AQP3, p-AQP2, and AQP2 expression is reduced in polyuric rats with hypercalcemia: prevention by cAMP-PDE inhibitors

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1The Water and Salt Research Center, University of Aarhus, DK-8000 Aarhus C, Denmark; 2Department of Physiology, School of Medicine, Dongguk University, Kyungju 780–714, Korea; and 3Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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Wang, Weidong, Chunling Li, Tae-Hwan Kwon, Mark A. Knepper, Jørgen Frøkiær, and Søren Nielsen. AQP3, p-AQP2, and AQP2 expression is reduced in polyuric rats with hypercalcemia: prevention by cAMP-PDE inhibitors. Am J Physiol Renal Physiol 283: F1313–F1325, 2002. First published July 30, 2002; 10.1152/ajprenal.00040.2002.—The purpose of this study was to evaluate whether hypercalcemia is associated with downregulation of renal aquaporins (AQPs), including AQP1, AQP2, phosphorylated AQP2 (p-AQP2), AQP3, and AQP4, and if this is the case, to test whether cAMP-phosphodiesterase (PDE) inhibitor treatment can prevent AQP downregulation and prevent the development of polyuria. Vitamin D-induced hypercalcemia in rats was associated with increased urine output and reduced urine osmolality, consistent with previous findings (Levi M, Peterson L, and Berl T. Kidney Int 23: 489–497, 1983). Semiquantitative immunoblotting revealed a significant reduction in the abundance of inner medullary AQP2 (52 ± 6% of control levels), consistent with previous studies, and of AQP2, which is phosphorylated at the PKA phosphorylation consensus site serine 256 (p-AQP2; 36 ± 8%). Moreover, AQP3 abundance was also significantly decreased (45 ± 7 and 61 ± 6% of control levels in inner medulla and whole kidney, respectively). Consistent with this, immunohistochemistry demonstrated reduced AQP3 immunolabeling along the entire collecting duct. AQP4 expression was not reduced. Surprisingly, total kidney AQP1 abundance was also reduced (60 ± 6%). AQP1 expression was reduced in the cortex and outer stripe of the outer medulla (48 ± 7%; i.e., in proximal tubules). In contrast, AQP1 levels were not changed in the inner stripe of the outer medulla or in the inner medulla (i.e., descending thin limbs and vasa recta). Treatment with the cAMP-PDE inhibitors rolipram and milrinone in combination (inhibiting PDE IV and PDE III isoenzymes) at day 2 and onward completely prevented the hypercalcemia-induced downregulation of AQP2 and AQP3 (but not AQP1) and completely prevented the development of polyuria. In conclusion, AQP3, AQP2, and p-AQP2 are downregulated and are likely to play critical roles in the development of polyuria associated with vitamin D-induced hypercalcemia. Moreover, PDE inhibitor treatment significantly prevented the reduced expression of collecting duct AQPs and prevented the development of polyuria.

nephrogenic diabetes insipidus; urinary concentration mechanism; vasopressin

IT IS WELL KNOWN THAT HYPERCALCEMIA in both humans and experimental animals is associated with a urinary concentrating defect and significant polyuria (11, 20, 30). Despite considerable experimental work (2, 3, 10), the precise pathogenic mechanisms involved in the impaired urinary concentration remains only partly understood and it is likely to be multifactorial. Several lines of evidence have pointed to a significant defect in countercurrent multiplication in hypercalcemia, which is associated with decreased medullary hypertonicity (2, 28, 29) and to a defect in collecting duct water reabsorption. As delineated below, three previous studies have been aimed at defining the molecular explanation for this at the level of involved channels and transporters.

To address whether the defect in the countercurrent multiplication system is due to dysregulation of key sodium transporters in the thick ascending limbs (TALs), we previously examined the expression of the rat type 1 bumetanide-sensitive cotransporter Na-K-2Cl cotransporter (BSC-1), Na-K-ATPase, and type 3 Na/H exchanger (NHE3). It was found that apical plasma membrane expression levels and protein abundance of the Na-K-2Cl cotransporter in TAL is markedly decreased in vitamin D-induced hypercalcemia, suggesting a major role of BSC-1 in the urinary concentration defects associated with hypercalcemia. In contrast, no change was found in NHE3 and Na-K-ATPase expression. The abundance of ROMK, a key channel in modulating the efficiency of BSC-1-mediated sodium reabsorption, was also reduced, further...
substantiating a significant defect in TAL function in hypercalcemia (38).

There is reduced responsiveness of the collecting duct to vasopressin in hypercalcemia as demonstrated by several lines of evidence, such as changes of adenylate cyclase activities, reduced cAMP levels, and reduced protein kinase activity (1, 4, 12, 27) in collecting ducts from hypercalcemic animals. Consistent with this, Earm et al. (7) and Sands et al. (31) recently showed that aquaporin-2 (AQ2P2) protein abundance was significantly reduced in rats with vitamin D-induced hypercalcemia and that AQ2P2 levels in the apical plasma membrane were somewhat reduced. This suggested that dysregulation of collecting duct AQ2P2 might play some roles in the development of polyuria induced by hypercalcemia.

However, several important questions have remained unanswered. First, it is important to investigate whether other AQPs are downregulated, with special emphasis on collecting duct AQ3P. Water transport across the basolateral membrane of collecting duct cells is thought to be mediated by AQ3P and AQ4 (8, 36). It could be speculated that hypercalcemia may be associated with significant alterations in expression of AQ3P. This becomes important to investigate because AQ3P expression is known to be regulated by vasopressin (8) and recently it was shown that mice lacking AQ3P have very severe polyuria (21).

Second, it is important to investigate whether AQ4 expression is reduced because mice lacking AQ4 have a mild urinary concentrating defect and reduced osmotic water permeability of the inner medullary collecting duct (IMCD) in response to vasopressin (5).

Third, mice lacking AQ1P1 have a severe urinary concentrating defect and the osmotic water permeability of proximal tubules and descending thin limbs (DTLs) is dramatically reduced. The lack of AQ1P1 in DTLs is thought to play a key role by compromising the countercurrent multiplication process (22, 33). Thus it is important to determine whether there are changes in AQ1P1 expression, and if this is the case, to define the tubule segments in which this occurs. Fourth, it is important to develop strategies to prevent or treat the downregulation of key renal transporters causing functional defects. This could be on the basis of the known signaling cascades involved in channel and transporter regulation. Such modulations may later become important in developing novel treatments for such disorders. Because earlier studies have shown reduced cAMP production or decreased vasopressin-stimulated adenylate cyclase activity in rats with vitamin D-induced hypercalcemia (1, 23, 27), it is very likely that this directly causes the decrease in collecting duct AQ2P2 expression and, in part, causes the development of significant polyuria. Thus we now hypothesize that both vasopressin-regulated collecting duct AQPs (i.e., AQ2P and AQ3P) are downregulated [similar to what has been seen in lithium-induced nephrogenic diabetes insipidus (NDI)] (19). Moreover, we hypothesize that phosphodiesterase (PDE) III and PDE IV inhibitor treatment of hypercalcemic rats may increase the levels of intracellular cAMP in collecting duct cells and that this may prevent the downregulation of key collecting duct AQPs and moreover prevent the development of polyuria.

Thus the purposes of the present study were the following: 1) to investigate whether vitamin D-induced hypercalcemia is associated with a reduction in the expression of AQ3P; 2) to investigate whether vitamin D-induced hypercalcemia is associated with a reduction in the expression of AQ4P; 3) to investigate whether vitamin D-induced hypercalcemia is associated with a reduction in the expression of AQ1P1 and, if this is the case, to define the tubule segment in which this occurs; and 4) to examine whether downregulation of collecting duct AQPs can be prevented by treatment of hypercalcemic rats with key PDE inhibitors. Prevention of the downregulation of collecting duct water channels and prevention of the development of severe urinary concentrating defect would substantiate a role for collecting duct water channels in hypercalcemia-induced NDI. In addition, it would provide support for a potential strategy of treating such urinary concentrating defects.

METHODS

Experimental Animals and Protocol

Studies were performed in male Wistar rats initially weighing 230–260 g. The animals were maintained on standard rat chow (Altromin, Lage, Germany) and had free access to tap water throughout the experiment. During the entire experiment, rats were kept in individual metabolic cages, with a 12:12-h light-dark cycle, a temperature of 21 ± 2°C, and humidity of 55 ± 2%.

The following protocols were performed. Protocol 1. After a period of acclimation, the animals were randomized into two groups matched for body weight: hypercalcemic group (HC; n = 20) and control group (CON, n = 18). To produce hypercalcemia, rats were fed for 8 days with rat chow containing 8.5 mg dihydrotycachysterol (DHT; Sigma D-9257)/kg of dry food. During the experimental period, each rat in the HC group had a consistent intake of 18–19 g of rat chow/day, corresponding to 153–162 μg of DHT/day. In the CON group, rats were fed with rat chow without DHT for 8 days and they were offered the amount of food corresponding to the mean intake of food in the HC group of rats during the previous day; thus the food intake was matched between the two groups.

Protocol 2. This protocol involved rats treated with DHT (protocol 1) and CON animals without DHT treatment (n = 5). The DHT-treated animals were divided into two groups: HC rats not treated with PDE inhibitors (HC = 5) and HC rats treated with rolipram and milrinone (PDEi-HC; n = 5). For this purpose, osmotic minipumps (model 2001, Alzet) were filled, one with rolipram (0.72 mg·kg⁻¹·day⁻¹) dissolved in 50% DMSO and the other with milrinone (9 mg·kg⁻¹·day⁻¹) dissolved in 30% lactic acid. The osmotic minipumps were implanted subcutaneously and intraperitoneally, respectively, under anesthesia on the second day after DHT treatment. CON and HC rats that did not receive inhibitors were implanted with osmotic minipumps filled with vehicle.

Protocol 3. To determine whether the urinary concentrating defect is recovered in response to PDE inhibitor treat-
ment of rats with vitamin D-induced hypercalcemia, another three groups of rats were used: CON, DHT-treated, and DHT plus PDE inhibitor treated; n = 6 in each of the three groups. The treatments were identical to those described in protocol 2 except that rats were deprived of water for 24 h between days 7 and 8. At day 8, rats were killed and urine was collected after thirsting for determination of osmolality.

**Clearance Studies**

Daily urine output and water intake were determined throughout the study. Urine volume, osmolality, creatinine, sodium, and calcium concentration were measured. Plasma was collected from the abdominal aorta at the time of death and on days 7 and 8. The treatments were identical to those described in protocol 2 except that rats were deprived of water for 24 h between days 7 and 8. At day 8, rats were killed and urine was collected after thirsting for determination of osmolality.

**Primary Antibodies**

For semiquantitative immunoblotting, previously characterized polyclonal antibodies were used and are summarized as follows:

1. AQP2 (LL127, 1:3,000): an affinity-purified polyclonal antibody to AQP2 (24).
2. Phosphorylated AQP2 (p-AQP2; AN244-pp-AP, 1:200): an affinity-purified polyclonal antibody to p-AQP2 (6).
3. AQP3 (LL178, 1:300): an affinity-purified polyclonal antibody to AQP3 (8).
4. AQP4 (LL182, 1:1,000): an affinity-purified polyclonal antibody to AQP4 (36).
5. AQP1 (LL266, 1:3,000): an affinity-purified polyclonal antibody to AQP1 (26).

**Membrane Fractionation for Immunoblotting**

Whole kidneys or dissected inner medulla, inner stripe of outer medulla, outer stripe of outer medulla, and cortex were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) with an ultraturrax T8 homogenizer (IKA Labortechnik), and the homogenate was centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

**Electrophoresis and Immunoblotting**

Samples of membrane fractions together with molecular markers were run on 12% polyacrylamide minigels (Mini Protein II, Bio-Rad) for AQP2, p-AQP2, AQP3, AQP4, and AQP1. For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading. A 10% acrylamide gel was used for AQP1. For each gel, an identical gel was run in parallel and subjected to enhanced chemiluminescence system (Amersham). The labeling density was determined for blots where samples from HC or/and PDEi-HC rats were run on each gel with samples from respective CON rats. The labeling density was corrected by densitometry of Coomassie-stained gels.

**Preparation of Tissue for Immunocytochemistry**

Kidneys from CON, HC, and PDEi-HC rats were fixed by retrograde perfusion via the aorta with 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). For immunoperoxidase, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-embedded tissue was cut at 2 μm on a rotary microtome (Leica). The staining was carried out by using indirect immunoperoxidase. The sections were dewaxed and rehydrated, endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 10 min at room temperature. To reveal antigens, sections were treated with 1 mmol/l Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and were heated with a microwave oven for 10 min. Nonspecific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl for 30 min followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After rinsing with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 3 × 10 min, the sections were incubated in horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (P448, DAKO) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. The microscopy was carried out with a Leica DMRE light microscope.

**Statistical Analyses**

Values are presented as means ± SE. Comparisons between groups were made by unpaired t-test. P values <0.05 were considered significant.

**RESULTS**

**Rats with Hypercalcemia Were Associated with Altered Renal Water Handling**

Rats treated with vitamin D for 8 days developed significant hypercalcemia with an increase in total plasma calcium levels: 3.0 ± 0.06 vs. 2.4 ± 0.04 mmol/l in controls (P < 0.05, Table 1). As previously demonstrated (7, 31), hypercalcemia in rats was associated with marked alteration in water balance. In the basal period, urine output was not different between the two groups (Fig. 1A). Three days after DHT treatment, urine output from HC rats was significantly higher than that from CON rats (P < 0.05, Fig. 1A) and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HC (mean ± SE)</th>
<th>CON (mean ± SE)</th>
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</thead>
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<tr>
<td>Number of rats</td>
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<td>12</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH2O</td>
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<td>298 ± 1.6</td>
</tr>
<tr>
<td>Plasma calcium, mmol/l</td>
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<td>2.4 ± 0.04</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/l</td>
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<td>28 ± 0.8</td>
</tr>
<tr>
<td>Urine-to-plasma osmolality ratio</td>
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<td>Creatinine clearance, ml/min·kg⁻¹</td>
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<td>7.6 ± 0.5</td>
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Values are means ± SE. *P < 0.05, compared with control group.
increased progressively during the whole period of treatment. Consistent with this, 8 days after DHT treatment, urine output was significantly increased in HC rats: 98 ± 8 vs. 47 ± 5 μL·min⁻¹·kg⁻¹ in controls (P < 0.05, Fig. 1A). In parallel, the marked polyuria was associated with increased water intake: 140 ± 7 vs. 89 ± 4 μL·min⁻¹·kg⁻¹ in controls (P < 0.05). The marked increase in urine output was also associated with decreased urine osmolality (888 ± 66 vs. 1,749 ± 166 mosmol/kgH₂O in controls, P < 0.05, Fig. 1B) as well as urine-to-plasma osmolality ratio (3.0 ± 0.2 vs. 5.6 ± 0.7 in controls, P < 0.05; Table 1), indicating that hypercalcemia is associated with an impaired urinary concentration. In contrast, urine output was unchanged in CON rats (Fig. 1A).

Rats with Hypercalcemia Had Marked Reduction in p-AQP2 Abundance in Kidney

As shown in Fig. 2, semiquantitative immunoblotting of membrane fractions from whole kidneys of the HC rats revealed a significant decrease in AQP2 abundance to 57 ± 11% of control levels (100 ± 13%, P < 0.05; Fig. 2, A and B; Table 2) after 8 days on the DHT-containing diet. Moreover, densitometric analysis revealed a marked decrease in AQP2 abundance in inner medulla to 52 ± 6% of control levels (100 ± 9%, P < 0.05; Fig. 2, C and D; Table 2), consistent with previous studies (7, 31). Consistent with this, immunoperoxidase microscopy confirmed the reduction of AQP2 abundance in the kidney collecting duct principal cells of HC rats (Fig. 3). In CON rats, strong AQP2 labeling was associated with the apical domains of cortical collecting duct (not shown) and IMCD cells (arrows, Fig. 3A). In contrast, in kidneys of HC rats, there was a marked reduction of AQP2 labeling in the apical domains of cortical collecting duct (not shown) and IMCD principal cells (arrows, Fig. 3B).

Immunoblotting with antibodies that selectively recognize AQP2, which is phosphorylated in the PKA phosphorylation consensus site (serine 256) demonstrated that p-AQP2 was markedly decreased in HC rats (Fig. 2, E and F), further substantiating a defect in vasopressin regulation of collecting duct water reabsorption. As shown in Fig. 2F, densitometric analysis of immunoblots revealed a marked reduction in inner medullary p-AQP2 abundance to 36 ± 8%, compared with CON rats (100 ± 8%, P < 0.05; Table 2). Consistent with this, immunohistochemistry showed that p-AQP2 labeling was markedly reduced in IMCD principal cells in kidney of HC rats (arrows, Fig. 3E), whereas CON rats had abundant labeling of p-AQP2 in apical domains of cortical collecting duct (not shown) and medullary collecting duct principal cells (arrows, Fig. 3D).

Rats with Hypercalcemia Had Marked Reduction of Kidney AQP3 but Not AQP4 Abundance

AQP3 and AQP4 are basolateral water channels in collecting duct principal cells, and there is considerable evidence that they represent basolateral exit pathways for water absorbed apically via AQP2. Densitometric analysis of all the immunoblots of kidney from HC rats revealed a marked decrease of AQP3 abundance in inner medulla to 45 ± 7% of control levels (100 ± 8%, P < 0.05; Fig. 4, A and B; Table 2), thus paralleling the reduction in AQP2 abundance. Moreover, there was a parallel significantly decreased whole kidney AQP3 abundance in HC rats: 61 ± 6% of control levels (100 ± 16%, P < 0.05; Table 2).

Immunocytochemical analysis also showed reduced AQP3 labeling in collecting duct principal cells in kidney of HC rats, and this was seen along the entire length of the collecting duct. In CON rats, an intense AQP3 labeling was seen in basolateral domains of cortical collecting duct (arrowheads, Fig. 5A) and IMCD (arrowheads, Fig. 5C) principal cells, consistent with previous observation (8). In contrast, HC rats exhibited markedly reduced labeling of AQP3 in cortical collecting ducts (arrowheads, Fig. 5B), outer medullary collecting ducts (OMCDs; not shown), and IMCDs (arrowheads, Fig. 5D). This is consistent with the decreased abundance determined by semiquantitative immunoblotting. The results strongly suggest that reduced expression of both the apical AQP2 and the basolateral AQP3 in the collecting duct principal cells may importantly contribute to the polyuria and de-
creased urine concentration encountered in hypercalcemia-induced NDI.

AQP4 is another basolateral water channel that is mainly expressed in the IMCD principal cells (36). AQP4 abundance is not sufficient in whole kidney homogenates to assess its renal abundance by using immunoblotting; however, immunoblotting using membrane fractions of inner medulla revealed no difference in AQP4 abundance (Fig. 6, A and B; Table 2). This indicates that AQP4 is not likely to be involved in the urinary concentrating defect associated with hypercalcemia. This was confirmed by immunohistochemistry. In both CON and HC rats, an intense AQP4 labeling was seen in basolateral domains of IMCD principal cells (arrowheads, Fig. 7, A and B), and no detectable difference of AQP4 labeling was found between the two groups.

**Rats with Hypercalcemia Had Reduced Proximal Tubule but Maintained DTL AQP1 Protein Expression**

AQP1 is expressed in proximal tubules and DTL of the loop of Henle in kidney and plays critical roles in the constitutive water reabsorption in these segments. As shown in Fig. 8, A and B, whole kidney AQP1 abundance was significantly decreased in HC rats to 60 ± 6% of control levels (100 ± 9%, *P < 0.05; Table 2). To explore whether changes of whole kidney AQP1 abundance occur in the proximal tubules or descending limb of the loop of Henle, AQP1 levels in different kidney zones were examined by immunoblotting, and in addition, immunocytochemistry was performed. The results showed a significant decrease of AQP1 abundance in the cortex and outer stripe of the outer medulla.
Table 2. Densitometric analysis of immunoblots from hypercalcemic and control rats

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<tr>
<th>Parameter</th>
<th>n</th>
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<th>IM</th>
<th>ISOM</th>
<th>Cortex + OSOM</th>
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<tr>
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<td>100 ± 9%</td>
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<tr>
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Values are means ± SE. AQP1, AQP2, AQP3, and AQP4, aquaporin-1, -2, -3, and -4, respectively; HC, hypercalcemic rat group; CON, control rat group; IM, inner medulla; ISOM, inner stripe of the outer medulla; OSOM, outer stripe of the outer medulla; n, no. of rats. *P < 0.05, compared with control rats.

dulla (combined) from HC rats: 48 ± 7% of control levels (100 ± 13%, P < 0.05; Figs. 8, C and D), whereas there was no difference in AQP1 expression in the inner stripe of the outer medulla (80 ± 14% of control levels, not significant) and inner medulla (96 ± 18% of control levels, not significant) (Table 2). This strongly indicates that the decrease in expression is at the level of the proximal tubule and not the DTL. Immunocytochemical analysis of AQP1 expression also showed reduced AQP1 labeling in proximal tubule cells in kidney of HC rats (Fig. 9). In CON rats, the AQP1 labeling was mainly seen in apical domains of proximal tubule cells in cortex (arrows, Fig. 9A) and thin descending limb cells in inner medulla (arrows, Fig. 9C). In contrast, HC rats exhibited much weaker labeling of AQP1 in proximal tubule cells (arrows, Fig. 9B) but not in inner medullary thin descending limb cells (arrows, Fig. 9D), consistent with immunoblots (Table 2).
Rolipram and Milrinone Restored AQP2, p-AQP2, and AQP3 Abundance in Rats with Hypercalcemia

Selective inhibitors of PDE IV and III have been demonstrated to raise cAMP levels (13) in inner medulla collecting duct cells. cAMP levels are known to be critically involved in the AQP2 trafficking as well as in the regulation of AQP2 expression levels (25). We therefore tested whether rolipram and milrinone treatment affects the expression of collecting duct AQPs and

![Fig. 4. Semiquantitative immunoblotting of membrane fractions of rat kidney inner medulla. A: immunoblots were reacted with anti-AQP3 and revealed 27-kDa and 33- to 40-kDa AQP3 bands representing nonglycosylated and glycosylated forms of AQP3, respectively. B: densitometric analysis revealed a marked decrease in inner medullary AQP3 protein abundance in HC rats compared with CON rats. *P < 0.05.](image)

![Fig. 5. Immunoperoxidase microscopy of AQP3 using paraffin-embedded rat kidney tissues from CON rats (A and C) and HC rats (B and D). AQP3 labeling was associated with basolateral plasma membrane domains (arrowheads) in the cortical collecting duct (A) and IMCD (C) principal cells from CON rats. In a kidney from an HC rat, there was a marked decrease in overall AQP3 labeling (arrowheads) in the cortical collecting duct (B) and IMCD (D) principal cells. P, proximal tubule. Magnification: ×1,000.](image)

![Fig. 6. Semiquantitative immunoblotting of membrane fractions of kidney inner medulla. A: immunoblots were reacted with anti-AQP4 and revealed an ~32- to 34-kDa band as well as a higher molecular mass band representing oligomeric AQP4. B: densitometric analysis revealed that inner medullary AQP4 protein abundance was not changed in rats with hypercalcemia.](image)
the changes in urinary concentration in HC rats. Combined treatment with rolipram and milrinone in HC rats significantly reduced the degree of polyuria to control levels and restored the impaired urinary concentration, as manifested by increasing urine osmolality and urine-to-plasma osmolality ratio (Table 3). Consistent with this, semiquantitative immunoblotting of membrane fractions from the inner medulla of PDE-inhibitor treated rats revealed a marked prevention in the downregulation of AQP2 abundance (128 ± 21% in PDEi-HC vs. 100 ± 7% in control, not significant; Fig. 10, A and B; Table 4). Immunohistochemistry confirmed the prevention of downregulation of AQP2 expression in IMCD principal cells. AQP2 labeling in the kidneys of rats treated with PDE inhibitors demonstrated abundant apical AQP2 labeling in apical domains (Fig. 3C) similar to the labeling observed in controls (Fig. 3A). This finding indicates a potential role of intracellular cAMP in regulating AQP2 protein expression in hypercalcemia-induced NDI.

Similarly, semiquantitative immunoblotting of membrane fractions from the inner medulla of rats treated with PDE inhibitors showed a marked restoration in p-AQP2 abundance (149 ± 27% in PDEi-HC vs. 100 ± 11% in controls, not significant; Fig. 10, C and D). Immunohistochemistry revealed abundant apical labeling of p-AQP2 in IMCD principal cells from kidneys of rats treated with PDE inhibitors (Fig. 3F). This is consistent with the maintained abundance of p-AQP2 protein in kidneys of rats treated with PDE inhibitors as determined by semiquantitative immunoblotting. This strongly indicates that factors involved in regulating AQP2 abundance and AQP2 phosphorylation are critically dependent on intracellular cAMP levels.

The changes in the expression of inner medullary AQP3 and AQP1 in response to rolipram and milrinone treatment in HC rats were further examined to clarify whether the abundance of these AQPs are also regulated by intracellular cAMP levels. Interestingly, densitometric analysis of all the immunoblots of kidney from HC rats treated with PDE inhibitors revealed that inner medullary AQP3 abundance was normalized to control levels (116 ± 15% vs. 100 ± 12% in controls, not significant; Fig. 11, A and B; Table 4), whereas no changes of AQP1 abundance were observed (data not shown). This strongly suggests that both
AQP2 and AQP3 protein abundance is regulated by intracellular cAMP levels.

The urinary concentrating capacity was determined in HC, vitamin D-treated PDEi-HC, and CON rats (protocol 3) to assess whether PDE-inhibitor treatment recovered the urinary concentrating capacity in response to 24 h of water deprivation. At day 7 (before thirsting), a significant impairment in concentrating ability was evident in HC rats but not in PDE-inhibitor treated rats (Table 5), consistent with the results in the previous sets of rats (protocol 2, see above). To determine the urinary concentrating capacity, the rats were dehydrated for 24 h. This induced a marked increase in urine osmolality in CON rats and in vitamin D-treated rats but not in the group of rats that were treated with PDE-inhibitors (Table 5). Thus despite an increased urine osmolality and normalized AQP2 and AQP3 expression in response to PDE-inhibitor treatment, the treatment did not completely restore the urinary concentrating capacity because 24 h of dehydration did not lead to a significant increase in urine osmolality.

**DISCUSSION**

The results of the present study demonstrated that hypercalcemia is associated with substantial reduction in the expression of collecting duct AQP2 and AQP3 as well as a reduced expression of AQP2 that is phosphorylated in the PKA phosphorylation consensus site serine 256 of AQP2 (p-AQP2; i.e., “active AQP2”). This was seen in association with the progressive development of polyuria and decreased urinary osmolality. This strongly suggests that dysregulation of AQP3, as well as AQP2, plays an important role in the development of polyuria associated with hypercalcemia. In support of this conclusion, we also showed that treatment of rats that had vitamin D-induced hypercalcemia with the PDE inhibitors rolipram and milrinone, which are known to increase the intracellular cAMP level, prevented the downregulation of AQP2, p-AQP2, and AQP3 protein expression in kidneys of rats with hypercalcemia. Moreover, PDE inhibitor treatment of HC rats completely prevented the increase in urine osmolality.

**Table 3. Functional data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HC</th>
<th>PDEi-HC</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Urine output, μl·kg⁻¹·min⁻¹</td>
<td>47 ± 1.3††</td>
<td>25 ± 4.4</td>
<td>29 ± 2.7</td>
</tr>
<tr>
<td>Water intake, μl·kg⁻¹·min⁻¹</td>
<td>95 ± 4.6†</td>
<td>71 ± 9.6</td>
<td>86 ± 6.8</td>
</tr>
<tr>
<td>Plasma calcium, mmol/l</td>
<td>3.4 ± 0.04†</td>
<td>3.3 ± 0.1†</td>
<td>2.4 ± 0.03</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH₂O</td>
<td>298 ± 0.9†</td>
<td>305 ± 1.5†</td>
<td>294 ± 1.4</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH₂O</td>
<td>1042 ± 69††</td>
<td>1669 ± 157</td>
<td>1657 ± 109</td>
</tr>
<tr>
<td>Urine-to-plasma osmolality ratio</td>
<td>3.5 ± 0.2††</td>
<td>5.5 ± 0.5</td>
<td>5.6 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. PDEi-HC, hypercalcemic rats treated with phosphodiesterase inhibitors rolipram and milrinone. †P < 0.05, compared with control group. ††P < 0.05, compared with PDEi-HC group.
output and prevented the decrease in urine osmolality. This strongly supports the view that intracellular cAMP is a key factor in regulating both AQP2 and AQP3 expression levels and strongly supports the view that downregulation of AQP2 and AQP3 plays a significant role in the urinary concentrating defect associated with hypercalcemia.

Role of AQP2 and p-AQP2 Downregulation in Hypercalcemia-Induced NDI

It is well established that AQP2 is the chief target for vasopressin regulation of collecting duct water reabsorption and hence body water balance. This has been described in detail in several reviews (17, 25). In addition to downregulation of AQP2, we demonstrate here that the levels of AQP2 that are phosphorylated at the PKA consensus site serine 256 are also significantly reduced. This extends previous studies from our laboratory and others showing that hypercalcemia causes a substantial reduction of AQP2 expression in the IMCD and a reduced targeting to the apical plasma membrane, in association with the progressive development of polyuria and decreased urine osmolality (7, 31). Moreover, we have also shown that both abundance and apical plasma membrane expression levels of the Na-K-2Cl cotransporter BSC-1 were also significantly decreased in the TAL of HC rats (38). Therefore, these findings in part define the underlying molecular mechanisms of a decreased vasopressin-regulated water permeability of the collecting duct for osmotic equilibration and an impaired generation of hypertonic med-

Table 4. Immunoblotting and densitometric analysis of collecting duct aquaporin expression in kidney inner medulla

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HC</th>
<th>PDEI-HC</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>AQP2</td>
<td>48 ± 9%</td>
<td>128 ± 21%</td>
<td>100 ± 7%</td>
</tr>
<tr>
<td>p-AQP2</td>
<td>37 ± 12%</td>
<td>149 ± 27%</td>
<td>100 ± 11%</td>
</tr>
<tr>
<td>AQP3</td>
<td>55 ± 15%</td>
<td>116 ± 15%</td>
<td>100 ± 12%</td>
</tr>
<tr>
<td>AQP4</td>
<td>86 ± 6%</td>
<td>89 ± 9%</td>
<td>100 ± 7%</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, compared with control group. †P < 0.05, compared with PDEI-HC group.
The reduction in p-AQP2 expression observed in the present study demonstrates that the cAMP/PKA signal transduction pathway for regulation of AQP2 abundance is disturbed in hypercalcemia-induced NDI. This is highly consistent with the view that cAMP levels are markedly reduced in collecting duct principal cells in HC rats (1). Also the observation that treatment of HC rats with PDE inhibitors prevents downregulation of p-AQP2 levels supports this view and supports the view that downregulation of AQP2 and p-AQP2 is dependent on cAMP and that this downregulation plays a significant role in the development of polyuria. The role of intracellular cAMP in collecting duct principal cells and TAL cells in urinary concentrating defect associated with hypercalcemia is not completely elucidated. Early studies emphasized the role of decreased vasopressin-stimulated adenylate cyclase activity and cAMP production in hypercalcemia (1, 23, 27). Further work provided evidence that high calcium inhibits AVP-dependent cAMP production in mTAL (35) and that AVP fails to increase cAMP accumulation in mTAL of HC rats (2). However, vasopressin-stimulated adenylate cyclase activity was indistinguishable in OMCD and IMCD tubules dissected from HC and normocalcemic rats (2), suggesting a defect of a post-cAMP step in the signal transduction pathway might be involved in the decreased AVP-dependent water permeability of the collecting duct. Our present results demonstrating that PDE-inhibitor treatment of HC rats in part can prevent the urinary concentrating defect strongly support the view that cAMP is an important component in this. It should be emphasized that the thirsting of DHT-treated rats that were cotreated with PDE inhibitors failed to increase urine osmolarity in response to 24-h thirsting in contrast to controls. This demonstrates that PDE-inhibitor treatment only partly prevents the urinary concentrating defect.

Sands et al. (31) demonstrated that the IMCD water permeability did not increase in HC rats after AVP treatment, whereas urea permeability was significantly increased and this was accompanied by an increase in the abundance of the AVP-regulated urea transporter. This observation was somewhat puzzling because both are thought to occur via cAMP-mediated mechanisms (31). Thus after DHT-induced hypercalcemia, there is apparently a difference in the effect of cAMP in regulating urea and water permeability in IMCD. It may be speculated that this may reflect different cellular mechanisms underlying the regulation of these two channels/carriers; e.g., hypercalcemia may induce endocytosis of AQP2 or prevent exocytosis of AQP2, whereas recent evidence suggests that vasopressin does not regulate urea permeability by trafficking of urea transporters (16). A potential role of the calcium sensing receptor has been suggested to be involved in the downregulation of AQP2 (31, 32) and the water permeability of IMCDs in hypercalcemia. Additional studies are warranted to further explore the importance of this and the regulatory pathways involved.

### Role of AQP3 Downregulation in Hypercalcemia-Induced NDI

Our data demonstrated that hypercalcemia-induced NDI is associated with markedly decreased AQP3 abundance in kidney in addition to the observed downregulation of AQP2 and p-AQP2. Moreover, treatment with the PDE inhibitors rolipram and milrinone prevented the downregulation of AQP3 protein expression levels in HC rats and restored the decreased urine concentration. This indicates that low renal AQP3 abundance also contributes to the polyuria and urinary-concentrating defect in kidneys of HC rats. Moreover, this provides direct evidence that intracellular cAMP may be an important factor in regulating AQP3 expression levels.

The mechanisms underlying regulation of AQP3 expression is currently not well established. As with AQP2, AQP3 mRNA levels were induced by infusion with the selective V2 agonist DDAVP (37). Examination of the 5′ flanking DNA of AQP3 failed to reveal a cAMP response element; however, Sp1 and AP2 cis-regulatory elements are present (15), and these are known to be associated with cAMP-mediated transcriptional regulation (14, 34). AQP3 is localized in the basolateral plasma membrane domains of collecting duct principal cells (8). Immunelectron microscopy previously demonstrated a predominant labeling of AQP3 in the basolateral plasma membranes with little labeling of intracellular vesicles, suggesting that AQP3 is not regulated by vesicular trafficking, in contrast to the findings with AQP2. Immunoblotting has shown that thirsting of normal rats for 48 h or DDAVP treatment of Brattleboro rats for 5 days induces a marked increase in AQP3 expression. These conditions did not lead to observed changes in AQP1 or AQP4 protein expression, even in the severe polyuric condition induced by lithium treatment in rats (19).

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**Table 5. Changes in urine osmolality (protocol 3)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>6</td>
<td>1,181 ± 121</td>
<td>783 ± 107⁺</td>
<td>954 ± 83⁺⁺</td>
<td>1,763 ± 156⁺⁺</td>
</tr>
<tr>
<td>PDEi-HC</td>
<td>6</td>
<td>1,196 ± 106</td>
<td>1,028 ± 234</td>
<td>1,628 ± 209</td>
<td>1,918 ± 220⁺⁺</td>
</tr>
<tr>
<td>CON</td>
<td>6</td>
<td>1,275 ± 146</td>
<td>1,458 ± 187</td>
<td>1,369 ± 90</td>
<td>2,576 ± 200⁺⁺</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats. Urinary osmolality (mosmol/kgH2O) was measured after 24 h of dehydration from day 7 to day 8. ⁺P < 0.05, compared with control rats; †P < 0.05, compared with PDEi-HC rats; ‡P < 0.05, compared with day 7.
clear evidence that AQP3 regulation is related to changes in vasopressin and water balance. However, there are several examples showing a decoupling of AQP2 and AQP3 expression, indicating that more needs to be learned regarding the mechanism controlling AQP3 expression (25).

Unchanged AQP4 Abundance in Hypercalcemia-Induced NDI

AQP4 expression was unchanged in HC rats compared with CON rats despite the fact that both AQP3 and AQP4 are located in the basolateral membrane of collecting duct principal cells. Studies in transgene mice lacking either AQP3 or AQP4 have made it clear that AQP3 plays a major role as an exit pathway for water reabsorption in the collecting duct. This is on the basis of the observations that AQP3-deficient mice have a very severe urinary-concentrating defect with severe polyuria (21). In contrast, AQP4-deficient mice exhibit a much less severe urinary concentrating defect (5). In response to water deprivation, AQP4-deficient mice only have marginally reduced urinary concentrating ability. However, isolated perfused collecting duct experiments revealed that in tubules dissected from AQP4-deficient mice, there was a reduction in the water permeability in response to vasopressin treatment (5). There is a marked axial heterogeneity in the expression of AQP3 and AQP4 along the collecting duct (8, 36, 37). AQP4 is mainly present in the IMCD, whereas AQP3 is very abundant in the cortical collecting duct and OMCD as well as in the proximal parts of the IMCD but less abundant (although present) in the terminal IMCD. Thus it may be speculated that AQP3 is important for water reabsorption in the proximal part of the collecting duct in which by far the largest amount of water is reabsorbed. Thus reduction in AQP3 expression (as determined in this study) in the proximal segments of the collecting ducts would predict development of significant polyuria. Moreover, it may be speculated that AQP4 expression cannot compensate for the increased delivery of water to the terminal collecting duct. The mechanisms involved in regulating the expression of AQP4 are not well understood, but the present data indicate that they are distinct from those regulating AQP3 expression.

Reduced Proximal Tubule AQP1 Abundance in Hypercalcemia-Induced NDI

The total kidney expression of AQP1 is significantly reduced in rats with vitamin D-induced hypercalcemia. Examination of different kidney zones revealed that AQP1 expression is reduced in cortex but not in inner medulla, strongly suggesting that AQP1 expression is reduced in the proximal tubule but not in the DTL. This was confirmed by immunocytochemistry demonstrating reduced brush-border labeling and also reduction in the basolateral plasma membrane labeling in the proximal tubule. In contrast, no detectable change was observed of AQP1 immunolabeling in DTLs. The functional implication of these observations is unclear.

First of all, it has been argued that AQP1 expression in the DTL is essential for efficient countercurrent multiplication in the renal medulla (17, 18). Consistent with this observation, AQP1-knockout mice have dramatically reduced osmotic permeability of DTLs and exhibit a very severe urinary concentrating defect. The observed maintained expression of AQP1 in DTLs in rats with vitamin D-induced hypercalcemia indicates that DTL AQP1 is not involved in the development of polyuria. We observed a significant reduction in AQP1 expression in the proximal tubule. AQP1 expression in the proximal tubule plays a significant role for water reabsorption in the proximal tubule, consistent with the observations that proximal tubules from AQP1-deficient mice have dramatically reduced water permeability. In addition, a high expression of AQP1 in the proximal tubule may play a role in facilitating sodium reabsorption, e.g., by preventing back-leak of sodium due to instant osmotic equilibration (9). The functional consequence of a reduced expression of AQP1 in the proximal tubule during hypercalcemia is unclear. It is unlikely that this is involved in the polyuria. One argument supporting this notion is that PDE-inhibitor treatment of HC rats does not prevent downregulation of AQP1 but prevents completely the development of polyuria. However, it cannot be ruled out that the reduced AQP1 expression may have a bearing on the efficiency of water reabsorption or efficiency of sodium reabsorption in the proximal tubule. Future studies will be needed to establish this.

Summary

We have reported an altered expression of collecting duct AQPs (p-AQP2 and AQP3) in hypercalcemia-induced NDI in rats, accompanied by polyuria and urinary-concentrating defect. Moreover decreased AQP2, p-AQP2, and AQP3 expression was prevented by PDE inhibitors treatment, suggesting an important role of intracellular cAMP in regulating AQP2 and AQP3 expression in collecting duct. Moreover, it strongly supports the view that downregulation of AQP2, p-AQP2, and AQP3 plays a significant role in the development of polyuria associated with vitamin D-induced hypercalcemia.

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RENAL AQP EXPRESSION IN HYPERCALCEMIA


