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The Sodium Chloride Cotransporter (NCC) and Epithelial Sodium Channel (ENaC) Associate

Abinash C. Mistry^{a,*}, Brandi M Wynne^{a,*}, Ling Yu^{b,d}, Viktor Tomilin^{h,i}, Qiang Yue^b, Yiqun Zhou^b, Otor Al-Khalili^b, Rickta Mallick^a, Hui Cai^{a,b,c}, Abdel A Alli^b, Benjamin Ko^e, Alexa Mattheyses^g, Hui-Fang Bao^b, Oleh Pochynyukⁱ, Franziska Theilig^f, Douglas C Eaton^b, and Robert S. Hoover^{a,b,c,1}

^aDivision of Nephrology, Department of Medicine, Emory University, Atlanta, GA, 30322

^bDepartment of Physiology, Emory University, Atlanta, GA, 30322 ^cResearch Service, Atlanta Veteran's Administration Medical Center, Decatur, GA, 30033 ^dThe College of Resources and Environmental Science at Nanjing Agricultural University, Nanjing, China, 210095 ^eDepartment of Medicine, University of Chicago, Chicago, IL, 60637 ^fDepartment of Medicine, University of Fribourg, 1700 Fribourg, Switzerland ^gDepartment of Cell Biology, Emory University, Atlanta, GA, 30322 ^hInstitute of Cytology, Russian Academy of Sciences, St. Petersburg, Russian Federation ⁱDepartment of Integrative Biology and Pharmacology, The University of Texas Health Science Center at Houston, TX, 77030

Abstract

The thiazide-sensitive sodium chloride cotransporter (NCC) and the Epithelial Sodium Channel (ENaC) are two of the most important determinants of salt balance and thus systemic blood pressure. Abnormalities in either result in profound changes in blood pressure. There is one segment of the nephron where these two sodium transporters are co-expressed, the second part of the Distal Convulated Tubule. This is a key part of the aldosterone-sensitive distal nephron, the final regulator of salt handling in the kidney. Aldosterone is the key hormonal regulator for both of these proteins. Despite these shared regulators and co-expression in a key nephron segment, associations between these proteins have not been investigated. After confirming apical localization of these proteins, we demonstrated the presence of functional transport proteins and native association by Blue Native PAGE. Extensive co-immunoprecipitation experiments demonstrated a consistent interaction of NCC with alpha and gamma ENaC. Mammalian two-hybrid studies demonstrated direct binding of NCC to ENaC subunits. Fluorescence Resonance

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¹To whom correspondence should be addressed: Dr. Robert S. Hoover, Division of Nephrology, Department of Medicine, Emory University, 615 Michael St, rm. 601, Atlanta, GA, 30322, Telephone: 404-727-2525; Fax: 404-727-3425. robert.hoover@emory.edu.

*These authors contributed equally to this paper.

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Author Contributions: ACM and BMW conducted much of the experimentation, analyzed the results, interpreted the data and did some of the writing. LY, VT, QY, YZ, OA, RM, AAA, AM, HFB, and FT also conducted experimentation and interpreted results. BK, HC, OP, and DCE helped in interpretation of data and writing the paper. DCE also helped conceive the paper. RSH conceived the idea for the project and wrote most of the paper.

Energy Transfer and immunogold EM studies confirmed that these transport proteins are within appropriate proximity for direct binding. Additionally, we demonstrate that there are functional consequences of this interaction, with inhibition of NCC affecting ENaC function. This novel finding of an association between ENaC and NCC could alter our understanding of salt transport in the distal tubule.

SUMMARY STATEMENT

The epithelial sodium channel (ENaC) and sodium chloride cotransporter (NCC) are key regulators of blood pressure. The association of cotransporters and channels is rare in nature. Here we demonstrate the association of these key proteins.

Keywords

sodium channel; sodium transport; transporter; protein-protein interaction; fluorescence resonance energy transfer (FRET)

INTRODUCTION

Hypertension affects approximately 1 billion individuals worldwide (1). In the United States almost 1/3 of the adult population has hypertension (2). The key role of kidney sodium reabsorption in the development of hypertension is emphasized by the fact that all presently identified forms of Mendelian hypotension or hypertension involve genetic defects in sodium reabsorption (3). The thiazide-sensitive sodium-chloride cotransporter (NCC) is the salt reabsorptive pathway localized to the apical membrane of the mammalian distal convoluted tubule (DCT). The epithelial sodium channel (ENaC) is the sodium reabsorptive pathway localized to the second part of the DCT (DCT2), Connecting Tubule and Collecting Duct. It is composed of three subunits (α , β and γ). Genetic disorders of blood pressure have informed us that increases in activity of either protein result in hypertension and decreases in activity result in hypotension.(4–9) These proteins are the main sodium reabsorptive pathways of the aldosterone-sensitive distal nephron, the final regulator of sodium and volume homeostasis in the kidney. In rats, mice and humans NCC and ENaC are co-expressed in one segment of the distal nephron, the DCT2 (10–16). As this is the only part of the DCT that expresses the aldosterone-specifying enzyme, 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2) (17, 18), it is presumably the only part of the DCT that is responsive to aldosterone. Not surprisingly, both of these proteins are quite sensitive to aldosterone and are regulated by key intracellular mediators of aldosterone's actions.

Aldosterone stimulation of ENaC has been established for decades and its actions have been well-described (19). The determination that NCC is also a key regulatory target for aldosterone has only been recognized more recently. There are now a number of studies that have demonstrated stimulation of NCC by low salt diets or infused aldosterone (20–24). Additionally, two of the key intracellular mediators of aldosterone's actions, NEDD4-2 and SGK1 regulate both NCC and ENaC. These are the two primary sodium transporters that are utilized by aldosterone to maintain sodium homeostasis and thus blood pressure. Alterations of either result in clear changes in blood pressure. Despite their role in regulating blood

pressure, shared regulatory pathways, and co-expression in an aldosterone-sensitive nephron segment, interactions between ENaC and NCC have not previously been reported.

Now we report that these two critical proteins are bound together in the DCT2.

Understanding how these proteins interact and affect the function of each other could lead to a significant alteration of our understanding of salt handling in the kidney and thus blood pressure homeostasis.

EXPERIMENTAL PROCEDURES

Materials

Materials were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Confocal Microscopy in Mouse Kidney Cortex

To determine if γ -ENaC colocalizes with NCC, kidney slices from mice were stained with primary antibodies to NCC and γ -ENaC. Following treatment with fluorescent secondary antibodies, γ -ENaC (red) and NCC (green) were examined using confocal microscopy on an Olympus Fluoview 1000 confocal microscope. To examine co-localization, we first determined that there was no fluorescence bleed through from the green channel to the red channel or vice versa. Then we merged images from the green channel and the red channel; yellow in the merged image was an indication of co-localization. Primary antibodies were rabbit anti-NCC antibody raised against a mouse peptide antigen (25) and donkey anti- γ -ENaC antibody (F-20, Santa Cruz Biotechnology, cat # sc-22245). Secondary antibodies were Alexa Fluor 488 rabbit anti-goat (1:600) and Alexa Fluor 594 donkey anti-rabbit (1:600).

Cell Culture and Treatments

mDCT15 cells were plated on cell culture dishes and grown in growth medium containing a 50:50 mix of DMEM/F12, 5% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin/Neomycin (P/S/N), at 37°C. Experiments were conducted when the cells reached 90–95% confluence.

Electrophysiology

Gigaohm seals were obtained on individual mDCT15 cells grown on permeable supports and single channel activity recorded for up to 30 minutes (26). Channel density (N), Channel open probability (P_o), and the Current/Voltage (I/V) relationship were calculated as previously reported (27). To examine the amiloride-sensitivity of the current the tips of patch pipettes were filled with saline (about 5 mm from the tip). The rest of the pipette was filled with a saline solution containing 50 nM amiloride. After the patch was formed, normal channel activity was recorded prior to the diffusion of amiloride to the cell surface at the pipette tip (typically 5 to 10 minutes). Current tracings were recorded with no applied potential.

For the EP studies examining the effects of thiazides on ENaC the following methodology was used: mDCT15 cells were grown on cover-glasses to confluence in DMEM/F12

medium (Cellgro, USA), supplemented with 5% FBS, 100 I.U. /ml penicillin and 100 µg/ml streptomycin and 50 nM dexamethasone. Single channel activity of ENaC on the membrane of mDCT15 cells was determined under voltage-clamp conditions in outside-out configuration. Gap-free single channel current data from gigaOhm seals were acquired with an Axopatch 200B (Molecular Devices) patch clamp amplifier interfaced via a Digidata 1440 (Molecular Devices). Currents were low-pass filtered at 1 kHz with an eight-pole Bessel filter (Warner Instruments). Events were inspected visually prior to acceptance. Recording pipettes had resistances of 5–8 megaOhms. Bath and pipette solutions were (in mM): 140 NaCl, 10 EDTA, 10 Hepes (pH 7.31); and 150 mM CsCl, 10 BAPTA, 10 Hepes (pH 7.3). Channel activity (NP_o) and open probability (P_o) was assessed using Clampfit 10.5 software (Molecular Devices). For calculating P_o in paired experiments, N was fixed as the greatest number of active channels observed in control or experimental conditions. For representation, current traces were filtered at 200 Hz. For studies in CHO cells, recombinant mouse ENaC subunits ($\alpha\beta\gamma$) were overexpressed in CHO cells by transfecting 0.2 µg/subunit/9.6 cm² of the appropriate plasmid cDNAs using the Polyfect reagent (Qiagen; Valencia, CA) as described previously (28, 29). Single channels were recorded as detailed above.

Standard Immunoblotting

mDCT15 cells were incubated as above. The cells were harvested, lysed using lysis buffer containing protease inhibitor, and homogenized by sonication on ice. The cell lysates were centrifuged briefly and supernatant was collected. Proteins were transferred electrophoretically to PVDF membranes. After blocking with 3% BSA, the membranes were probed with corresponding primary antibodies; anti-NCC (25) 1:4000–1:8000, Actin (Santa Cruz, 1:1000) overnight at 4°C. All ENaC antibodies (1:200–1:1000 dilution) utilized for immunoblotting were from the lab of Douglas Eaton and have been validated (30–32). The NCC antibody (1:5000–1:8000) was developed and validated in the Hoover Lab (25). The blots were washed in TBST. Secondary antibodies were donkey anti-rabbit HRP-linked antibody (Amersham, 1:5000). Supersignal West Pico was used for chemiluminescence (Thermo Scientific). Chemiluminescence was detected with G:Box (gelbox) and analysis by Genetools software (Syngene).

Immunoprecipitation

mDCT15 cells or mouse kidney cortex was lysed and then immunoprecipitated with the indicated antibody according to the manufacturer's instructions (Pierce Co-Immunoprecipitation Kit, cat # 26149). Antibodies for immunoprecipitation were the same as used above for immunoblotting. Then lysates and immunoprecipitated protein were immunoblotted for the indicated proteins.

BN-PAGE

Blue Native (BN)-PAGE was performed using the Novex Native-PAGE Bis-Tris gel system (Invitrogen) according to the manufacturer's directions. Briefly, total cellular proteins were extracted from mDCT15 cells using non-denaturing lysis buffer in the presence of *n*-dodecyl-β-d-maltoside (DDM) at a final concentration of 1%. Samples were prepared with 2.5µL of G250 Coomassie additive. Samples were electrophoresed on Native-PAGE Novex

3–12% Bis-Tris gels using ‘light blue’ cathode buffer for 1hr at 150V, followed by 1hr at 250V and transferred to PVDF membranes in NuPAGE Transfer Buffer for 2hrs at 25V. Membranes were washed using tris-buffered saline (TBS)-tween and blocked with 5% milk (Bio-Rad) in TBS-T prior to immunoblotting. Primary antibodies were allowed to incubate overnight (4°, constant rocking), with secondary incubation times for 2hr at RT.

Animal Preparation and Experimentation

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. Mice were kept in cages with autoclaved bedding and received free access to water and a standard diet (Diet 5001; Purina, 0.4% Na). Mice were euthanized using carbon dioxide, as approved through Emory IACUC (IACUC protocol # DAR-2002607-012417BN). Kidneys were then harvested and the cortex dissected.

Preparation of Kidney Cortex

Mouse kidney cortex was homogenized in a glass tissue grinder in ice-cold RIPA buffer (1xProtease Inhibitors cocktail & 1xPhosphatase Inhibitor). After centrifugation at 13,000 rpm for 20 min at 4°C, the protein concentration was determined using BCA protein assay kit. The appropriate amount of each sample was diluted in a Trisglycine/ SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 3% β -mercaptoethanol, 0.02% bromophenol blue) and heated at 70°C for 15 min prior to loading for SDS-PAGE.

Mammalian Two-hybrid assay

We utilized the Matchmaker Mammalian Two-hybrid assay kit (Clontech). A full-length clone for mouse NCC was inserted into the DNA-binding domain (DNA-BD) plasmid vector, pM. Full-length clones of each subunit of ENaC were inserted into the activation domain (AD) plasmid, pVP16. These constructs were transfected into CHO cells along with the pG5CAT reporter plasmid. Each ENaC subunit was tested singly with NCC. The reporter gene will only be expressed if the protein encoded in the DNA-BD vector binds to the protein encoded in the AD vector. 48 hours after transfection cells were harvested and binding was assessed by measuring reporter gene expression (chloramphenicol acetyltransferase, CAT). Experiments were analyzed for the presence of chloramphenicol acetyltransferase (CAT) with a commercially available UV assay (FAST CAT Assay Kit, Thermo-Fisher, Molecular Probes, Cat # F-6616). Blots were visualized with a Kodak 2000M camera system (Eastman Kodak). Intensity of signal was detected with Image J.

Flouresence Resonance Energy Transfer (FRET) acceptor photobleaching

NCC-EGFP and α , β and γ ENaC-mCherry constructs were generated and cotransfected into COS-7 cells (50–60% confluency) using the Xfect system (Clontech, Mountainview, CA). These chromophores are an excellent donor/acceptor pair, which have been previously used in FRET studies (33, 34). FRET was measured by the acceptor photobleaching method. Live cell images were acquired during incubation at 37°C, 5% CO₂. The donor (EGFP) excitation/emission (488/509nm) and the acceptor (mCherry) excitation/emission (587/610nm) spectra were used for all images. Three single images were taken before and after 20 rounds photobleaching; these were used for before and after image analysis, where

increases in EGFP fluorescence intensity were assessed after depletion of mCherry. Images were acquired using the Nikon 1A1R TE2000 laser scanning/live cell confocal (60X, 1.4NA, oil) and acquired/analyzed using Elements by Nikon (Nikon Instruments, Melville, NY). Fluorescence images were acquired by a Nikon A1R TE2000 laser scanning confocal microscope. The FRET efficiency (%E) was calculated as: $((\text{donorpostbleach} - \text{donorprebleach}) / \text{donorpostbleach}) \times 100$.

Electron Microscopy

For this mouse kidneys were fixed by in vivo perfusion of 3% PFA/PBS, followed by post-fixation of kidney slices with 3% PFA/ 0.1% glutaraldehyde/PBS for 2 hours and cryoembedding. Ultrathin sections were blocked with serum-free protein block (Dako) followed by double staining with guinea pig anti-NCC antibody and rabbit anti- γ ENaC antibody (gifts of Johannes Loffing) (35). Secondary antibodies were colloidal gold labeled anti-guinea pig (12 nm) and anti-rabbit (6 nm) both from Jackson laboratories. Images were captured with a Phillips CM-100 TEM.

Statistical Analysis

Statistical analysis was performed using either SigmaPlot (Systat, San Jose) or GraphPad Prism (Graphpad Software, San Diego CA). Error bars represent SEM. Data were analyzed for statistical significance using a paired t-test, ANOVA (Holm-Sidak) or Mann-Whitney Rank Sum where appropriate. A p-value of less than 0.05 was taken as statistically significant.

RESULTS

NCC and ENaC co-localize to the apical membrane of DCT2 by confocal microscopy in mouse kidney cortex

Co-expression of NCC and all three subunits of ENaC in the DCT2 of mouse, rat and human kidney cortex has been shown many times (10–16). In fact, the DCT2 is often defined by the co-expression of NCC and ENaC. Those studies all used staining of consecutive sections but they all demonstrated apically oriented NCC and apically oriented ENaC. Lack of studies utilizing antibodies derived from different species has resulted in the absence of images demonstrating colocalization by confocal microscopy. Here, utilizing our rabbit anti-NCC antibody (25) and goat anti- γ ENaC antibody (Santa Cruz) we examined the coexpression of NCC and ENaC in mouse kidney cortex by confocal microscopy (figure 1). The merged images demonstrate co-localization of NCC and γ ENaC in the apical membrane of mouse DCT2. This confirms the previous reports of apically oriented NCC and ENaC as described above.

mDCT15 cells express functional ENaC channels

We have established a model of the DCT2 (mDCT15 cells) that natively expresses NCC, all three subunits of ENaC, NEDD4-2, SGK1, mineralocorticoid receptor (MR) and many of the other key proteins expressed in the second part of the DCT (24, 25, 36). This model has been extensively validated (25) and provides an excellent model to study the interaction of NCC and ENaC. Having previously established that these cells express functional NCC (24,

25, 37), we examined the function of ENaC in these cells by cell-attached patch clamp. Single channel current recordings demonstrated channels with long mean open and closed times characteristic of ENaC (Fig. 2A). Six out of twenty-five patches had similar channels and patches with channels usually had multiple channels (Fig. 2A). The Current/Voltage (I/V) curve inwardly rectified with a conductance of 6pS near zero applied voltage (Figure 2B), consistent with ENaC. Amiloridesensitivity of the current was then demonstrated (Figure 2C). These studies demonstrate the presence of functional ENaC channels in mDCT15 cells and establish our ability to measure ENaC function.

NCC and ENaC run in the same band in BN gel

Blue Native PAGE utilizes mild detergents to preserve protein-protein interactions, allowing the ability to assess native oligomeric structure and associated proteins. Therefore we sought to determine if NCC and ENaC are present in same oligomeric band in BN gels. NCC has recently been shown to run as an approximately 720 kDa band on BN gels (38). ENaC was previously shown to run in a band somewhere above 600 kDa (39). We ran membrane protein samples from mDCT15 cells on BN gels and probed the blots with antibodies for NCC and α ENaC. These studies confirmed that NCC and α ENaC both run at about 720 kDa (Figure 3). This demonstrated that in cells natively co-expressing NCC and ENaC, we see α ENaC in the same band as NCC. Interestingly, in contrast to NCC, α ENaC seems to run in multiple bands. This could be consistent with differential binding of NCC and ENaC depending on conditions such as phosphorylation or cleavage. These studies indicate that all of NCC and at least a major fraction of ENaC protein appear to exist in the same macromolecular complex in the native non-denatured conformation.

NCC co-immunoprecipitates (co-IPs) with ENaC in mDCT15 cells and rodent kidney cortex

Since these proteins are similarly regulated, expressed in the same location, and appear to exist in the same macromolecular complex we decided to assess whether they were bound together. In initial studies lysate from mDCT15 cells was immunoprecipitated with anti-NCC antibody and immunoblotted for γ and α ENaC. These studies demonstrate co-IP of the 65 kDa band of γ ENaC and the 65 kDa band of α ENaC in mDCT15 cells and mouse kidney cortex (figure 4A). IgG and resin only (no antibody) negative controls are included as well, demonstrating the specificity of this interaction. Then using mouse kidney cortex, we immunoprecipitated with γ ENaC antibody and probed for NCC, demonstrating that NCC associates with γ ENaC whether we IP with NCC ab or γ ENaC ab (figure 4B). In figure 4C we focus on α ENaC in mDCT15 cells to closely examine the characteristics and specificity of this interaction. Here we demonstrate that when we immunoprecipitated with anti-NCC antibody α ENaC associates with NCC and that no association is found in the absence of NCC antibodies. Additionally, neither the alpha subunit of the Sodium-Potassium ATPase nor GAPDH associates with NCC. Having established that immunoprecipitated NCC associates with α ENaC, we then went on to test whether immunoprecipitating with anti- α ENaC antibody brought down NCC. As seen in figure 4C, NCC does associate with immunoprecipitated α ENaC while neither GAPDH or Na-KATPase do. Thus NCC and ENaC remain in a specific complex whether we immunoprecipitate with NCC or ENaC. We then went on to demonstrate that the same holds true in mouse kidney cortex (fig 4D). The controls demonstrate that Sodium-Potassium ATPase appears to have a small amount of non-

specific binding to the Pierce Co-IP kit resin, unchanged in the presence of antibody. Interestingly, when we IP with NCC antibody we seem to preferentially pull down cleaved ENaC. This is consistent with the Native PAGE data indicating that a portion of ENaC runs in the same band as NCC, but a portion runs in other bands. This extensive examination of the association of α and γ ENaC with NCC in DCT2 cells and in rodent kidney cortex reveals a strong and consistent interaction between these proteins.

NCC directly binds ENaC in Mammalian Two-hybrid assay

Since NCC is bound in a complex with ENaC in mammalian tissue and in mDCT15 cells, we decided to test whether ENaC subunits directly bind NCC utilizing a Mammalian Two-hybrid assay. This is a variation of the Yeast Two-hybrid system used to assess direct binding between proteins. However, in this system cDNAs encoding mammalian proteins are transfected into mammalian cells (as opposed to yeast) allowing for native conformation and post-translational modification. The mammalian two-hybrid system produces quantitative estimates of the strength of interaction between two proteins. NCC-DNABD plasmid and ENaC-AD plasmids were transfected into CHO cells along with the pG5CAT reporter plasmid and binding was assessed by measuring reporter gene expression (Fig 5). Each ENaC subunit was tested singly with NCC. Table 1 indicates what is loaded in each lane. The HA tag on NCC appears to non-specifically enhance binding so only untagged NCC was analyzed. A graphical representation of multiple replicates of the experimentation indicates that γ ENaC and α ENaC bind NCC directly, with β ENaC trending toward binding when compared to control.

NCC and ENaC FRET

To further demonstrate the close association of these proteins we examined their relationship in living cells through Fluorescence Resonance Energy Transfer (FRET). NCC-EGFP and α , β and γ ENaC-mCherry constructs were generated and cotransfected into COS-7 cells (Figure 6). FRET was measured by the acceptor photobleaching method (33). The positive control was a fused EGFP-mCherry construct (Figures 6A and D). Negative controls are tagged proteins cotransfected with untagged fluorophore only (Figures 6A and B). FRET performed on COS-7 cells expressing EGFP-tagged NCC and mCherry-tagged α , β and γ ENaC demonstrated increased EGFP fluorescence after acceptor photobleaching when compared to respective α , β and γ ENaC-mCherry + EGFP negative controls (Figures 6A and C). α and β ENaC also demonstrate increased EGFP fluorescence after acceptor photobleaching compared to the NCC-EGFP + mCherry negative control. While this doesn't prove direct binding, this does indicate that the proteins are close enough (within 10 nm) to directly bind to each other (39, 40). Negative controls do not exhibit significant FRET. Also shown are representative images of a negative control (Fig 6B), an experimental group (6C) and the positive control (6D).

NCC and γ ENaC co-localize by Electron Microscopy in DCT2

Finally, to further examine the association of NCC and ENaC in native tissue we utilized immunogold labeling and Electron Microscopy (Fig. 7). Ultrathin sections from mouse kidney cortex were examined for coexpression of NCC and ENaC to identify DCT2. Images were taken from the apical portion of DCT2 cells. 12 nm gold labeled NCC and 6 nm gold

labeled γ ENaC were both found to be coexpressed on the apical surface. EM immunogold labels only a portion of expressed proteins. Given that only a subset of ENaC and NCC proteins are labeled, a smaller subset of associations of NCC and ENaC will be captured. Nonetheless, the magnified insert shows the larger immunogold labeled NCC and the smaller ENaC labeled immunogold particle separated by about the diameter of the NCC labeled particle. Interestingly, the NCC-labeled particle appears closer to the ENaC-labeled particle than it does to its probable dimer partner NCC-labeled particle. This demonstrates that NCC and γ ENaC appear to be within approximately 12 nm of each other in the apical membrane of the DCT2. While this does not prove direct binding this proximity is consistent with it.

Inhibition of NCC results in decreased ENaC function

In order to ascertain whether this interaction has any functional impact on these transport proteins we assessed the effect of inhibiting NCC on ENaC function in mdct15 cells (Fig. 8). We began to examine the baseline functional implications of this relationship by measuring amiloride-sensitive uptakes as a measurement of ENaC activity with and without inhibitors of NCC. Here we modified our well-established protocol for measuring NCC function by thiazide-sensitive $^{22}\text{Na}^+$ uptakes in mDCT15 cells (24, 25, 41) to assess amiloride-sensitive $^{22}\text{Na}^+$ uptakes. We simply substituted amiloride into the protocol instead of metolazone. Amiloride-sensitive uptakes were then performed in the presence and absence of .1mM metolazone (Fig 8a). This indicated that amiloride-sensitive uptakes were significantly decreased in the presence of thiazide. While this could indicate a decrease in ENaC function with inhibition of NCC, it could also reflect a change in amiloride-sensitivity in the presence of thiazides. Additionally, this does not address whether the number of channels or the open probability is altered. Therefore, we went on to examine this more closely through the use of electrophysiology. Using mDCT15 cells, single channel recordings were obtained in excised outside-out patches with and without HCTZ applied to the apical surface. In figure 8b we show a representative continuous experiment demonstrating ENaC activity in the control and after HCTZ. Area 1 is a channel recording without HCTZ and area 2 is with HCTZ. ENaC open probability clearly significantly decreases with HCTZ. Figure 8c shows the compilation of data from six separate experiments, clearly demonstrating a consistent effect of decreased open probability of ENaC with NCC inhibition. While it is historically accepted, both clinically and experimentally, that thiazides have no significant effect on ENaC when expressed alone, we examined whether this was true in our hands. Heterologous expression of ENaC in a model system that does not express NCC (CHO cells) demonstrated no effect of 100uM HCTZ on ENaC function (Figure 9). These studies confirm that the effect of thiazides on ENaC is an NCC-specific effect.

These findings could indicate that when bound together, inhibiting activity of one inhibits activity of the other. Perhaps when bound together they operate only in parallel. Interestingly, this effect is not explained by decreased NCC sodium transport resulting in decreased intracellular sodium, as that would increase the driving force for ENaC. Alternatively, this could indicate that ENaC function is lessened when thiazide binds to the complex through NCC. Perhaps this engenders a conformational change that results in more

closed ENaC channels. Either way, a NCC specific signal is being transmitted which is resulting in functional changes in ENaC. Having now examined the impact of NCC inhibition on ENaC activity with two different methodologies it is clear that there are significant functional implications of the association of NCC and ENaC.

DISCUSSION

Using multiple techniques we have now demonstrated a novel and previously unsuspected interaction between ENaC and NCC, both key proteins for maintaining blood pressure homeostasis. This interaction has a clear impact on function of these transport proteins and occurs in the only part of the DCT that is presumably sensitive to aldosterone. Although the sensitivity of the DCT1 and the DCT2 to aldosterone has never been explicitly investigated the expression pattern of 11 β -hydroxy-steroid dehydrogenase type 2 (11 β HSD2), the aldosterone-sensitizing enzyme, would suggest that only the DCT2 is aldosterone sensitive. While mineralocorticoid receptors are expressed in DCT1 and DCT2 (17, 42–44), 11 β HSD2 is only expressed in DCT2 (17, 18). While there has recently been reported some species variation concerning the expression of 11 β HSD2 in the DCT2 (45), it is clear that in all species there is no 11 β HSD2 in the DCT1. The localization of 11 β HSD2 to DCT2 only would suggest that the reported large changes in NCC protein expression induced by chronic aldosterone (21–23) are only taking place in the DCT2. These studies were largely done with immunoblotting of samples containing both segments of the DCT so segment-specific changes remain unknown. But if a segment that makes up 1/3 of the DCT is causing a 3 fold change in protein expression of NCC in the whole DCT, this would imply that there are changes on the order of 9 fold in this segment. This presumably exquisitely aldosterone-sensitive segment is also the only segment that co-expresses NCC and ENaC. Interestingly, recent data has suggested that DCT1 and DCT2 may have quite different physiological roles. As detailed above, the DCT2 appears to be largely responsible for mediating the effects of aldosterone in the DCT. However, a number of recent studies have suggested that the primary physiological role of the DCT1 may be related to potassium homeostasis. Acute potassium loading rapidly dephosphorylates NCC, resulting in kaliuresis (46–48). This effect is independent of aldosterone and is likely predominantly occurring in DCT1. Conversely, inhibiting NCC acutely by thiazides (which inhibits NCC in DCT1 and DCT2) does not appear to promote kaliuresis (49), perhaps suggesting that NCC inhibition must be confined to the DCT1 to induce acute kaliuresis. Additionally, the expression of NCC in the DCT1 has recently been determined to be dependent on the presence of a basolateral K channel (50). This apparent interdependence of potassium handling and NCC regulation in DCT1 may suggest a primary role for DCT1 in aldosterone-independent potassium regulation. In contrast, the DCT2 appears to have the primary role of mediating the DCT effects of aldosterone. As we have accumulated more data, it has now become clear that these two DCT segments are distinct in expression of proteins and perhaps even physiological role.

In addition to being localized to the aldosterone-sensitive DCT segment, ENaC and NCC also share a similar aldosterone-induced regulatory network. NEDD4-2 and SGK1 have been identified as key mediators of aldosterone's effects on ENaC. The E3 ubiquitin ligase NEDD4-2 binds to the PY (proline-tyrosine) motif in ENaC subunits and ubiquitinates it,

resulting in endocytosis and degradation of ENaC (19). SGK1 phosphorylates NEDD4-2 resulting in accumulation of ENaC at the cell surface. A recent publication examined the regulation of NCC by NEDD4-2, demonstrating that an inducible renal tubule-specific NEDD4-2 knockout mouse had increased NCC protein (36). Additionally, it was shown that NEDD4-2 binds and ubiquitinates NCC, resulting in decreased NCC protein. Gene silencing of NEDD4-2 in mDCT15 cells resulted in increased total and cell surface NCC protein (36). A paper detailing the phenotype of this mouse surprisingly reported that while NCC protein expression was increased, functional ENaC was not (51). While β ENaC and γ ENaC expression is increased they are mostly cytoplasmically located and α ENaC expression is unchanged. Thiazides, but not amiloride, corrected the salt-sensitive hypertension in these animals, suggesting that the hypertension was mostly due to increased NCC function. This finding was in contrast to the constitutive global NEDD4-2 knockout which showed increased ENaC expression and unchanged NCC (52). Recent evidence also points to NCC being regulated by SGK1 similarly to ENaC. Phosphorylation of NEDD4-2 by SGK1 appears to prevent the association of NCC and NEDD4-2, resulting in increased NCC protein (36). SGK1 also appears to regulate ENaC and NCC independently of NEDD4-2. (53) An inducible renal tubule specific SGK1 knockout mouse was hypotensive and had decreased expression of NCC and all three subunits of ENaC (54). All of these findings led us to hypothesize that these proteins were bound together in a complex. Our discovery that this cotransporter and channel directly bind to one another may suggest that their association is critical to the regulation of sodium homeostasis in this nephron segment.

Although the association of these particular proteins is novel, direct interaction between other ion channels and cotransporters is also rare. While it is reasonably common for different channels to bind, interact and even combine to form heteromeric channels, it is rare for this to happen with channels and transporters (exchangers, cotransporters or other non-channel transport proteins). Most of the reported direct interactions of transporters with channels involve the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR binds to and alters the function of several members of the SLC26a family of anion exchangers (55–57). An extensive review of the literature did not reveal any other reported direct interactions of transporters with channels. As implied by its name, it has been thought that CFTR's size and many regulatory domains make it equal parts regulatory element and channel. Others have felt that CFTR was more transporter than channel. Given these properties it may have been unique in its ability to form complexes with transporters. Therefore, to our knowledge, this is the first reported direct association of a "true" channel with a non-channel transport protein.

While the novelty and somewhat surprising nature of these findings has necessitated the use of multiple techniques and systems to simply prove the interaction and demonstrate functional significance, this has left many intriguing questions to investigate. For example examination of the IP blots (fig 4) reveals that ENaC associated with NCC typically has a molecular weight below 75 kDa. This would seem to suggest that NCC may preferentially bind the cleaved, activated form of ENaC. The appearance of ENaC in multiple bands in the Native PAGE with NCC running only in the higher band could also suggest that the binding of the two proteins is conditional. A full investigation of the factors that weaken or strengthen the association is an important area of future study. In particular, determining the

role of aldosterone in strengthening or weakening the association will be a critical study. Another important study is examining the importance of the interaction to the transmission of aldosterone signaling via intracellular signaling proteins such as NEDD4-2 and SGK1. NEDD4-2 binds NCC but NCC does not have the necessary PY motif to directly bind NEDD4-2 (36). ENaC directly binds NEDD4-2 and now we know that ENaC directly binds NCC. Is ENaC necessary for the NEDD4-2 effect on NCC? The presence of ENaC in the only part of the DCT that is presumably aldosterone-sensitive may not be happenstance. It is possible that ENaC is necessary to mediate aldosterone effects on NCC. So examining the impact of the relationship of NCC and ENaC on aldosterone signaling and the impact of aldosterone signaling on the relationship will be an intriguing and likely complicated puzzle to unravel. We feel that understanding the relationship between these two key proteins could significantly alter our understanding of salt handling in the kidney and thus blood pressure.

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ABBREVIATIONS

NCC	Sodium Chloride Cotransporter
ENaC	Epithelial Sodium Channel
DCT	Distal Convolute Tubule
FRET	Fluorescence Resonance Energy Transfer
NEDD4	neural precursor cell expressed developmentally down-regulated protein 4
SGK1	serum and glucocorticoid- regulated kinase
CFTR	cystic fibrosis transmembrane conductance regulator

EGFP enhanced green fluorescent protein

11 β HSD2 11 β -hydroxysteroid dehydrogenase type 2.

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Figure 1. γ ENaC Co-localizes with NCC in Mouse Kidney Cortex by Confocal Microscopy. A. Merged image of NCC (from panel B, green) and γ ENaC (from panel C, red) immunostaining. B. NCC immunostaining in mouse kidney cortex with rabbit anti-NCC antibody (1:1000). C. γ ENaC immunostaining with donkey anti- γ ENaC antibody (1:200). D. Brightfield image. Similar images were obtained on 2 other occasions.

Figure 2.

mDCT15 cells express functional ENaC. A. Single channel records from a cellattached patch with no applied potential. “c” marks the closed levels and “o1-o5” mark the levels of one to five channels open. B. I–V relationship for the channel. X-axis voltages are minus the pipette potential; i.e., the values are displacements from the resting potential of the cell. The current/voltage (I/V) curve inwardly rectified with a conductance of 6pS near zero applied voltage, consistent with ENaC. The current is fit by the Goldman current equation. The fit predicts a membrane potential between –10 and –20 mV and a relatively high intracellular sodium (about 20 mM) consistent with NCC activity. C. Current tracing with no applied potential demonstrating amiloride sensitivity. To examine the amiloride sensitivity of the current the tips of patch pipettes were filled with saline (about 5 mm from the tip). The rest of the pipette was filled with a saline solution containing 50 nM amiloride. After the patch was formed, normal channel activity was recorded prior to the diffusion of amiloride to the cell surface at the pipette tip (typically 5 to 10 minutes).

Figure 3.

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) detects NCC and ENaC in same macromolecular complex. mDCT15 cell lysate run on non-denaturing Bis-Tris gel (Invitrogen) and immunoblotted with Anti-NCC (1:5000) and anti- α ENaC (1:500) antibodies. Representative image of 3 replicates.

Fig. 4. Co-immunoprecipitation Reveals Association of NCC and ENaC. A. Lysate of mDCT15 cells was immunoprecipitated with anti-NCC antibody and probed with anti-α and anti-γ ENaC antibodies. IgG and resin only (no antibody) negative controls are also displayed. B. IP of mouse kidney cortex lysate with γENaC antibody probed with NCC antibody. C. mDCT15 lysate immunoprecipitated with NCC antibody and probed with α-ENaC antibody (1st panel) or NCC antibody (2nd panel). mDCT15 lysate immunoprecipitated with α-ENaC antibody and probed with NCC antibody (3rd panel). No antibody lanes contain lysate with resin only. Blots of NaK-ATPase and GAPDH are included as negative controls (panels 4 and 5). D. Experiments as done in C, but on mouse kidney cortex lysate instead of mDCT15 cells. Each of the demonstrated Co-IP experiments was replicated a minimum of 3 times.

Physical Association of ENaC subunits with NCC

Figure 5.

Protein-Protein interaction between NCC and ENaC subunits in Mammalian Two-hybrid Assay. NCC-DNABD construct and each ENaC-AD construct were transfected into CHO cells. Each ENaC subunit was tested singly with NCC. After 48 hrs cells were harvested and reporter gene expression (CAT) was assessed. Table 1 indicates the transfected components for each lane. Lanes 1–4: appropriate controls as indicated in Table 1. Lanes 2 and 3 are the negative controls, indicating that the protein encoded by DNA-BD plasmid does not bind the protein encoded by the AD plasmid. Lane 4 is a positive control. Lanes 5–10: Each ENaC subunit with WT and HA-tagged NCC as indicated in Table 1. Graphical representation (n=3,* = p<.05, error bars are SEM.).

Figure 6.

Fluorescence resonance energy transfer (FRET) acceptor photobleaching demonstrates close proximity of NCC and ENaC subunits. A. COS-7 cells were transfected with fluorescently labeled NCC-EGFP and/or ENaC (α , β or γ)-mCherry (experimental groups), or empty vectors EGFP and mCherry (control groups). A fused EGFP-mCherry vector was used for positive control. Single color images taken before and after 20 rounds of acceptor (mCherry) photobleaching were assessed for an increase in fluorescence of the donor fluorophore, EGFP. Graph represents FRET efficiency as fluorescence intensity [((donorpostbleach

$-\text{donorprebleach})/\text{donorpostbleach}) \times 100]$. Negative controls do not exhibit significant FRET. Data are mean $\pm$ SEM, n=6–8, (** p<0.05, *** p<0.0001). B. Representative image of a negative control (empty vector EGFP+ α ENaC-mCherry) before and after acceptor (mCherry) photobleaching. Before and after Single channel EGFP and single channel mCherry images are shown. Pre-photobleaching merged image is also displayed. C. Representative image of an experimental group (NCC-EGFP + α ENaC-mCherry), demonstrating an increase in EGFP fluorescent intensity following mCherry photobleaching. D. Representative image of the positive control (fused EGFP-mCherry).

Figure 7.

Electron Microscopy (EM) detects close association of NCC and ENaC. Immunogold labeling of NCC (12 nm particles) and γ ENaC (6 nm particles) in mouse DCT2. The insert shows a magnified view of closely associated NCC and γ ENaC. Similar associations were observed on at least 3 other occasions.

Figure 8. Inhibition of NCC results in decreased ENaC function. A. Amiloride-sensitive $^{22}\text{Na}^+$ uptakes were performed in mDCT15 cells with and without .1mM metolazone (n=14, * = p<.05). B. A representative single channel recording of ENaC in excised outside-out patches with and without 100 nM HCTZ in mDCT15 cells. Area 1 is a channel recording without HCTZ and area 2 is with HCTZ. C. Graph of data from six separate single channel recording experiments (n=6, * = p<.05 vs. control).

Figure 9.

Thiazides have no effect on ENaC function in the absence of NCC. A. Graph of data from four separate single channel recording experiments ($n=4$, not significantly different from control). B. A representative single channel recording of ENaC in excised outside-out patches with and without 100 nM HCTZ in CHO cells transfected with mouse ENaC ($\alpha\beta\gamma$). Area 1 is a channel recording without HCTZ and area 2 is with HCTZ.

Table 1

Transfection	DNA-BD(5ug)	AD-plasmid(5ug)	Reporter (2ug)
1.Untransfected	none	None	None
2.Basal ctrl	pM	pVP16	pG5CAT
3. Negative control	pM	pVP16-CP	pG5CAT
4.Positive control	pM-53	pVP16-T	pG5CAT
5. Experiment 1	pM-NCC	pVP16-alphaENaC	pG5CAT
6.Experiment 2	pM-NCC-HA	pVP16- alphaENaC	pG5CAT
7. Experiment 3	pM-NCC	pVP16- betaENaC	pG5CAT
8. Experiment 4	pM-NCC-HA	pVP16- betaENaC	pG5CAT
9. Experiment 5	pM-NCC	pVP16-gammaENaC	pG5CAT
10. Experiment 6	pM-NCC-HA	pVP16- gammaENaC	pG5CAT