted in a reduced rate of AQP2 internalization from the apical plasma membrane (13).

In all of the studies shown in this paper, there was a high level of AQP2 phosphorylation even in the control period without vasopressin addition, and the phosphorylation did not suppress with oral water loading even though urinary osmolality was markedly decreased. Thus we conclude that, at the time points examined in our studies, a high level of Ser256 phosphorylation does not necessarily require a high vasopressin level. Consistent with the results in this paper, using a Ser269 phospho-specific antibody it has previously been shown that 2 h after dDAVP administration to either Wistar rats or Brattleboro rats there is no increase in the abundance of pSer256-AQP2, whereas apical plasma membrane pSer256-AQP2 increased 10-fold (3). Furthermore, the same studies demonstrated that there is a high level of pSer256-AQP2 in intracellular vesicles in kidneys from untreated Brattleboro rats, indicating that Ser256 was phosphorylated, even in the absence of vasopressin. Previous studies, of course, have demonstrated that under some conditions Ser256 phosphorylation does increase with vasopressin administration (15, 20). The findings imply that whereas Ser256 phosphorylation of AQP2 depends somewhat on vasopressin, it also depends on other unknown factors, which may under some circumstances overshadow the effects of vasopressin. The findings may provide an explanation for observations by Lankford et al. (12) that baseline water permeability in isolated, perfused inner medullary collecting ducts can be very high independently of vasopressin’s short-term action. One possibility is that Ser256 can be phosphorylated by kinases other than PKA as suggested by Brown (2). Such kinases may be controlled by other than the canonical pathways implicated in vasopressin signaling.

Our previous studies indicated that phosphorylation of Ser256 is necessary for phosphorylation at Ser264 and Ser269.
Thus at first glance this conclusion may appear to be at odds with the present results establishing independence of the two phosphorylation events. However, a likely explanation is that an increase in pSer256 is not necessary for vasopressin to increase Ser269 phosphorylation if Ser256 phosphorylation is already high without vasopressin. Thus we propose that a high baseline level of Ser256 phosphorylation is sufficient to allow Ser269 phosphorylation, independently of whether the level is changed by vasopressin. In general, the current findings are consistent with our previous observations that vasopressin regulates Ser269 phosphorylation (5, 14).

Similar conclusions may derive from observations in another biological model exploited in this paper, viz. mpkCCD cells. The fact that increased phosphorylation of AQP2 at Ser256 in these cells is not necessary for vasopressin to increase Ser269 phosphorylation in AQP2 is consistent with previous findings in mpkCCD cells (22). In fact, that study, like the present one, showed that vasopressin-mediated apical redistribution of AQP2 can occur without a change in Ser256 phosphorylation. Overall, we suggest from these studies that Ser269 phosphorylation may be a more consistent indicator of vasopressin action than Ser256 phosphorylation.

One advantage of the methodology described is the ability to calculate absolute numbers of AQP2 molecules per inner medulla, or indeed per IMCD cell. A sample order of magnitude calculation (see Supplementary file; supplementary material for this article is available on the web site) estimated there to be \( \sim 5.4 - 8.4 \) nmol of AQP2/rat inner medulla, equating to \( \sim 4.4 - 6.7 \times 10^8 \) AQP2 molecules/IMCD cell. In principle, application of a similar technique would allow absolute quantification of any protein of interest in the inner medulla.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES