Supporting Information

Gunaratne et al. 10.1073/pnas.1007424107

SI Materials and Methods

Animals. All experiments were conducted in accord with animal protocol H-0110R1 approved by the Animal Care and Use Committee of the National Heart, Lung and Blood Institute. For isolated mTAL experiments, pathogen-free male Sprague-Dawley (Taconic Farms) weighing 120–150 g were used. For experiments involving short-term in vivo dDAVP infusion, pathogen-free male homozygous Brattleboro rats (Harland Sprague-Dawley) weighing 120–150 g were used.

mTAL Suspension Preparation. A method for isolating viable rat medullary thick ascending limb (mTAL) cells was adapted from the previously described protocol of Eveloff et al. (1). Sprague-Dawley rats were injected with furosemide (2.5 mg i.p.) and heparin (250 USP units) for 15 min before euthanization by decapitation. Retrograde perfusion of the kidneys was performed via the abdominal aorta with 10 mL of cold Hank's balanced buffer salt solution (20 mM Hepes, 135 mM NaCl, 0.43 mM NaH_2PO_4, 0.21 mM Na_2HPO_4, 5 mM KCl, 1.0 mM $CaCl_2$, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 5.55 mM glucose, 10 μM furosemide, 300 mOsm, pH 7.4, bubbled with 100% O_2 for 30 min before use), followed by 10 mL of prewarmed digestion enzyme solution containing 230 U/mL collagenase B (Roche Diagnostics) and 0.5 mg/mL hyaluronidase (Worthington Biochemical) dissolved in Hanks' solution. After removal of the kidneys, the outer medulla (inner stripe) was excised, minced into ≈1-mm cubes, and digested for 30 min in 10 mL of the digestion enzyme solution at 37 °C in an Erlenmeyer flask, with gentle continuous stirring and 100% O_2 applied to the solution surface. Samples were aspirated every 10 min using a large-bore glass Monstr-pette (Chase Scientific Glass). After digestion, the supernatant was collected and subjected to a low-speed centrifuge spin ($60 \times 70 \times g$, 30 s) to gently pellet the heavier mTAL tubules from the non-mTAL components (proximal tubules, outer medullary collecting duct tubules, thin descending limb tubules, and vasa recta). The resulting pellet was washed three times with cold Hanks' buffer, resuspended and aliquoted for hormone treatment.

Assessment of mTAL Suspension Purity. The purity of the isolated mTAL suspensions was evaluated by immunoblotting to compare the relative enrichment of NKCC2-characterized mTAL tubule structures, AQP1-characterized proximal tubule structures, and AQP2-characterized collecting duct structures in the isolated mTAL fraction vs. the total outer medulla and non-mTAL fractions.

mTAL Hormone Treatment. Isolated mTAL tubule suspensions were treated for 15 min with the V2-receptor selective vasopressin analog dDAVP (1 nM; Bachem, #H7675), glucagon (100 nM; Sigma-Aldrich), parathyroid hormone (10 nM; Sigma-Aldrich), and calcitonin (1 μM; Sigma-Aldrich) either individually (for cAMP assay) or in combination with one another [for cAMP assay and mass spectrometry (MS) profiling]. Hormone concentrations were culled from previous TAL studies (2–4). Before treatment with the respective hormones, mTAL suspensions were preincubated for 15 min with the phosphodiesterase inhibitor IBMX (0.5 mM; Sigma-Aldrich). For later experiments that aimed to examine the effect of dDAVP alone, mTAL suspensions were not preincubated with IBMX before hormone treatment. In all cases, the mTAL tubule suspensions were treated at 37 °C in a closed chamber under the application of 100% O_2. Following treatment, samples were pelleted by brief centrifugation and lysed in the appropriate buffer depending on the particular method of analysis (either mass spectrometry or immunoblotting).

cAMP Assay. A cAMP enzyme immunoassay (GE Healthcare) was used to quantify the expected increases in intracellular cAMP levels after mTAL tubule suspensions were subjected to treatment with dDAVP, glucagon, parathyroid hormone, and calcitonin as described in *Results*.

In-Solution Protease Digestion and Phosphopeptide Enrichment. In preparation for phosphoproteomic analysis by mass spectrometry, hormone-treated mTAL tubule suspensions were pelleted by brief centrifugation and resuspended in lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris·HCl) containing a protease/phosphatase inhibitor mixture (Thermo Scientific). The cells were sonicated with 0.5-s pulses for 30 cycles. Protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce), and ≈500 μg of protein/sample was used for quantitative phosphoproteomic profiling. Sample reduction was performed with 10 mM DTT for 1 h, followed by alkylation with 40 mM iodoacetamide for 1 h under dark conditions. Samples were diluted with 25 mM ammonium bicarbonate such that the final urea concentration was less than 1 M before protein digestion with trypsin GOLD (Promega) at a wt/wt ratio of 1:20 for 16 h at 37 °C. Following protease digestion, peptide samples were desalted with 1-cc HLB cartridges (Waters). Phosphopeptide enrichment was performed using immobilized metal affinity chromatography with a Ga^{3+} matrix (Phosphopeptide Isolation Kit; Pierce). The samples were then desalted with C18 ZipTips (Millipore) and resuspended in 0.1% formic acid.

LC-MS/MS Analysis on LTQ-Orbitrap. All samples were analyzed on an LTQ-Orbitrap (Thermo-Fisher Scientific) interfaced with a nano-LC 1D plus system (Eksigent Technologies). Fragmentation was conducted via collision-induced dissociation. Briefly, samples were loaded onto an Agilent Zorbax 300SB-C18 trap column (0.3 mm i.d. × 5 mm length, 5-μm particle size) at a flow rate of 10 μl/min for 15 min. Reversed-phase C_{18} chromatographic separation of trapped peptides was carried out on a prepacked BetaBasic C_{18} PicoFrit column (75 μm i.d. × 10 cm length; New Objective) at 300 nL/min using the following gradient: 2–5% solvent B for 2 min; 5–45% solvent B for 45 min; 45–50% solvent B for 5 min; 50–95% solvent B for 5 min (solvent A: 0.1% formic acid in 98% water, 2% acetonitrile; solvent B: 0.1% formic acid in 100% acetonitrile). Eluted peptides were sprayed into the LTQ-Orbitrap mass spectrometer equipped with a nano-spray ion source. LTQ-Orbitrap settings were as follows: spray voltage—1.5 kV; temperature of ion transfer tube—180 °C; and full MS mass range—300–2000 m/z. The LTQ-Orbitrap was operated in a data-dependent mode such that a single survey MS^1 scan for precursor ions was followed by three data-dependent MS^2 scans for precursor ions above a threshold ion count of 2,000 with collision energy of 35%. An MS^3 scan was triggered if a specified neutral loss fragment (−32.67, −49, or −98 from the precursor ion) was detected in an MS^2 scan. Survey MS scans were acquired in the Orbitrap component with a resolution of 30,000, and MS^2 and MS^3 scanning were performed in the linear ion trap.

Data Repository. Mass spectrometric raw data have been deposited in the Tranche repository to facilitate data sharing and validation and can be downloaded at http://www.proteomecommons.org/ (hash: F+7Jv1/Py0TMAp5w+spCDGMMJ06Ki965Ad1rsc0DV

k457/LxujZziDhPKcYKUst960wKR+Jlsfv55OAG0caHw0l8d8oAAAAAAAAHsQ==).

Data Searching, Scoring, and Quantification. MS spectra were searched using three different search algorithms: InsPecT (5), SEQUEST (6), and OMSSA (7). For MS^2 spectra, the fixed modification was carbamidomethylation of cystein, whereas the variable modifications were phosphorylation of Ser, Thr, and Tyr and oxidation of methionine with a maximum of four modifications. For MS^3 spectra, the variable modification of neutral loss of water (−18 Da for Ser and Thr) was added. A total of three missed cleavages were allowed per peptide. Searches were conducted against the most recent *Rattus norvegicus* RefSeq Database [National Center for Biotechnology Information (NCBI)] using the target-decoy approach described in previous work (8). Both OMSSA and InsPecT searches were performed on the National Institutes of Health Biowulf cluster (http://biowulf.nih.gov). All MS datasets were filtered for a <2% false discovery rate. Quantification of phosphopeptide abundance (area under the curve of the reconstructed ion chromatogram elution profile) was achieved using QUOIL, an in-house software program designed for quantification of label-free peptides by LC-MS (9, 10). Phosphorylation site assignment was carried out using the following scoring algorithms: Ascore (11) and PhosphoScore (12) for SEQUEST data and Phosphate Localization Score for InsPecT data (13). To assign a phosphorylation site correctly, at least one score had to pass the given threshold (Ascore ≥ 19, PhosphoScore was "passed" or "OneChoice"; PLS score ≥8). Final assignment of phosphorylation sites was performed using the in-house programs, National Heart, Lung and Blood Institute Promatch and Phosphosite (8).

Sequence Logo Analysis. The open access web tool enoLOGOS (14) was used to generate weighted sequence logos from the aligned sequences of the up- and down-regulated phosphopeptides. Specific parameters used were as follows: logo plot method—relative entropy; scale letters by prob—"on"; and log base—"2." Values for individual amino acid frequencies in the rat proteome were calculated using the most recent *R. norvegicus* RefSeq Database (NCBI) and entered manually.

Phospho-Specific NKCC2 Antibodies. Rabbit polyclonal phospho-specific NKCC2 antibodies recognizing Ser(p)-126 and Ser(p)-874 were generated against synthetic phosphopeptides and affinity purified (PhosphoSolutions). The phosphopeptides used for immunization and purification were 11-mer symmetrically flanking the target sites (RefSeq: NP_062007). To evaluate the specificity of the phospho-NKCC2 antibodies, dot blotting was performed against the same synthetic NKCC2 phosphopeptides for Ser(p)-126 and Ser(p)-874 used for antibody generation, along with the corresponding nonphosphorylated peptides for each NKCC2 site.

Preadsorbed R5 NKCC2 Antibodies. Aliquots of the R5 antibody (gift from B. Forbush, Yale University, New Haven, CT), which detects phosphorylation at Thr(p)-96 and/or Thr(p)-101 in rat NKCC2 (15), were preadsorbed separately with synthetic peptides (AnaSpec) singly phosphorylated at either site to enable selective detection of phosphorylation at the unblocked phosphorylation site. The sequences of these synthetic phosphopeptides correspond to amino acids 92–107 (YYLRTFGHNTMDAVPR) in rat NKCC2, with monophosphorylation at either threonine. Preadsorption was carried out at an antibody:peptide molar ratio of 1:10 at 4 °C for 24 h.

Other Antibodies. A rabbit polyclonal antibody (H7644) against total NHE3 was generated against a synthetic peptide corresponding to amino acids 621–640 of rat NHE3 (RefSeq: NP_036786) and affinity purified (Lofstrand Labs). Specificity of this antibody was confirmed by both immunoblotting and immunohistochemistry in comparison with previous NHE3 antibodies. Other antibodies used in immunoblotting experiments included the L320 rabbit polyclonal antibody against total NKCC2 (16), the K5007 rabbit polyclonal antibody against total AQP2 (17), and the LL266 rabbit polyclonal antibody against total AQP1 (18). The C20 chicken polyclonal antibody against total NKCC2 was used in immunofluorescence experiments (19). Commercially available antibodies used in immunoblotting experiments included those for total β-catenin (Cell Signaling, #2677), β-catenin-(p)Ser552 (Cell Signaling, #9566), NHE3-(p)Ser552 (Novus, NB110-81529), and acetyl-CoA-carboxylase-(p)Ser79 (Cell Signaling, #3661).

Short-Term dDAVP Treatment of Brattleboro Rats. Perfusion-fixed kidney tissue samples were prepared from vehicle- and dDAVP-treated Brattleboro rats. Briefly, Brattleboro rats were given intramuscular injections of 2 nmol dDAVP (Bachem, H7675), and vehicle-injected Brattleboro rats served as controls. After 1 h, the rats were anesthetized and surgically prepared for retrograde perfusion of the kidneys via the abdominal aorta. The kidneys were perfused first with PBS for 10 s to wash out the blood, followed by perfusion with ice-cold 4% paraformaldehyde for 5 min. The fixed kidneys were embedded in paraffin and sectioned (4 μm) for immunofluorescence studies. For immunoblot analysis, homogenates from the outer medulla were also prepared from vehicle- and dDAVP-treated Brattleboro rats, following the same treatment protocol described. These samples were homogenized in Laemmli buffer (1.5% SDS, 10 mM Tris, pH 6.8) containing a protease/phosphatase inhibitor mixture (Pierce, #78440). Following protein concentration determination by the BCA method (Pierce), samples were stored in the presence of 6% glycerol and 40 mM DTT after heating at 60 °C for 15 min. In all Brattleboro rat studies, urine osmolarity was measured before and after dDAVP/vehicle treatment using a vapor pressure osmometer (Vapro 5520; Wescor).

Immunohistochemistry. Immunostaining was performed as previously described (20). In brief, paraffin-embedded whole kidney sections were dewaxed using xylene and rehydrated subsequently in 100%, 95%, 90%, and 70% ethanol. Antigen retrieval was performed with microwave treatment for 5 min in TEG buffer (10 mM Tris and 0.5 mM EGTA, pH 9.0) followed by neutralization in 50 mM NH4Cl in PBS. Blocking was performed using 1% BSA, 0.2% gelatin, and 0.05% saponin in PBS. Incubation with the primary antibody (diluted in 0.1% BSA and 0.3% Triton X-100 in PBS) was performed overnight (4 °C). After wash with 0.1% BSA, 0.2% gelatin, and 0.05% saponin in PBS, tissue sections were incubated for 1 h with secondary antibody [conjugated with either Alexa 488 or Alexa 568 (Invitrogen)] diluted in 0.1% BSA and 0.3% Triton X-100 in PBS. The sections were then preserved in fluorescence mounting medium (S3023; Dako North America). Sections were also incubated without primary antibody as a negative control. Confocal fluorescence images were acquired using a Zeiss LSM 510 META microscope and software (Carl Zeiss MicroImaging).

Semiquantitative Immunoblotting. After solubilization in Laemmli buffer, proteins were resolved by SDS/PAGE on 4–15% gradient Criterion polyacrylamide gels (Bio-Rad) at 200 V for 1 h and transferred electrophoretically onto nitrocellulose membranes at 250 mA for 1 h. Blots were blocked for 30 min with a proprietary blocking buffer (Li-Cor, #927–40000), rinsed, and probed with the respective antibodies (in Licor blocking buffer containing 0.1% Tween-20) overnight at 4 °C. After a 1-h incubation with secondary antibody [Alexa fluor 680 goat anti-rabbit IgG (Invitrogen)] at a 1:5,000 dilution, sites of antibody–antigen reaction were detected using the Odyssey Infrared Imager (Li-Cor).

In Vitro Protein Kinase Assay. Nonphosphorylated peptides corresponding to the sequences surrounding Ser-126 and Ser-874 of rat NKCC2 were synthesized (AnaSpec). The sequence of the

synthetic peptide for Ser-126 contained amino acids 119–136 of rat NKCC2 (biotin-GPKVNRPSLQEIHEQLAK) and that for Ser-874 contained amino acids 866–890 of rat NKCC2 (biotin-TKPAPKKDSNISTIQSMHVGEFNQK). Both peptides (0.4 nmol) were incubated with purified active protein kinase-α (PRKACA) or purified active AMP-activated kinase (AMPK)-α2β1γ1 (with or without 0.1 mM AMP) at a kinase:peptide molar ratio of 1:32 in kinase reaction buffer supplemented with 200 μM ATP for 1 h at 37 °C (all components from Cell Signaling). The rat acetyl-CoA carboxylase–derived SAMS peptide (biotin-HMRSAMSGLHLVKRR; Enzo Life Sciences) was used as an established substrate for AMPK phosphorylation (21). Negative control experiments were conducted in the same manner using all reagents except the purified kinase. Following incubation, the reaction volume was divided into separate halves for independent analysis by LC-MS/MS and immunoblotting. For the immunoblotting fraction, the kinase reaction was terminated by the addition of 5× Laemmli buffer, and the sample was heated at 60 °C for 10 min. In the LC-MS/MS fraction, the kinase reaction was terminated by addition of 8 M urea to yield a concentration of 6 M. The reaction mixture was then diluted using 25 mM ammonium bicarbonate (such that the final urea concentration was <1 M) before digestion with trypsin GOLD (Promega) for 16 h at 37 °C. Samples were then desalted using HLB cartridges, resuspended in 0.1% formic acid, and analyzed on an LTQ-Orbitrap mass spectrometer.

Statistical Analysis. Data are presented as mean ± SE. All statistical comparisons were made by *t* tests. $P < 0.05$ was considered significant.

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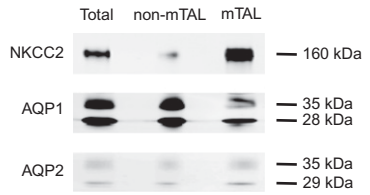

Fig. S1. Technical controls. Immunoblots showing relative abundance of NKCC2 (mTAL marker), AQP1 (descending limb marker), and AQP2 (collecting duct marker) in total outer medulla, non-mTAL, and mTAL tubule fractions.

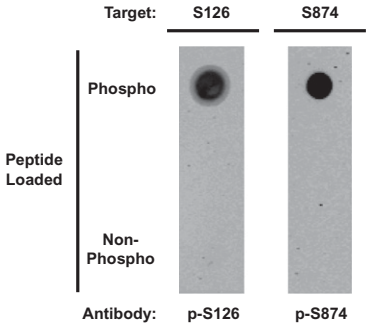

Fig. S2. NKCC2 phospho-antibodies. Dot blots illustrating specificity of the phospho-antibodies against NKCC2 phosphorylated at Ser126 (*Left*) and Ser874 (*Right*). Dot blotting was performed against the synthetic NKCC2 phosphopeptides for Ser(p)-126 and Ser(p)-874 used for antibody generation, along with the corresponding nonphosphorylated peptides for each NKCC2 site.

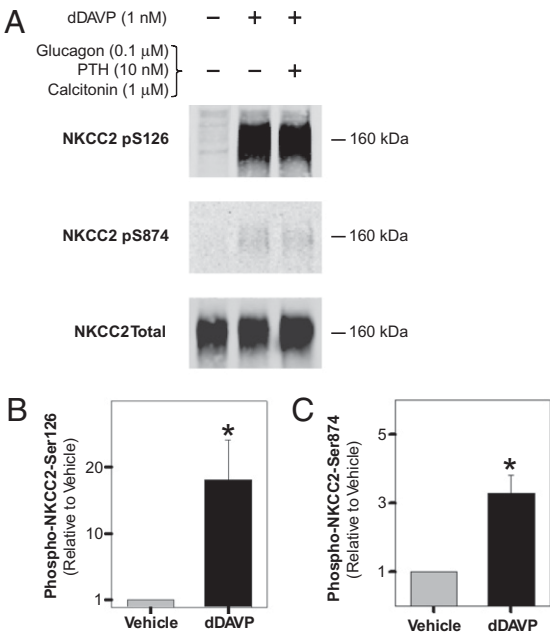

Fig. S3. Vasopressin effects on phosphorylation of NKCC2 in vitro. (*A*) Immunoblotting with phospho-specific antibodies for NKCC2 phosphorylation at Ser126 (*Top* blot) and Ser874 (*Middle* blot) in paired vehicle- and hormone (either dDAVP alone or hormone mixture)-treated mTAL tubule suspensions. Total NKCC2 protein was also probed (*Bottom* blot). (*B* and *C*) Results of densitometry analysis of immunoblots ($n = 3$) quantifying phosphorylation of NKCC2 at Ser126 (*B*) and Ser874 (*C*) in paired vehicle- and dDAVP (alone)-treated mTAL tubule suspensions. Error bars indicate SEM. Asterisks indicate statistical significance ($P < 0.05$).

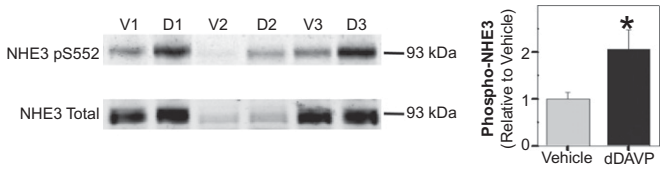

Fig. S4. Vasopressin effects on phosphorylation of NHE3 in vitro. Immunoblotting for NHE3 phosphorylation at Ser552 in paired vehicle- and dDAVP-treated mTAL tubule suspensions. Total NHE3 protein was also probed (*Bottom* blot). Bar graph shows results of densitometry analysis of immunoblots. Error bars indicate SEM (n = 3). The asterisk indicates statistical significance ($P < 0.05$). V, vehicle; D, dDAVP.

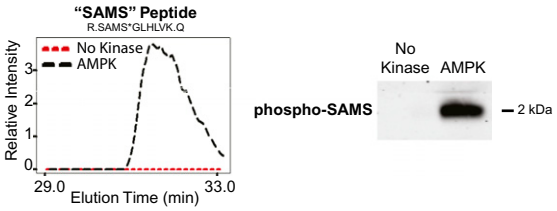

Fig. S5. AMPK phosphorylation of SAMS peptide in vitro. Representative MS[1] time-course curves (*Left*) quantifying phosphorylation of the rat acetyl-CoA carboxylase–derived SAMS peptide (HMRSAMSGLHLVKRR) after incubation with purified active AMPK-$\alpha 2\beta 1\gamma 1$. Immunoblotting confirmation (*Right*) of SAMS peptide phosphorylation using a pSer79-specific rat acetyl-CoA carboxylase antibody.

Other Supporting Information Files

Table S1 (PDF)