Adenylate Cyclase 6 Determines cAMP Formation and Aquaporin-2 Phosphorylation and Trafficking in Inner Medulla

Timo Rieg,*† Tong Tang,*† Fiona Murray,‡ Jana Schroth,† Paul A. Insel,‡ Robert A. Fenton,§ H. Kirk Hammond,*† and Volker Vallon*†‡

*Department of Medicine, University of California San Diego, La Jolla, California; †Veterans Affairs San Diego Healthcare System, San Diego, California; ‡Department of Pharmacology, University of California San Diego, La Jolla, California; and §The Water and Salt Research Center, Department of Anatomy, University of Aarhus, Aarhus, Denmark

ABSTRACT

Arginine vasopressin (AVP) enhances water reabsorption in the renal collecting duct by vasopressin V2 receptor (V2R)-mediated activation of adenylyl cyclase (AC), cAMP-promoted phosphorylation of aquaporin-2 (AQP2), and increased abundance of AQP2 on the apical membrane. Multiple isoforms of adenylyl cyclase exist, and the roles of individual AC isoforms in water homeostasis are not well understood. Here, we found that levels of AC6 mRNA, the most highly expressed AC isoform in the inner medulla, inversely correlate with fluid intake. Moreover, mice lacking AC6 had lower levels of inner medullary cAMP, reduced abundance of phosphorylated AQP2 (at both serine-256 and serine-269), and lower urine osmolality than wild-type mice. Water deprivation or administration of the V2R agonist dDAVP did not increase urine osmolality of AC6-deficient mice to the levels of wild-type mice. Furthermore, AC6-deficient mice lacked dDAVP-promoted inner medullary cAMP formation and phosphorylation of serine-269 and had attenuated increases in both phosphorylation of serine-256 and apical membrane AQP2 trafficking. In summary, AC6 expression determines inner medullary cAMP formation and AQP2 phosphorylation and trafficking, the absence of which causes nephrogenic diabetes insipidus.


The anti-diuretic hormone arginine-vasopressin (AVP) is the primary regulator of water reabsorption in the renal collecting duct (CD) and is critically involved in the regulation of water balance and maintenance of plasma osmolality.1 AVP acts on the CD through the Gs protein–coupled vasopressin V2 receptor (V2R) to stimulate adenylyl cyclase (AC) and thus the synthesis of cAMP.2 cAMP activates protein kinase A (PKA), which phosphorylates the water channel aquaporin-2 (AQP2) in its COOH-terminal tail on serine residue 256 (S256), thereby resulting in apical plasma membrane accumulation of AQP2.3–6 In addition, cAMP-independent activation of AQP2 has been reported, which may involve V2R action of phosphoinositide-specific phospholipase C.7 Other non–PKA-targeted AQP2 phosphorylation sites include S261, S264, and S269.8 Phosphorylation of AQP2 at S264 and S269 requires prior phosphorylation of S256.9 Immunohistochemistry showed that pS269-AQP2 localizes exclusively in the apical plasma membrane.
membrane of the connecting tubule and CD. AVP-stimulated AQP2 phosphorylation thus induces plasma membrane accumulation and retention of the channel. Genetic defects in the V₉R or AQP2 impair AVP-induced increases in tubular water permeability and cause nephrogenic diabetes insipidus (NDI). In comparison, less is known about the role of genetic variation of the proteins involved in signaling from V₉R to AQP2.

Generation of cAMP involves the activation of ACs, of which nine different membrane-bound isoforms have been identified (AC1 to 9). Studies on AC isoform expression in the kidney have been almost exclusively confined to the rat, in which mRNA analyses have shown that all membrane-bound isoforms, except for AC1 and 8, are expressed. The same pattern was found for mRNA expression of ACs in inner medullary CD (IMCD) suspensions of rats.

Based on in situ hybridization studies and the relative expression of AC6 mRNA in CD principal cells of rats, as well as studies using small interfering RNA designed to knock down AC6 in primary cultured mouse IMCD, it has been proposed that this AC isoform may contribute to AVP-stimulated AC6 mRNA in CD principal cells of rats, as well as studies using small interfering RNA designed to knock down AC6 in primary cultured mouse IMCD, it has been proposed that this AC isoform may contribute to AVP-stimulated cAMP formation, although studies in rat IMCD have indicated that AVP-promoted Ca²⁺/calmodulin-dependent cAMP accumulation involves AC3 activity. In these experiments, we examined mice that lack AC6 (AC6⁻/⁻) to gain insights regarding the role of AC6 in the formation of cAMP, phosphorylation of AQP2 at S256 and S269, and urinary concentration in vivo. The results indicate an important contribution of AC6 to AVP action and urinary concentration in vivo and that AC6⁻/⁻ mice have NDI.

RESULTS

Basal Analysis of AC6⁻/⁻ Mice with Free Access to Fluid

Urine osmolality was significantly lower in AC6⁻/⁻ versus WT mice in spontaneously voided urine (Figure 1A). This was associated with an approximately threefold greater brain AVP mRNA expression and greater fluid intake (measured in home cages) in AC6⁻/⁻ compared with wild-type (WT) mice. Plasma osmolality, GFR (both shown in Figure 1A) and hematocrit (WT: 43.9 ± 0.8% and AC6⁻/⁻: 45.0 ± 1.3%, not significant) were not significantly different between genotypes, indicating intact fluid balance in AC6⁻/⁻ mice. Food intake was slightly higher in AC6⁻/⁻ versus WT mice (5.2 ± 0.2 versus 4.3 ± 0.2 g/d, P < 0.05) and was associated with greater urinary excretion of K⁺ (58 ± 5 versus 46 ± 2 mmol/mmol creatinine, P < 0.05) and a tendency for greater Na⁺ excretion (46 ± 3 versus 40 ± 2 mmol/mmol creatinine, P = 0.088). Urine collections for 24 hours in metabolic cages in a separate set of mice showed that AC6⁻/⁻ mice were polyuric compared with WT mice (7.2 ± 0.9 versus 1.0 ± 0.2 ml/day per mouse, n = 5; P < 0.05). The latter analysis may have overestimated the difference between genotypes because, due to evaporation, the rate of recovery is less with low compared with higher urine flow rates.

Under basal conditions, lower urine osmolality in AC6⁻/⁻ mice (compared with WT mice) was associated with reduced inner medullary levels of cAMP (Figure 2, A and B) and inner medullary abundance of AQP2 phosphorylated at serine 256 (p-S256) compared with WT mice; serine 269 (p-S269) is not detectable (ND) in AC6⁻/⁻, whereas total AQP2 is not different. For physiologic parameters, n = 8/group; for mRNA expression, n = 5/group; for protein abundance, n = 3/group. *P < 0.05 versus WT.

Figure 1. Reduced urine osmolality and AQP2 phosphorylation in AC6⁻/⁻ mice with free water access. (A) Despite having greater brain arginine-vasopressin (AVP) mRNA expression, AC6⁻/⁻ mice have lower urinary osmolality associated with greater fluid intake than do wild-type mice (WT). Intact water balance is indicated by similar plasma osmolality and GFR in AC6⁻/⁻ and WT mice. (B) Membrane fractions of inner medulla were obtained by differential centrifugation; densitometric analysis of Western blots was performed using β-actin expression as reference. Lower urine osmolality in AC6⁻/⁻ mice is associated with lower amounts of AQP2 phosphorylated at serine 256 (p-S256) compared with WT mice; serine 269 (p-S269) is not detectable (ND) in AC6⁻/⁻, whereas total AQP2 is not different. For physiologic parameters, n = 8/group; for mRNA expression, n = 5/group; for protein abundance, n = 3/group. *P < 0.05 versus WT.
similar between genotypes and both pS256-AQP2 and pS269-AQP2 were detected in the IMCD of AC6-/- mice (Figure 3; Supplemental Figure S4).

The urine-to-plasma ratio for osmolality was reduced by approximately 60% in AC6-/- compared with WT mice (2.9 ± 0.3 versus 6.6 ± 0.5, n = 8; P < 0.05). Plasma levels of urea were not different between genotypes (Table 1), but urinary urea concentration (AC6-/-: 0.51 ± 0.04 versus WT: 1.1 ± 0.07 mol/L, n = 8; P < 0.05) was reduced in the same proportion as urinary osmolality (Figure 1A). As observed for the total osmoles, the urine-to-plasma ratio for urea was reduced by approximately 60% in AC6-/- mice, indicating that, in contrast to mice lacking the urea transporter UT-B, AC6-/- mice have no “urea-selective” urinary concentrating defect.

Response to Water Deprivation in Inner Medullary AC Isoform Expression and in AC6-/- mice
Under basal conditions, inner medullary mRNA expression of the AC6 isoform was predominant among membrane-bound AC isoforms in WT mice (Figure 4A). All other isoforms were less abundant; AC8 was not detectable (see also Figure S2). Moreover, inner medullary AC6 mRNA expression in WT mice was inversely related to fluid intake, unlike what was observed for several other AC isoforms in response to fluid intake (Figure 4B).

AC6-/- mice increased their urine osmolality in response to 18-hour water deprivation (Figure 5). However, despite having greater AVP mRNA expression in brain and greater urinary AVP/creatinine ratios (Figure 5), AC6-/- mice did not reach the level of urinary concentration induced by water deprivation in WT mice (Figure 5). This was associated with lower ratios of urinary cAMP/AVP, lower total urinary cAMP concentration (126 ± 4 versus 196 ± 16 μmol/L, n = 8; P < 0.05), and greater body weight loss and increase in plasma osmolality in AC6-/- versus WT mice (Figure 5). The relationship between plasma osmolality and urinary AVP seemed intact in AC6-/- (Figure 5), suggesting unaltered regulation and release of AVP.

In inner medulla of WT mice, AC1 mRNA expression, like AC6, was inversely related to fluid intake, whereas AC7 mRNA expression changed in parallel with fluid intake. AC6-/- mice had greater inner medullary expression of AC7 than did WT mice (Figure S1A). Studies on inner medullary mRNA expression of cAMP-degrading phosphodiesterases (PDEs) indicated that PDE1A is the most abundant isofrom in WT mice under basal conditions (Figures S1 and S3); none of the strongly expressed PDEs in inner medulla were significantly down- or up-regulated in AC6-/- mice (Figure S3A). Studies on inner medullary mRNA expression of membrane-bound AC isoforms in AC6-/- mice subjected to water deprivation showed significantly higher expression of AC4 and lower expression of AC1 compared with WT mice (Figure S1B). Inner medullary mRNA expression of PDE isoforms was not altered in AC6-/- mice compared with WT mice under these conditions (Figure S3B).

Effect of 1-Desamino-8-D-Arginine Vasopressin on cAMP Formation, AQP2 Abundance, Phosphorylation and Trafficking, and Urinary Concentration in AC6-/- Mice
Addition of forskolin to isolated IMCD from untreated mice increased cAMP formation in WT mice, but had only a very minor effect in AC6-/- mice, indicating that the majority of membrane-bound AC activity in IMCD is mediated by AC6 (Figure 2A). Addition of 1-desamino-8-D-arginine vasopressin (dDAVP) to isolated IMCD increased cAMP formation in WT mice, but this response was absent in AC6-/- mice (Figure 2, A and B), showing the key role of AC6 in dDAVP-induced cAMP formation in IMCD.

To study the in vivo response to V2R activation, we water-loaded mice by oral gavage to suppress the endogenous AVP system and administered dDAVP or vehicle (by intraperitoneally application) before collecting urine for 2 hours in metabolic cages. Urinary osmolality was not significantly different between genotypes in response to water loading plus application of vehicle (Figure 6A). Administration of dDAVP along with water loading increased urinary osmolality in both genotypes, but urine osmolality was significantly lower in AC6-/- versus WT mice (Figure 6A).

In another set of mice, we performed Western blot analysis to assess the abundance and phosphorylation of AQP2 in inner medulla in response to dDAVP or vehicle after suppression of endogenous AVP by water loading overnight. Treatment with vehicle after water loading was associated with lower total AQP2 and pS256-AQP2 abundance in AC6-/- versus WT mice, whereas pS269-AQP2 was not detectable in both genotypes (Figure 6). Twenty minutes after treatment with dDAVP (a time known to be associated with changes in AQP2 phosphorylation2,21), the in-

Figure 2. Impaired forskolin and dDAVP-induced cAMP formation in freshly isolated inner medullary collecting ducts of AC6-/- mice. (A) Stimulation of cAMP formation by forskolin (Forsk; 10 μM) is attenuated, and the response to dDAVP is absent in AC6-/- mice. (B) Increase in cAMP formation in response to dDAVP relative to control values. Protein concentration was adjusted to 60 μg/vial. Experiments were performed with phosphodiesterase inhibition (0.5 mM 3-isobutyl-1-methylxanthine), n = 5/group. *P < 0.05 versus WT, #P < 0.05 versus control same genotype.
crease in total AQP2 abundance was similar between genotypes. However, the dDAVP-induced increase in pS269-AQP2 in IMCD, observed in WT mice, was not detectable in AC6−/− mice, and the increase in pS256-AQP2 abundance was attenuated in AC6−/− mice compared with WT mice (WT: 0.39 ± 0.07 versus 1.51 ± 0.44 arbitrary units; P < 0.05; Figure 6).

Immunohistochemistry showed that systemic dDAVP treatment of WT mice increased the abundance of total AQP2, pS256-AQP2, and pS269-AQP2 in the apical plasma membrane of IMCD (Figure 3). In contrast to WT mice, in AC6−/− mice, the apical plasma membrane abundance of pS269-AQP2 did not increase in IMCD after dDAVP treatment, and the increase in the abundance of total and phosphorylated AQP2 at the apical plasma membrane (arrows) of WT mice and AC6−/− mice (J–L) to a similar extent. Right panel, inner medulla. In WT mice under basal conditions, total AQP2 is predominantly intracellular in the IMCD (A), pS269-AQP2 is detected on the apical plasma membrane (B, arrows), and pS256-AQP2 is intracellular and in apical membrane domains (C). (D and E) In AC6−/− mice, although total AQP2 abundance is not different from WT mice, pS269-AQP2 is virtually absent (asterisk), and pS256-AQP2 is less abundant on the apical plasma membrane (F). (G–I) Systemic application of dDAVP after acute water loading increased the abundance of total and phosphorylated AQP2 at the apical plasma membrane (arrows) of WT mice, but this was attenuated in AC6−/− mice (J–L).

Table 1. Basal parameters in WT and AC6−/− mice

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<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>AC6−/−</th>
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<tr>
<td>Body weight (g)</td>
<td>29.3 ± 0.5</td>
<td>30.0 ± 0.9</td>
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<tr>
<td>Plasma Na⁺ (mmol/L)</td>
<td>153 ± 2</td>
<td>155 ± 2</td>
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<tr>
<td>Plasma K⁺ (mmol/L)</td>
<td>4.5 ± 0.4</td>
<td>4.6 ± 0.3</td>
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<tr>
<td>Plasma urea (mmol/L)</td>
<td>9.1 ± 0.6</td>
<td>10.0 ± 0.9</td>
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<tr>
<td>Plasma glucose (mg/dl)</td>
<td>172 ± 7</td>
<td>181 ± 4</td>
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<tr>
<td>Plasma aldosterone (pg/ml)</td>
<td>909 ± 90</td>
<td>824 ± 94</td>
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Values are mean ± SEM of measurements in n = 8 mice/genotype.

DISCUSSION

Much has been learned about the molecular determinants of AVP-regulated water transport in the CD, including the multiple phosphorylation sites on AQP2 and mutations in V₂R or AQP2 leading to NDI. However, little is known about the role and molecular identity of the AC isoform(s) responsible
with NDI, however, AC6 electrolyte disturbances or growth retardation. Consistent mRNA and protein expression and lower urine osmolality.\textsuperscript{23,24}

\textbf{Figure 4.} AC6 is the highest expressed isoform and inversely regulated by fluid intake in renal inner medulla. mRNA expression of AC isoforms of WT mice. (A) Basal levels of mRNA expression of AC isoforms (1 to 9) in mice with free water access. Results are shown relative to AC6, which have the highest expression level. ND, not detectable. (B) Changes in AC isoform expression in response to water loading and water deprivation relative to free water access (see Methods section for details). Relative to water loading, water deprivation increased mRNA expression of AC1 and AC6 but reduced that of AC7. n = 5 per condition. §P < 0.05 versus water load.

for the formation of cAMP involved in this signaling cascade. These studies showed for the first time that, in mouse IMCD, almost all of the membrane-bound AC activity and all of the dDAVP-induced cAMP formation is mediated by AC6. More importantly, we showed that AC6 plays a role in urinary concentration and that mice deficient in AC6 expression have NDI. The defect in urinary concentration is associated with, and potentially the consequence of, reduced cAMP formation and AQP2 phosphorylation and apical redistribution in IMCD.

AC6\textsuperscript{-/-} mice are in fluid balance and show no signs of electrolyte disturbances or growth retardation. Consistent with NDI,\textsuperscript{22} however, AC6\textsuperscript{-/-} mice have lower urinary osmolality despite elevated AVP mRNA expression in the brain and greater urinary AVP levels in response to water deprivation. NDI is also indicated by the lower urine osmolality in response to dDAVP. These results argue against polydipsia, resulting from defective osmoregulation of thirst, as the primary cause of lower urine osmolality in AC6\textsuperscript{-/-} mice. We water-loaded mice while testing for the effect of dDAVP to prevent the need for homeostatic water retention and balance: under these conditions, dDAVP significantly increased urine osmolality in AC6\textsuperscript{-/-} mice, but those mice produced less concentrated urine than WT mice. Our results indicate that AC6 activity contributes to, but does not fully explain, AVP-induced urinary concentration.

Do changes in PDE expression compensate for the loss of AC6? Mice with genetically increased activity of PDE3 and PDE4 show a fourfold higher PDE activity in microdissected IMCD and cortical CD and lack an increase in cAMP in response to V\textsubscript{2}R activation, which is associated with lower AQP2 mRNA and protein expression and lower urine osmolality.\textsuperscript{23,24} Pharmacologic studies have argued against a role of PDE3 in urine osmolality.\textsuperscript{25} However, treatment of mutant AQP2 het-
eurozygous knock-in mice or mice with hereditary NDI with the PDE4 inhibitor, rolipram, increases urine osmolality and restores AVP-dependent cAMP accumulation in IMCD, indicating a role for PDE4 in the regulation of cAMP and urinary concentration. Sildenafil, an inhibitor of PDE5 (which selectively hydrolyzes cGMP), induces apical accumulation of AQP2 in CD principal cells in rats\textsuperscript{26} but fails to increase urine osmolality in mice.\textsuperscript{25} We find relatively low expression of PDE3–5 mRNA in inner medulla of WT mice compared with that of the most abundant isoform, PDE1A, and expression of those groups of PDE isoforms did not consistently change with water intake or deletion of AC6 (Figure S3B).

Do other AC isoforms compensate for the loss of AC6? AC6 is the most abundant isoform in rat kidney, and mRNA expression levels of AC4, AC5, and AC9 are <10% of that of AC6 in whole kidney.\textsuperscript{19} Our studies yield a similar pattern of expression of those AC isoforms (Figure 4). The second most abundant isoform in rat IMCD is AC3.\textsuperscript{27} Moreover, it has been speculated that Ca\textsuperscript{2+} mobilization\textsuperscript{2} and signaling mechanisms involving calmodulin/AC3 are important in CD water transport of the rat and mouse.\textsuperscript{17,18} In comparison, AC3 is not among the most abundant isoforms in the mouse inner medulla based on mRNA expression and is not regulated by variation in fluid intake in WT or AC6\textsuperscript{-/-} mice (Figure 4; Figure S1). Furthermore, mice that lack AC3 show apparently normal urinary flow rates and urine osmolalities.\textsuperscript{28} AC5 expression is found in medulla and cortical and outer medullary CD of rats.\textsuperscript{16,29,30} We found that expression of AC5 mRNA does not change with variation in fluid intake and AC5 is not up-regulated in AC6\textsuperscript{-/-} mice. Moreover, in preliminary studies, we found that lack of AC5 does not alter basal urine osmolality (WT: 1988 ± 81 and AC5\textsuperscript{-/-} mice: 2089 ± 53 mmol/kg, not significant; n = 4; genotype; mice were kindly provided by P. L. Han\textsuperscript{31}). Inner medullary mRNA expression of AC4 and AC7 is increased in AC6\textsuperscript{-/-} mice under basal conditions; however, this does not restore agonist-induced accumulation of cAMP. The results of other functional studies also imply that those AC isoforms do not compensate for the lack of AC6 in inner medulla. A possible explanation for this unique importance of AC6 is its compartmentation in membrane microdomains (e.g., rafts/caveolae) in IMCD cells in a manner akin to what has been noted in other cell types or perhaps to bind snapin, which, in turn, controls the trafficking of AQP2.\textsuperscript{32–34}

Mice with a mutation in S256-AQP2 (S256L) can not phosphorylate AQP2 at S256 because of the amino acid change and show reduced apical membrane accumulation (analyzed in outer medullary CD), resulting in NDI.\textsuperscript{35} In contrast to AC6\textsuperscript{-/-} mice, these mice do not increase urine osmolality in
response to dDAVP. We propose that the ability of AC6−/− mice to concentrate their urine is caused in part by the maintenance of phosphorylation and apical redistribution of AQP2 in renal cortex and outer medulla, where the majority of the osmotic water reabsorption occurs. In contrast, we showed that, in inner medulla, dDAVP-induced up-regulation of cAMP formation and phosphorylation of AQP2 at S269-AQP2 are lacking; moreover, increases in pS256-AQP2 and AQP2 trafficking into the apical membrane are attenuated in IMCD of AC6−/− mice compared with WT mice, which may explain the defect in urinary concentration. The results may imply that phosphorylation of S256 primes the subsequent phosphorylation at S269. Alternatively, AC6 may play a primary role in the signaling cascade leading to AVP-induced phosphorylation of AQP2 at S269 with secondary consequences on the net amount of pS256-AQP2 detectable in the membrane fraction. Notably, AC6−/− mice up-regulate the amount of inner medullary AQP2 phosphorylated at S256 in response to systemic dDAVP, whereas the latter does not stimulate cAMP formation in isolated IMCD in these mice. This might reflect cAMP-independent activation of AQP2 in AC6−/− mice, which may involve V2R activation of phosphoinositide-specific phospholipase C and PKA-independent AQP2 phosphorylation at S256.36,37 These mechanisms may also contribute to intact AQP2 regulation in renal cortex of AC6−/− mice.

A prevalent dysfunctional missense single nucleotide polymorphism of AC6 with a reduced ability to form cAMP has been described with a frequency of approximately 3% in a white population.38 The current results warrant further studies to determine whether these single nucleotide polymorphisms have potential consequences on urinary concentration. In summary, these results showed that expression of AC6 determines cAMP formation and AQP2 phosphorylation and trafficking in the IMCD. Furthermore, we showed that these effects are functionally relevant for urinary concentration. Mice lacking AC6 are thus a new model of NDI.

**CONCISE METHODS**

All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and was approved by the local Institutional Animal Care and Use Committee. Age-matched adult male and fe-
Figure 6. Reduced urine osmolality and inner medullary abundance and phosphorylation of AQP2 in AC6−/− mice in response to vasopressin V2 receptor activation by dDAVP. (A) Acute water loading (3% of body weight by oral gavage followed by urine collection for 2 hours) lowered urine osmolality to similar levels in both genotypes. dDAVP increased urinary osmolality in AC6−/− mice, but urine osmolality was lower than in WT mice. \( n = 8 \)/group. *\( P < 0.05 \) versus WT. (B) Endogenous AVP was suppressed overnight (5% dextrose/1% ethanol in drinking water). dDAVP or vehicle were applied, and inner medullae were harvested 20 minutes later. Western blot analysis was performed as described in Figure 1. dDAVP increased the abundance of (B) total and (C) S256-phosphorylated AQP2 (p-S256) in AC6−/− mice, but the increase in p-S256 was lower than that observed in WT mice. (D) The dDAVP-induced phosphorylation of AQP2 at S269 (p-S269) observed in WT mice is not detectable in AC6−/− mice. \( n = 2 \) and \( n = 4 \)/group for vehicle and dDAVP treatment, respectively. *\( P < 0.05 \) versus WT; ND, not detectable.

**Inner Medullary Expression of AC Isoforms in WT mice under Basal Conditions and in Response to Water Loading or Deprivation**

RT-PCR was performed to determine expression of AC isoforms in inner medulla under control conditions, with mice having free access to food and fluid, and in response to water loading for 2 hours (by oral gavage, 10 mM glucose solution, 3% of body weight) and water restriction for 18 hours, respectively. Mice were euthanized, and renal inner medullae and brain were rapidly removed. Total RNA from inner medullae and brain was isolated and reverse transcribed into cDNA. Quantitative PCR (Bio-Rad Opticon 2 using qPCR MasterMix Plus SYBR Green Kit; Eurogentec, San Diego, CA) was performed using 8 ng RNA/reaction and 100 nM sense/anti-sense primers. Primer sets were designed for each of the membrane-bound AC isoforms, PDE isoforms, and AVP (primer sequences provided by request). All primers were validated using mouse reference cDNA as the template (Stratagene, 50 ng). To determine primer efficiency, a standard curve was constructed for each primer using the following dilutions of the template: 1, 1:8, 1:64, and 1:512. The linearity and sensitivity of the reaction were determined by plotting log cDNA concentration versus Ct number. Primers were deemed suitable for real-time PCR if the efficiency of the PCR was between 90 and 100%. The efficiency of all primers used was comparable.

**Analysis of AC6−/− Mice with Free Access to Fluid**

Male mice were kept in standard rodent cages with free access to food and water. Fluid intake and food intake were measured over 3 days and averaged. Spontaneously voided urine was collected for determination of Na+, K+, creatinine, urea, and osmolality. Blood was drawn from the retroorbital plexus for determination of hematocrit, plasma Na+, K+, urea, and osmolality. Osmolality was measured by vapor pressure (Vapor; Wescor, Salt Lake City, UT), Na+ and K+ by flame photometry (Cole-Parmer Instrument, Vernon Hills, IL), and creatinine and urea using commercial enzymatic assays (Thermo Fisher Scientific, Waltham, MA). GFR was determined in conscious mice using plasma kinetics of FITC-inulin after a single-dose intravenous injection. Brains were har-
vested to determine AVP mRNA by RT-PCR. Inner medullary abundance of total AQP2 protein and AQP2 phosphorylated at S256 and S269 was determined as described below. In a different set of mice, 24-hour metabolic cage experiments with free access to food and water were performed to measure fluid excretion.43

**Response to Water Deprivation**

Water deprivation was performed overnight (18 hours), followed by determination of changes in body weight and collection of spontaneous voided urine to measure urinary osmolality and AVP concentrations (by RIA; IBL, Hamburg, Germany); results were normalized to urinary creatinine. Blood was taken for assay of hematocrit and osmolality. mRNA from inner medullae and brain was isolated as described above for determination of AC isoforms and AVP, respectively.

**Effect of dDAVP on Urinary Concentration and AQP2 Abundance and Phosphorylation after Suppressing Endogenous AVP**

Mice were randomized to acute water loading (10 mM glucose solution, 3% of body weight) given by oral gavage, immediately followed by intraperitoneally injection of vehicle (sterile water) or dDAVP (0.1 μg/kg; Sigma-Aldrich, St. Louis, MO). This dose of dDAVP prevents water load–induced diuresis in WT mice.45 The mice were placed in metabolic cages for quantitative urine collections over 2 hours without access to food or water. Urine was analyzed as described above.

In another set of mice, endogenous AVP levels were suppressed by providing them with 5% dextrose/1% ethanol solution overnight. The next day, the mice were injected with vehicle or dDAVP (as described above). Mice were euthanized 20 minutes after injection, and inner medullae were isolated to determine abundance of total AQP2 and AQP2 phosphorylated at S256 and S269 (see below).

**Inner Medullary Formation of cAMP**

IMCD were isolated using a modification of the method of Chou et al.46 To prevent degradation of cAMP formed during incubation with dDAVP and forskolin, IMCD aliquots were incubated at 37°C for 10 minutes with 0.5 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Forskolin (10 μM; Sigma-Aldrich) or the V₂R agonist dDAVP (10 pM to 10 nM; Sigma-Aldrich) was added, and the incubation continued for 15 minutes. Reactions were terminated by addition of ice-cold 10% TCA, and the cAMP content of samples was assessed by RIA.40,44

**Inner Medullary Abundance of Total AQP2 and AQP2 Phosphorylated at S256 and S269**

Kidneys were removed, and inner medullae were dissected and prepared for Western blotting. Proteins were transferred to ni-
trocellulose membranes and immunoblotted with AQP2 (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), pS256-AQP2 (1:3000), and pS269-AQP2 (1:1000) (the latter two were generous gifts from J. Hoffert and M. Knepper, National Institutes of Health). Chemiluminescent detection was performed with ECL Plus (Amer- sham, Piscataway, NJ). Densitometric analysis was performed by ImageJ Software (National Institutes of Health, Bethesda, MD). β-Actin was used as a loading control (dilution 1:5000; Sigma-Aldrich).

**Immunohistochemistry and Preparation of Tissue for Light Microscopy**

All procedures have been described in detail previously. Labeling was visualized by use of a peroxidase-conjugated secondary antibody for light microscopy (P448; Dako, Glostrup, Denmark).

**Immunogold Electron Microscopy**

All tissue processing and staining procedures have been described previously. Briefly, for qualitative observations, sections from a minimum of three control or three AC6−/− mice, assessed under control or dDAVP-treated conditions, were compared, with a minimum of five cells per animal analyzed from sections oriented approxi- mately at right angles to the apical cell membrane and showing neg- ligible background over mitochondria and nuclei.

**Statistical Analysis**

The data are expressed as mean ± SEM. An unpaired t test was per- formed, as appropriate, to analyze for statistical differences between groups, with P < 0.05 considered statistically significant.

**ACKNOWLEDGMENTS**

Antibodies for pS256-AQP2 and pS269-AQP2 were kindly provided by Jason Hoffert and Mark Knepper (National Institutes of Health). We thank Tracy Guo for technical assistance. This work was sup- ported by a Grant-in-Aid from the American Heart Association Western Affiliate (08514F), National Institutes of Health Grants GM66232, DK56248, DK28602, P30DK079337, SPO1HLO66941, HL083741, HL088426, HL94728, and K99HL091061, Merit Review Awards from the Department of Veterans Affairs, and an ASN Carl W. Gottschalk Research Grant (to T.R.). Some of these data have been presented in abstract form at the Annual Meeting of the American Society of Nephrology, San Diego, CA, October 27 through November 1, 2009 and Experimental Biology, New Orleans, LA, April 18 through 22, 2009.

**DISCLOSURES**

None.

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Supplemental information for this article is available online at http://www.jasn.org/.
Fig. S1. mRNA expression of AC isoforms in renal inner medulla of WT and AC6−/− in response to access to water and water deprivation. (A) Free water access was associated with significantly higher expression of AC7 in AC6−/− than wild-type mice (WT). (B) In response to water deprivation, expression of AC1 was reduced and expression of AC4 was increased in AC6−/− compared to WT. n=4/group; *P<0.05 vs. WT. ND, not detectable.
Data supplement 2

Fig. S2. Adenylyl cyclase 8 (AC8) and 18S mRNA expression in renal inner medulla (IM) and brain (B) of wild-type mice by real-time PCR detection. Compared to whole mouse brain, where AC8 mRNA is well expressed (~23 cycles), specific expression is not detectable in mouse renal inner medulla (>35 cycles). 8 ng cDNA per reaction. 179 base pairs for AC8 and 150 base pairs for 18S cDNA.
Data supplement 3

(A) Basal expression levels in WT mice with free water access. Results are shown relative to PDE1A, which had the highest expression level.

(B) Under basal conditions with free water access, no significant differences were observed in AC6-/- vs. WT mice. (C) In response to water deprivation, the members of none of these PDE isoform groups were consistently down-regulated in AC6-/- mice. 

Fig S2. Inner medullary mRNA expression of phosphodiesterase isoforms (PDE) in wild-type (WT) and adenylyl cyclase 6 knockout mice (AC6-/-). (A) Basal expression levels in WT mice with free water access. Results are shown relative to PDE1A, which had the highest expression level. (B) Under basal conditions with free water access, no significant differences were observed in AC6-/- vs. WT mice. (C) In response to water deprivation, the members of none of these PDE isoform groups were consistently down-regulated in AC6-/- mice. 

n=5/group. Top of each graph indicates the substrate specificity (cAMP and/or cGMP) of each PDE isoform. ND, not detectable.
Fig. S3. Immunohistochemistry of AQP2 in AC6<sup>−/−</sup> mice. In mice subjected to acute water loading followed by dDAVP, total AQP2 is apparent throughout the collecting duct system (arrows). In contrast, pS269-AQP2 is detected in outer medullary collecting ducts (arrows), but not in IMCD. The dashed line represents the border between inner stripe outer medulla and inner medulla.