Adenylyl Cyclase 6 Enhances NKCC2 Expression and Mediates Vasopressin-Induced Phosphorylation of NKCC2 and NCC

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Arginine vasopressin (AVP) affects kidney function via vasopressin V₂ receptors that are linked to activation of adenylyl cyclase (AC) and an increase in cyclic adenosine monophosphate formation. AVP/cyclic adenosine monophosphate enhance the phosphorylation of the Na-K-2Cl cotransporter (NKCC2) at serine residue 126 (pS126 NKCC2) and of the Na-Cl cotransporter (NCC) at threonine 58 (pT58 NCC). The isoform(s) of AC involved in these responses, however, were unknown. Phosphorylation of S126 NKCC2 and T58 NCC, induced by the V₂ receptor agonist (1-desamino-8-D-arginine vasopressin) in wild-type mice, is lacking in knockout mice for AC isoform 6 (AC6). With regard to NKCC2 phosphorylation, the stimulatory effect of 1-desamino-8-D-AVP and the defect in AC6/C0/C0 mice seem to be restricted to the medullary portion of the thick ascending limb. AC6 is also a stimulator of total renal NKCC2 protein abundance in medullary and cortical thick ascending limb. Consequently, mice lacking AC6 have lower NKCC2 expression and a mild Bartter syndrome-like phenotype, including lower plasma concentrations of K⁺ and H⁺ and compensatory upregulation of NCC. Increased AC6-independent phosphorylation of NKCC2 at S126 might help to stabilize NKCC2 activity in the absence of AC6. Renal AC6 determines total NKCC2 expression and mediates vasopressin-induced NKCC2/NCC phosphorylation. These regulatory mechanisms, which are defective in AC knockout mice, are likely responsible for the observed mild Bartter syndrome. (Am J Pathol 2013, 182: 96e106; http://dx.doi.org/10.1016/j.ajpath.2012.09.014)
The Na-K-2Cl co-transporter 2 (NKCC2) is expressed in the medullary thick ascending limb (mTAL) and the cortical thick ascending limb (cTAL) of the loop of Henle, whereas the Na-Cl co-transporter (NCC) is localized to the distal convoluted tubule (DCT). Inactivating mutations in both NKCC2 and NCC are of clinical relevance and cause Bartter and Gitelman syndromes,6 respectively. Both segments express the V2R as shown by immunohistochemistry, mRNA expression, and protein abundance.7–11 Moreover, AVP has been shown to stimulate NKCC2 and NCC activity,12–14 including micropuncture studies in rats in which the V2R agonist 1-desamino-8-D-arginine vasopressin (dDAVP) stimulated NaCl transport in both thick ascending limb (TAL)15 and DCT.16

Gimenez and Forbush17 demonstrated that short-term activation of NKCC2 by dDAVP involves NKCC2 phosphorylation in the mouse kidney. The use of an antibody (R5) that targets double phosphorylated NKCC2 at threonine residues 96 and 101 (T96 and T101), indicated a 55% increase of NKCC2 membrane abundance on dDAVP stimulation. More recently, Gunaratne et al18 demonstrated that T96, but not T101, of NKCC2 is phosphorylated by dDAVP stimulation. Extensive phosphoproteomic profiling identified two additional serine phosphorylation sites of rat NKCC2, S126, and S874, both showing dDAVP-dependent phosphorylation in a protein kinase A-dependent manner.18

Multiple phosphorylation sites have been identified in NCC including residues T53, T58, and S71 (rodent nomenclature), and all three phosphorylation sites were shown to be phosphorylated by dDAVP stimulation in rats.19,20 Based on our prior studies, as previously discussed,4,5 in addition to in situ hybridization studies on the relative expression of AC6 mRNA in the kidney,21,22 we tested the hypothesis that AC6 contributes to NKCC2 and NCC expression and phosphorylation under both basal conditions and after V2R stimulation. We examined mice that lack AC6 (AC6−/−) to gain further insights. The results indicate an important contribution of AC6 for basal NKCC2 and NCC expression, as well as dDAVP-induced NKCC2 and NCC phosphorylation and expression. Moreover, mice lacking AC6 show features of Bartter syndrome.

Materials and Methods

Animals

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, 3-month-old to 6-month-old male and female AC6−/− and wild-type (WT) littermates were generated, as described,23 using heterozygous breeding. Their genetic background is a mix of 129Sv/J and C57BL/6, both sexes were used for experiments.

Mice with free access to food and water were euthanized and kidneys removed. Kidneys were prepared for Western blotting and immunohistochemistry (see as follows for details).

Role of AC6 for dDAVP-Stimulated NKCC2 and NCC Expression and Phosphorylation

To study acute effects of dDAVP on NKCC2 and NCC expression and phosphorylation, short-term water loading (3% of body weight by oral gavage) was performed followed by application of vehicle (sterile water, 2 μL/g body weight i.p.) or dDAVP (0.1 μg/kg i.p., Sigma-Aldrich, St. Louis, MO). This dose of dDAVP prevents water-load induced diuresis in WT mice24 and induces a comparable urine osmolality observed after 18-hour water restriction.4 One hour after injection, mice were euthanized and kidneys were prepared for immunohistochemistry and confocal laser scanning microscopy, described as follows.

To more strongly suppress endogenous vasopressin levels, mice were provided with 5% dextrose/1% ethanol solution overnight.4,17 The next day, mice were injected with vehicle (sterile water, 2 μL/g body weight i.p.) or dDAVP (0.1 μg/kg i.p., Sigma-Aldrich) and euthanized 20 minutes after injection. Kidneys were prepared for Western blotting, described as follows.

Western Blot Analyses

Renal tissue was homogenized in buffer containing protease inhibitor cocktail (250 mmol/L sucrose, 10 mmol/L triethanolamine, Sigma-Aldrich; Roche Applied Science, Indianapolis, IN, respectively) and Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). The homogenate was centrifuged at 4000 g for 15 minutes. Pellets were resuspended and used for Western blotting. Equal lane loading (total NKCC: 30 μg, pS126 NKCC2: 30 μg, total NCC: 20 μg, and pT58 NCC: 20 μg) was achieved using a Bio-Rad DC Protein assay (Bio-Rad Laboratories, Richmond, CA). Samples were resolved on 4% to 12% NuPAGE gels in MOPS buffer (Invitrogen, Carlsbad, CA). Gel proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies against NKCC2 (dilution 1:20,000, generous gift from Jan Loffing, University of Zurich),25 pS126 NKCC2: 1:1000) (generous gift from Chung-Lin Chou and Mark Knepper, NIH),16 NCC (1:2000, Millipore, Billerica, MA),26 and pT58-NCC (1:200, provided by Shinichi Uchida).27,28 Chemiluminescent detection was performed with ECL (Amersham, Piscataway, NJ). Densitometric analysis was performed by using ImageJ Software v1.46 (NIH, Bethesda, MD). β-actin was used as a loading control (dilution 1:5000, Sigma-Aldrich).
Immunofluorescent Labeling of Kidney Sections, Confocal Laser Scanning Microscopy, and Image Quantification

Tissue preparation, sectioning, and labeling were performed as previously described.\textsuperscript{29} Primary antibodies used were against NCC (1:4000), pT58 NCC (1:70,000), total NKCC2 (1:100), and pS126 NKCC2 (1:2000). Goat anti-rabbit Alexa 488 conjugated secondary antibodies (Invitrogen) were used for visualization of labeling. Sections were mounted using glycerol-based mounting medium containing anti-fade reagent (Dako, Carpinteria, CA). A Leica TCS SL confocal microscope with an HCX PL APO 63× oil objective lens (numerical aperture: 1.40) was used for imaging of labeled sections (Leica Microsystems, Ballerup, Denmark). For the semiquantitative imaging, six individual images from a labeled section per experimental mouse were acquired (30 images per genotype/experimental condition). Images were obtained using identical microscope settings (PMT offset and gain, sampling period, and averaging) for each tissue section labeled with a specific antibody. The dynamic range of the image acquisition was set so that the sections with the most intense fluorescence signal only had a few saturated pixels. The digital images were analyzed using ImageJ v1.46 software (NIH). Briefly, threshold intensity was set per image to highlight the specific labeling of an individual tubule with negligible background. This image was converted to binary and background noise (speckles) removed. The integrated labeling density of this image was obtained, providing a measurement of the total image area with specific labeling signal. As the length of labeled tubule varies between images, the binary image was also used to mask and then quantify the specific labeling pixel intensity from the corresponding original raw image. Using these values, the total integrated pixel intensity of each image in relation to the total area of labeled DCT or TAL was calculated, providing semiquantitative data of the labeling intensity, and thus abundance of protein, per tubule. This technique has been previously used with alternative antibodies and control studies performed by using serial sections and serial dilution of the primary antibody.\textsuperscript{30} The apical-to-basolateral pixel distribution was assessed similarly. Multiple regions of interest (five per labeled tubule, five tubules per animal), representing 4-μm single cross-sections through labeled DCT and mTAL cells, were selected, and distribution of pixel intensities from the apical cell pole through to the basolateral side was assessed using ImageJ v1.46 software (NIH). Using the plot profile function, the total pixel intensities in the initial 0.5 μm (representing the most apical NKCC2/NCC labeling) were analyzed by calculating the area under the curve using GraphPad Prism v5 (La Jolla, CA).

Immunohistochemistry

All procedures have been previously described in detail.\textsuperscript{29} Labeling was visualized by use of a peroxidase-conjugated secondary antibody for light microscopy (P448, Dako, Glostrup, Denmark) and visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (Kemen Tek, Copenhagen, Denmark). Light microscopy was performed with a Leica DMRE (Leica Microsystems). All microscope and camera settings were identical when obtaining images from different experimental conditions.

Statistical Analysis

The data are expressed as means ± SEM and analyzed using SigmaPlot v11.0 (San Jose, CA) software. Unpaired Student’s t-test and the analysis of variance, followed by the Bonferroni post test were performed, as appropriate, to analyze statistical differences between the groups with \( P < 0.05 \) considered to be statistically significant.

Results

NKCC2 Expression Is Decreased in AC6\(^{-/-}\) Mice

Under basal conditions, Western blot analysis showed that total renal NKCC2 expression was \( \sim 50\% \) lower in AC6\(^{-/-}\) versus WT mice (Figure 1A). This was associated with a \( \sim 1.8\)-fold greater expression of pS126 NKCC2 in AC6\(^{-/-}\) compared to WT mice (Figure 1B). Qualitative immunohistochemistry after short-term water loading (control for dDAVP), to suppress endogenous AVP levels, did not change the cellular localization of NKCC2 (Figure 2). By using immunofluorescence labeling and confocal analysis with identical conditions, however, we confirmed a reduced total of NKCC2 staining in kidneys of AC6\(^{-/-}\) mice compared to WT in both mTAL and cTAL (Figure 3). The abundance of NKCC2 phosphorylated at S126 was low in mTAL of WT mice and not detectable in AC6\(^{-/-}\) mice. In comparison, no apparent differences were observed in labeling intensity of the cTAL between genotypes, which had overall greater labeling intensity than the mTAL (Figure 4). Quantification of pS126 NKCC2 confirmed significantly lower abundance in mTAL of AC6\(^{-/-}\) versus WT mice (Figure 5, A and B); again, no differences were observed in labeling intensity of cTAL between genotypes (Figure 5, A and C). The short-term water loading may have reduced pS126 NKCC2 to 

\[ 0.05 \text{ mmol/L, } n = 9-10/\text{genotype}; \ P < 0.05 \] and a higher plasma pH (AC6\(^{-/-}\): 7.443 ± 0.004; WT: 7.419 ± 0.009, \( n = 9-10/\text{genotypes}; \ P < 0.05 \) in AC6\(^{-/-}\) mice, both pathognomonic findings in Bartter syndrome, consistent with the lower total NKCC2 expression and membrane labeling on the whole kidney level.
dDAVP-Induced mTAL Phosphorylation of S126 NKCC2 Is Absent in AC6−/− Mice

Quantitative laser-scanning confocal microscopy indicated that dDAVP treatment after short-term water loading did not change the labeling intensity for total NKCC2 in mTAL and cTAL of either group (Figure 3). In comparison, labeling of pS126 NKCC2 significantly increased in the mTAL of WT mice in response to dDAVP treatment (Figure 5). This response was absent in AC6−/− mice. dDAVP did not affect labeling intensity of pS126 NKCC2 in cTAL of WT or AC6−/− mice (Figure 5). The signal intensity of total NKCC2 labeling in the mTAL was greater in the first 500 nm of the apical plasma membrane domain in dDAVP-treated WT compared to AC6−/− mice (Figure 6).

Figure 1 Total Na-K-2Cl cotransporter 2 (NKCC2) expression is decreased in mice lacking AC6, and water loading (WL) overnight unmasks dDAVP effects on renal membrane expression of total NKCC2 and NKCC2 phosphorylated at serine 126 (pS126 NKCC2). A: Renal protein expression of NKCC2 was significantly lower in AC6−/− compared to wild-type (WT) mice under basal conditions. This was associated with enhanced phosphorylated serine 126 NKCC2 (pS126 NKCC2) expression in AC6−/− versus WT (n = 3/group). B: Compared with regular fluid intake, WL suppressed total NKCC2 expression, and the expression remained higher in AC6−/− versus WT mice. Treatment with dDAVP increased total renal expression of NKCC2 to comparable levels in both genotypes. The dDAVP-induced increase in renal pS126 NKCC2 observed in WT, however, was lacking in AC6−/− mice (n = 2 and n = 4/group) for WL and dDAVP treatments, respectively. *P < 0.05 versus WT, †P < 0.05 versus control same genotype.

Figure 2 Reduced total Na-K-2Cl cotransporter 2 (NKCC2) labeling in medullary mTAL and cortical thick ascending limb cTAL of AC6−/− knockout mice. Diaminobenzidine stained panels show representative images of kidney sections from wild-type (WT) and AC6−/− under control (acute water loading) or dDAVP-treated conditions. Original magnification: ×4 (insets).

Overnight Suppression of Endogenous Vasopressin Reveals a Role of AC6 for the dDAVP-Induced Increase in pS126 NKCC2 and Total NKCC2

In another set of mice, we performed Western blot analyses to assess the expression and phosphorylation of total NKCC2 and pS126 NKCC2 in response to dDAVP after water loading overnight. Compared with regular fluid intake (Figure 1A), water loading overnight (control) appeared to suppress total NKCC2 expression, and the expression remained higher in AC6−/− versus WT mice (Figure 1B). Treatment with dDAVP increased total renal expression of NKCC2 to comparable levels in both genotypes (Figure 1B). Of note, dDAVP treatment increased total renal NKCC2 expression in WT by 13 ± 3-fold, but only by 1.9 ± 0.3-fold in AC6−/− mice. The abundance of pS126 NKCC2 was not different under control conditions between genotypes. The dDAVP-induced increase, however, in renal pS126 NKCC2 observed in WT was lacking in AC6−/− mice (Figure 1B).

NCC Expression, But Not T58 Phosphorylation, Is Increased in AC6−/− Mice

Western blot analyses under basal conditions revealed that NCC was readily detectable as monomers, as well as polymers (Figure 7A). The latter were proposed to be of functional importance.31 Bartter syndrome is typically associated with an increased NCC expression.25,32 This is possibly a consequence of a reduced NKCC2 expression/activity and a greater delivery of NaCl from the TAL or secondary to renal NaCl loss. We found that total renal NCC expression was ~1.9-fold greater in AC6−/− versus WT mice; however, expression of...
pT58 NCC was not different between genotypes (Figure 7A). Qualitative immunohistochemistry after short-term water loading (control for dDAVP), did not change the cellular localization of NCC (Figure 8). By using immunofluorescence labeling and confocal analysis under identical conditions, however, we confirmed a more intense staining of total NCC in AC6/−/− versus WT mice in the DCT (Figure 9A and B), and that labeling of pT58 NCC was comparable between genotypes (Figure 9A and C).

**dDAVP-Induced Phosphorylation of T58 NCC Is Absent in AC6/−/− Mice**

Qualitative immunohistochemistry and quantitative laser-scanning confocal microscopy indicated that dDAVP treatment after short-term water loading did not change the labeling intensity for total NCC in DCT of either group (Figure 8 and Figure 9, A and B). Labeling of pT58 NCC significantly increased in response to dDAVP in WT, but not in AC6/−/− mice (Figure 8 and Figure 9, A and C). Total NCC labeling after dDAVP treatment in the DCT showed no difference in signal intensity and distribution in the region of the apical plasma membrane between genotypes (Figure 10).

**Overnight Suppression of Endogenous Vasopressin Reveals a Role of AC6 for the dDAVP-Induced Increase in pT58 NCC and Total NCC**

As observed under basal conditions and after short-term water loading (see previously), total NCC expression was significantly higher in AC6/−/− versus WT after water-loading overnight (control); dDAVP treatment significantly increased total renal NCC expression in WT with these conditions (Figure 7B). This effect was reversed in AC6/−/−, with dDAVP decreasing total renal NCC expression (Figure 7B). After water-loading overnight, renal pT58 NCC expression was higher in AC6/−/− versus WT; however, as observed after short-term water loading, treatment with dDAVP increased renal pT58 NCC expression only in WT with these conditions (Figure 7B).
Discussion

For the first time, the present studies show that AC6 is a determinant of basal expression of NKCC2 and NCC, as well as dDAVP-stimulated phosphorylation of S126 NKCC2 and T58 NCC in the mouse kidney. To our knowledge, this is the first time showing the functionality of both phospho-specific antibodies in the mouse. With regard to NKCC2 phosphorylation, the stimulatory effect of dDAVP and the role of AC6 seem to be restricted to the medullary segment of the TAL. At the latter site, dDAVP-induced phosphorylation of NKCC2 in WT mice was

Figure 5  Quantitative laser-scanning confocal microscopy indicates absence of NKCC2 phosphorylation in response to dDAVP at serine 126 (pS126 NKCC2) in medullary thick ascending limb (mTAL) of AC6−/− mice (A). Panels show representative images of kidney sections from wild-type (WT) and AC6−/− mice under control (acute water loading) or dDAVP-treated conditions (Images have been digitally altered to improve brightness/contrast for publication purposes). B: Labeling intensity of pS126 NKCC2 in mTAL is significantly lower in AC6−/− compared to WT control. dDAVP significantly increases pS126 NKCC2 in WT, but not AC6−/− mice. C: No significant differences were observed in labeling intensity of pS126 NKCC2 in cortical TAL between the groups, and dDAVP had no significant effect in either genotype. Scale-bar, mTAL, 10 μm; cTAL, 25 μm. *P < 0.05 versus WT, †P < 0.05 versus control same genotype. AU, signal intensity; cTAL, cortical thick ascending limb.

Figure 6  Greater membrane signal intensity of NKCC2 in the first 500 nm of medullary thick ascending limb (mTAL) in wild-type compared to AC6−/− mice after dDAVP treatment. A: Panels show representative images, analyzed by quantitative laser scanning microscopy of kidney sections from wild-type (WT) and AC6−/− mice after short-term water loading followed by dDAVP treatment. Colored lines represent regions of interest examined for NKCC2 labeling distribution in lower panels (Images have been digitally altered to improve brightness/contrast for publication purposes). B: Distribution of NKCC2 labeling. Signal intensity (AU) is measured from the apical pole of the cell with each trace representing an alternative cell. The mean amplitude of signal intensity is greater in the first 500 nm of WT mice, indicating greater NKCC2 labeling of the apical plasma membrane.
associated with a greater signal intensity for NKCC2 in the outermost 500 nm of the luminal membrane compared with mice lacking AC6.

We have previously shown that AC6 determines cAMP formation and aquaporin 2 phosphorylation and trafficking in the inner medullary collecting duct, and that mice lacking AC6 are more prone to develop nephrogenic diabetes insipidus. The role of AC6, however, in the expression and function of other renal transporters, including NKCC2 and NCC, has not been studied. Knockout of NKCC2 or a tubulus-specific knockdown of a kinase that regulates its function, oxidative stress-responsive kinase-1, causes Bartter syndrome. We found that the renal abundance of pS126 NKCC2 in AC6 knockout mice may protect the kidney from developing a more severe salt-losing phenotype. The increased pS126 NKCC2, however, was not seen in our immunohistochemical studies or with semiquantitative laser scanning microscopy after short-term water loading, which might have suppressed S126 NKCC2 phosphorylation to a greater extent in AC6 knockout mice. The molecular mechanism increasing pS126 NKCC2 abundance in AC6 mice under basal conditions remains unknown, but the findings indicate an AC6-independent mechanism of NKCC2 phosphorylation. Another common finding in Bartter syndrome is an increased expression of NCC, as well as hypotension. Consistent with these findings, AC6 knockout mice have higher renal NCC expression and membrane NCC labeling (current study), and a lower blood pressure. We previously found a tendency for higher urinary Na+ excretion in AC6 knockout mice, an effect that may be due to reduced transport in the TAL and might have been dampened by a lower blood pressure, and therefore reduced pressure natriuresis. Also, urinary K+ excretion was higher in AC6 knockout mice. Overall, AC6 deficiency causes only a mild salt-losing phenotype compared with the massive salt-losing nephropathy associated with PKD1.

![A](image1.png)  ![B](image2.png)

**Figure 7** Total Na-Cl cotransporter (NCC) expression is increased in mice lacking AC6, and water loading overnight (WL) unmasks dDAVP effects on renal membrane expression of NCC and NCC phosphorylated at threonine 58 (pT58 NCC). A: Renal protein expression of NCC was significantly higher in AC6 knockout compared to wild-type (WT) mice under basal conditions. Renal expression of phosphorylated threonine 58 NCC (pT58 NCC) was not significantly different between genotypes (n = 3/group). B: Total NCC expression was significantly higher in AC6 knockout versus WT after WL and dDAVP treatment significantly increased total renal NCC expression in WT under these conditions. This effect was reversed in AC6 knockout mice. After WL renal pT58 NCC expression was higher in AC6 knockout versus WT; however, treatment with dDAVP increased renal pT58 NCC expression only in WT (n = 2 and n = 4/group) for WL and dDAVP treatment, respectively. *P < 0.05 versus WT, †P < 0.05 versus control same genotype.

![Figure 8](image3.png)

**Figure 8** Absent NCC phosphorylation in response to dDAVP at threonine 58 (pT58 NCC) in the distal convoluted tubule of AC6 knockout mice. Diaminobenzidine stained panels show representative images of cortical kidney sections from wild-type (WT) and AC6 knockout mice under control (acute water loading) and dDAVP-treated conditions. Original magnification: ×4 (insets).
observed in mice with inactivation of all NKCC2 isoforms.32

Does AC6 contribute to dDAVP-stimulated NKCC2 phosphorylation and membrane expression? We observed a dDAVP-induced increase of total renal NKCC2 expression, as well as S126 NKCC2 phosphorylation in WT mice, effects that were reduced in AC6−/− mice. The observed effect of dDAVP-induced increase in total NKCC2 expression was only detected after water-loading overnight, which is expected to strongly suppress plasma AVP and maybe as a consequence, NKCC2 expression. Thus, the experimental condition might have enabled us to unmask such a response.

Figure 9  Quantitative laser-scanning confocal microscopy identifies greater total NCC labeling in AC6−/− mice, but the phosphorylation response to dDAVP at threonine 58 (pT58 NCC) is absent. A: Panels show representative images of cortical kidney sections from wild-type (WT) and AC6−/− mice under control (acute water loading), or dDAVP treated conditions. B: Total NCC labeling intensity is significantly greater in AC6−/− compared to WT control, but no effects of dDAVP were observed in either group. C: Basal labeling intensity of pT58 NCC is not significantly different between the groups. dDAVP significantly increases pT58 NCC labeling in WT, but not AC6−/− mice. Scale-bar, 25 μm. *P < 0.05 versus WT, †P < 0.05 versus control same genotype. AU, signal intensity.

Figure 10  Comparable membrane distribution of total NCC in the distal convoluted tubule between wild-type (WT) and AC6−/− mice after dDAVP treatment. A: Panels show representative images, analyzed by quantitative laser scanning microscopy, of kidney sections from WT and AC6−/− mice after short-term water loading followed by dDAVP treatment. Colored lines represent regions of interest examined for NCC labeling distribution in lower panels. B: Distribution of NCC labeling. Signal intensity (AU) is measured from the apical pole of the cell with each trace representing an alternative cell. The mean amplitude of signal intensity is not different between genotypes, indicating a comparable amount of NCC at the apical plasma membrane.
Immunohistochemistry and immunofluorescence confirmed the defect in dDAVP-induced phosphorylation of S126 NKCC2 in AC6−/− mice; of note, this effect of dDAVP in WT and the defect in AC6−/− mice were only present in the mTAL and not in the cTAL. The role of vasopressin in NKCC2 phosphorylation and translocation was shown by Gimenez and Forbush17 and confirmed by others.36–38 There is now an accumulating body of evidence that NKCC2 activation by the V2R involves cAMP-stimulation of protein kinase A.38 Our data expand this knowledge and add AC6 in the mTAL as a key enzyme in this signaling pathway. Gimenez and Forbush,17 by using electron microscopy, showed that 5% to 6% of total NKCC2 immunolabeling is found within 70 nm from the apical membrane, and 45% of total NKCC2 is located within the first 140 nm. In the mTAL, we observed a shift of NKCC2 toward the tubule lumen in dDAVP-stimulated WT compared with AC6−/− mice. This might reflect an important role of AC6 in the regulation and phosphorylation of transport proteins in cell membrane microdomains (eg, lipid rafts/caveolae) in a manner akin to what has been noted for aquaporin-2,4 and/or is related to proposed actions of snapin,39 which controls protein kinase A-stimulated, and soluble N-ethylmaleimide-sensitive factor attachment protein receptor-mediated NKCC2 exocytosis.40

The role of AC6 in dDAVP-induced regulation of total renal NKCC2 expression and phosphorylation at S126 appears to be restricted to the medullary portion of the TAL. Of note, we previously found a similar pattern for dDAVP-induced phosphorylation and regulation of aquaporin-2 along the collecting duct.4 In the medulla, AC6 is required for full phosphorylation of aquaporin-2 at S256 and S269. In contrast, phosphorylation of aquaporin-2 was unaffected in the renal cortex of AC6−/− mice.4 These findings are consistent with the concept that specific AC isoforms mediate nephron segment-specific hormonal effects. AC6 is also involved in the signaling cascade of AVP-induced phosphorylation of NCC. Previous studies indicated that the expression of NCC phosphorylated at residue T58 is restricted to the apical plasma membrane; electron microscopy studies showed weak labeling of pT58 NCC in AVP-deficient Brattleboro rats and dDAVP treatment increased the abundance of pT58 NCC only in the apical membrane.19 Our studies indicate that AC6 is not critical for the basal phosphorylation of NCC at T58 or NCC membrane expression. In fact, we find a higher, possible compensatory increase in total NCC and, in addition, in pT58 NCC after water-loading overnight. The dDAVP-induced increase in renal pT58 NCC abundance and membrane staining in the DCT, however, was completely absent in AC6−/− mice, and thus required AC6. The signaling pathway involved in vasopressin-induced NCC phosphorylation is not known, but in addition to AC6, might involve the “with-no-[Lys] kinases”/STE20/SPS1-related proline/alanine-rich kinase/oxidative stress-responsive kinase-1 pathway.19 The dDAVP-induced increase in total renal NCC expression, which was only observed in WT mice after water-loading overnight, was also dependent on AC6. A previous study reported an increase in renal NCC expression after chronic dDAVP administration.14

In contrast to a proposed effect on the trafficking of NKCC2 and aquaporin 2, the effect of dDAVP-induced phosphorylation on NCC trafficking remains unclear; it may rather be required for full intrinsic NCC activity. Western blotting and immunogold labeling indicated unchanged membrane NCC expression and no trafficking in response to dDAVP administration when stimulated for 60 minutes.20 Another study found that a 30-minute dDAVP administration increased plasma membrane NCC, as determined by confocal microscopy, immunogold labeling, and Western blotting, possibly indicating increased apical trafficking of the transporter,20 although an increased membrane retention may also have contributed. Both studies used Brattleboro rats, and the reason for these different findings remained elusive but might relate to differences in the time course. We could not detect an increase in membrane signaling intensity of total NCC in response to dDAVP after short-term water loading in WT mice when analyzed by immunohistochemistry or confocal immunofluorescence microscopy. Further studies are needed to better understand the regulation and trafficking of NCC.

In summary, the current results demonstrate that AC6 mediates vasopressin-induced phosphorylation of NKCC2 at S126 and of NCC at T58 in the kidney. AC6 is also a stimulator of renal NKCC2 protein abundance in mTAL and cTAL. As a consequence, mice lacking AC6 have lower NKCC2 expression and present with a mild Bartter syndrome-like phenotype, including lower plasma concentrations of K+ and H+ and upregulation of downstream NCC expression. In addition to the latter, increased AC6-independent phosphorylation of NKCC2 at S126 might help to compensate in the absence of AC6. Vasopressin-induced NKCC2 phosphorylation at S126 and the role of AC6 herein were restricted to the medullary portion of the TAL. The segment-specific use of different AC isoforms might increase the regulatory versatility of hormone-induced signaling along the nephron.

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References