Effects of ACE inhibition and ANG II stimulation on renal Na-Cl cotransporter distribution, phosphorylation, and membrane complex properties

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The renal distal convoluted tubule (DCT) is a key control point for extracellular volume and blood pressure regulation. The Na-Cl cotransporter (NCC), expressed in the apical membrane of the DCT, is the target of thiazide diuretics prescribed for hypertension and disorders of extracellular fluid volume. Theoretically, NCC activity in the DCT apical plasma membrane (APM) can be altered by changing any one of the following: NCC synthesis or degradation rates, NCC redistribution between APM and subapical cytoplasmic vesicles (SCV), or by covalent or allosteric modification of NCC. All of these mechanisms appear to operate in the DCT, as summarized below.

Total NCC abundance is decreased by high-salt diet, mineralocorticoid blockade and during aldosterone escape (34, 44, 48, 55), and increased by low-salt diet or chronic ANG II treatment (4, 23, 52). These studies have examined NCC abundance after hours or days.

Our in vivo studies (by both immuno-EM and density gradient membrane fractionation) demonstrated trafficking of NCC between APM and SCV during high-salt diet in response to ACE inhibitor treatment (to block ANG II production) or acute hypertension (17, 44, 45, 59), and net redistribution from SCV into APM in response to acute non-pressor ANG II infusion (45), indicating that these responses can occur rapidly. Acute stimulation with vasopressin has been reported to have no effect on distribution of NCC (36) and also reported to increase NCC traffic to the APM (31).

Regarding covalent or allosteric regulation, NCC can be phosphorylated at a number of sites by the WNK-SPAK cascade (39). In vivo, NCC is phosphorylated (8), likely mediated by the WNK-SPAK cascade, in response to low-salt diet (5, 51), chronic ANG II or aldosterone infusion (4, 52, 53), acute vasopressin (31, 36), insulin treatment (47), and treatment with the calcineurin inhibitor tacrolimus (13). That NCC phosphorylation increases NCC activity in the plasma membrane (27). Thus, understanding the cellular mechanisms that regulate NCC activation are central to understanding and treating hypertension and disorders of extracellular fluid volume.

WNK (“with no lysine”) kinases, which directly or indirectly activate NCC, provoke familial hyperkalemic hypertension (12, 14, 58).Taken together, these findings suggest that conditions that increase NCC activity elevate blood pressure (which drives pressure natriuresis to match salt output to input) while conditions that decrease NCC activity lower blood pressure (by increasing salt excretion). However, transgenic over-expression of NCC does not itself cause familial hyperkalemic hypertension, indicating the importance of NCC activation by phosphorylation and/or trafficking to the plasma membrane (27).

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Ser71 with alanine did not eliminate activity. These findings suggest that phosphorylation of NCC at T58 is a primary determinant of NCC activity.

We aimed to investigate the acute regulation of NCC distribution by ACE inhibition and ANG II in vivo with a focus on the role of NCC phosphorylation (NCCp). Specifically, we asked whether acute infusion of the ACE inhibitor captopril decreased NCCp in the APM, whether subsequently adding ANG II to the captopril infusion increased NCCp in the APM, and whether either treatment redistributed NCCp between APM and SCV. In addition, we investigated the membrane domain properties and mobilities in native gels of NCC total versus NCCp. The results provide evidence that, in vivo, NCC phosphorylation is not increased by acute non-pressor ANG II treatment, that NCCp is restricted to the APM yet redistributes within the APM in response to captopril or ANG II treatment, and that NCC and NCCp exist in multimeric complexes in distinct membrane domains.

**Glossary**

ACEI angiotensin-converting enzyme inhibitor

ANG II angiotensin II

AP2 clathrin adapter protein 2

APM apical plasma membrane

BN-PAGE Blue Native polyacrylamide gel electrophoresis

DCT distal convoluted tubule

DDM n-dodecyl-β-D-maltoside

IB immunoblot

ICM intracellular membrane

IP immunoprecipitation

MAP mean arterial pressure

NCC renal distal tubule Na-Cl cotransporter

NCCp phosphorylated NCC

NCCpS71 NCC phosphorylated at serine 71

NCCpT53 NCC phosphorylated at threonine 53

NCCpT58 NCC phosphorylated at threonine 58

NKCC thick ascending limb Na-K-2Cl cotransporter

NKCCpT96pT101 NKCC phosphorylated at threonines 96 and 101

PBS phosphate-buffered saline

PM plasma membrane

PV parvalbumin

SCV subapical cytoplasmic vesicle

SPAK STE20/SPS1-related protein/alanine-rich kinase

SPAKpS373 SPAK phosphorylated at serine 373

TAL thick ascending limb

WNK with no lysine kinases

**METHODS**

**Animal Protocols**

All animal experiments were approved by the University of Southern California Keck School of Medicine Institutional Animal Care and Use Committee and were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were performed on male Sprague-Dawley rats (300–350 g body wt) that were kept under diurnal light conditions and had free access to food and water. Rats were anesthetized intraperitoneally with Inactin (Sigma; 100 mg/kg). During surgery, body temperature was maintained thermostatically at 37°C. Euvolemia was maintained by infusion of 4% BSA in 0.9% saline into the jugular vein at 20–50 μl/min. Blood pressure was measured via a polyethylene catheter (PE-50) placed in the carotid artery. The left ureter was cannulated with a Surflo IV catheter (Terumo) for measurement of urine output. At the completion of surgery, there was a 30- to 60-min period to establish baseline before infusion of drugs and collection of in vivo data. During protocols, urine output rate was determined gravimetrically and mean arterial pressure (MAP) was continuously recorded (MAP = [t2 × diastolic] + [systolic]/3). At the end of each protocol, organs were removed while animals remained under deep Inactin anesthesia. Animals were euthanized by pneumothorax.

Protocol 1. Protocol 1, from our previous study (45), examined the effects of the angiotensin-converting enzyme inhibitor (ACEI) captopril versus effects of non-pressor ANG II coinfused with captopril via the jugular: 1) control infusion of vehicle (4% BSA in 0.9% saline), 2) captopril infusion in vehicle at 12 μg/min for 20 min, and 3) following 20 min captopril infusion (12 μg/min), ANG II (20 ng·kg⁻¹·min⁻¹) in vehicle coinfused with captopril (12 μg/min) for an additional 20 min. We combined samples from a previous analysis of effects of ANG II on proximal tubule (41; no. 2554; n = 5) with samples newly prepared using the identical protocol (n = 4–5).

Protocol 2. Protocol 2 examined the effects of acute ANG II infusion (without coinfusion of captopril) versus angiotensin 1 receptor (AT1R) blockade (n = 4 each group): 1) control infusion of vehicle; 2) ANG II in vehicle, 50 ng·kg⁻¹·min⁻¹; and 3) losartan infusion in vehicle, 10 mg/kg bolus over 30 min at 20 μl/min.

Protocol 3. Protocol 3 examined the effect of feeding rats a sodium-depleted diet for 1 wk as previously described (44; n = 5 each group): 1) control vivarium chow (0.4% Na) and 2) Na-depleted chow (0.07% Na).

Animals were euthanized at the end of all procedures while under deep anesthesia by cutting the diaphragm.

**Immunoelectron Microscopy**

As described previously (25, 45), at the end of the experimental protocol, kidneys were perfusion-fixed with a 4% paraformaldehyde solution containing 0.1 M sodium cacodylate at pH 7.2 and excised. Tissue blocks were trimmed from the cortex and fixed for 2 h, rinsed in PBS, and infiltrated with 2.3 M sucrose, mounted on holders, and frozen in liquid nitrogen.

Immuo-EM was performed on thin (~70 nm) cryosections prepared with random orientation from the frozen cortical tissue on a Reichert Ultracut S cryoultramicrotome (Leica), as previously described (25). The cryosections were first blocked by incubation in PBS containing 0.05 M glycine and 0.1% skim milk powder. Sections were labeled with one of the following: polyclonal antiserum against NCC total [D. Ellison, Oregon Health and Science University (OHSU), Portland, OR] at 1:800, or with anti-NCCpT53 (36) at 1:2,000. The anti-rabbit antibodies were visualized using goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles (GAR EM10, BioCell Research Laboratories, Cardiff, UK), diluted 1:50 in PBS with 0.1% skim milk powder and polyethylene glycol (5 mg/ml), and the anti-sheep IgG was visualized with donkey anti-sheep IgG conjugated to 5 or 15 nm colloidal gold particles (BioCell Research Laboratories) diluted 1:50 in PBS with 0.1% skim milk powder and polyethylene glycol (5 mg/ml). For simultaneous labeling with both anti-NCC total and anti-NCCpT53 antisera, sections were probed with sheep anti-NCCpT58 at 1:800 detected with donkey anti-sheep IgG conjugated to 15 nm colloidal Au and total NCC detected with rabbit antibodies at 1:1,600 probed with goat anti-rabbit IgG tagged with 5 nm Au. The cryosections were stained...
with 0.3% trunayl acetate in 1.8% methyl-cellulose for 10 min before examination in a FEI Morgani electron microscope. Electron micrographs were recorded at ×16,000–25,000 on Kodak 163 film and printed at ×50,000. Immunolabeling controls consisted of substitution of the specific primary antibody with nonimmune rabbit or sheep IgG or incubation without primary antibody.

Confoval Microscopy

As previously described in detail (41), kidneys were perfusion-fixed via the dorsal aorta with PLP fixative (2% paraformaldehyde, 75 mM lysine, and 10 mM Na-periodate, pH 7.4) at a rate that did not alter blood pressure (measured via carotid cannula), and were then post-fixed in PLP for another 2–3 h. The fixed tissue was cryoprotected by incubation overnight in 30% sucrose in PBS, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Cryosections (5 μm) were cut and transferred to Fisher Superfrost Plus-charged glass slides and air dried. For immunofluorescence labeling, the sections were rehydrated in PBS, followed by a 10-min wash in 50 mM NH4Cl in PBS, then incubated in 1% SDS in PBS for 5 min for antigen retrieval. Dual labeling was performed by incubating with polyclonal rabbit antiserum against NCC at 1:1,600 and polyclonal sheep antiserum against NCCpT58 also at 1:1,600. The sections were then incubated with a mixture of AlexaFluor 488-conjugated goat anti-rabbit (green) and Alexa 568-conjugated goat anti-sheep (red) (both from Molecular Probes) diluted 1:500 for 1 h and mounted in Prolong Antifade containing the nuclear dye DAPI (Molecular Probes, Invitrogen). Slides were viewed with a Zeiss LSM 510 microscope with differential interference contrast (DIC) overlay.

Homogenization

At the end of the protocols, kidneys were collected and renal cortices were dissected, diced, suspended in 5 ml isolation buffer [5% sorbitol, 5 mM histidine/imidazole buffer, pH 7.5, 0.5 mM disodium EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 9 μg/ml aprotinin, 5 μl/ml of a phosphatase inhibitor cocktail (Sigma P2850)], homogenized for 5 min at a low setting with an Ultra-Turrax T25 (IKA-Labortechnik), and centrifuged at 2,000 g for 10 min. The supernatant was saved, the pellet was rehomogenized in 5 ml isolation buffer and recentrifuged, and the two supernatants were pooled. This 2,000 g supernatant was equivalent to a homogenate; little or no NCC was detected in the final pellet (not shown). Protein concentration was measured for 5 min, and the protein concentration of the specific primary antibody with nonimmune rabbit or sheep IgG or incubation without primary antibody.

Detergent Solubility

The detergent solubility of NCC was assayed in density fractions from control untreated rat kidneys. Density gradient fractions were physically pooled into three windows: window 1 = fractions 4–5, window 2 = fractions 6–7, and window 3 = fractions 8–10. In brief, as described previously in detail (42), the protein concentration of each window was adjusted to 3 mg/ml, then aliquots were treated with or without 1% Triton X-100 in extraction buffer [50 mM MES (pH 6.4), 60 mM NaCl, 3 mM EGTA, and 5 mM MgCl2 and protease inhibitors (Roche Diagnostics, 1 pill/5.712 ml buffer)], incubated on ice for 1 h with frequent vortexing, then centrifuged at 100,000 g for 30 min. Supernatants (detergent-soluble fraction) and pellets (detergent-insoluble fraction) were collected, and the pellets were resuspended in the same volume as the supernatants. Equal volumes of detergent-soluble and insoluble fractions were assayed side by side by immunoblot to calculate the percentage of the total that was detergent soluble versus insoluble in each sample in each window.

Immunoblot Detection and Antibodies

SDS-PAGE and immunoblot analysis were used to determine relative abundance and phosphorylation of NCC and other proteins. In

Density Gradient Fractionation of Renal Cortex

Renal cortices were subjected to subcellular fractionation on sorbitol density gradients as previously described in detail (45). In brief, a 4-ml aliquot of kidney cortex 2,000 g supernatant homogenate was mixed with 6 ml 87.4% sorbitol with protease and phosphatase inhibitors, equilibrated on ice for 1 h, loaded between the two hyperbolic sorbitol gradients (35–55% sorbitol and 55–70% sorbitol), and centrifuged at 100,000 g for 5 h. Twelve fractions were collected, diluted in isolation buffer, pelleted at 250,000 g for 75 min, resuspended in 1 ml isolation buffer, and stored in aliquots at −80°C pending assay.

Immunoprecipitation Under Denaturing Conditions

Density gradient fractions enriched in NCC were denatured in 2% SDS and then diluted 10-fold (to 0.2% SDS) in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and protease and phosphatase inhibitor cocktails (according to the manufacturers’ guidelines; Roche and Sigma, respectively). The diluted membranes were preabsorbed with 30 μl of a 50% slurry of protein A/G agarose beads (Pierce) to reduce nonspecific protein binding (2 h at 4°C), spun at 3,500 g for 5 min, and the supernatant was incubated with polyclonal anti-NCC total antibody (1:5,000, D. Ellison, OHSU) or monoclonal anti-NKCC total antibody (1 μl/ml, C. Lytle, Univ. of Calif. Riverside). After overnight incubation, 30 μl of protein A/G beads were added, incubated at 4°C for 4 h, and pelleted (3,000 g, 15 min). The beads were washed four times in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% NaN3, and 0.1% Triton X-100, and a final wash with PBS. Proteins were eluted from the beads with 20 μl 2× Laemmli sample buffer (16) at 60°C for 20 min, separated from the beads using spin filters, then boiled for 5 min, resolved by SDS-PAGE, and subjected to immunoblot analysis.

Immunoprecipitation Under Nondenaturing Conditions

Density gradient fractions enriched in NCC were diluted 1:1 in a dilution buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Roche), treated with 1% Triton X-100 and incubated on ice for 1 h with frequent vortexing. The sample was further diluted up to 1 ml with the same dilution buffer plus 1% Triton X-100, then preabsorbed with 30 μl of a 50% slurry of protein A/G agarose beads (Pierce) to reduce non-specific protein binding, spun at 3,500 g for 5 min, and the supernatant was incubated with polyclonal anti-NCC total antibody (1:5,000, D. Ellison, OHSU). After overnight incubation, 30 μl of protein A/G beads were added and samples were incubated at 4°C for 4 h and pelleted (3,000 g, 15 min). The beads were washed four times in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% NaN3, and 0.1% Triton X-100, with a final wash with PBS. Proteins were eluted from the beads with 20 μl 2× Laemmli sample buffer at 60°C for 20 min, separated from the beads using spin filters, then boiled for 5 min, resolved by SDS-PAGE, and subjected to immunoblot analysis.
homogenates, PM or ICM fractions, a constant amount of protein/sample was assayed and compared on the same gel and blot. To determine protein distributions in density gradients, a constant volume of each fraction was assayed on the same gel and blot. All samples were denatured in Laemmli sample buffer for 20 min at 60°C, resolved on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-FL, Millipore), blocked for 1 h to overnight in blocking buffer (LI-COR, Lincoln, NE), and incubated for 2 h to overnight (phosphospecific antibodies incubated 2 h) with antibodies directed against the following: NCC total (1:1,000, D. Ellision, OHSU), NCCpT53 [1:5,000, (36)], NCCpT58 (1:1,000, Division of Signal Transduction Therapy, University of Dundee), NCCpT571 (1:1,500, S. Bachmann lab), NKCC (1:1,000, "T4," C. Lylte, Univ. of Calif. Riverside), NKCCpT69pT101 (1:2,000, "R5," B. Forbush, Yale Univ., New Haven, CT), parvalbumin (1:1,000, Santa Cruz Biotechnology), AP2 (1:3,000, Sigma Chemicals), villin (1:1,000, monoclonal ID2C3, Immunotech), γ-adducin (1:1,000, Santa Cruz Biotechnology), SPAK (1:1,000, E. Delpire, Vanderbilt Univ., Nashville, TN), and SPAKpS373/OSR1pS325 (6 μg/25 ml, preabsorbed with nonphosphorylated peptide, Division of Signal Transduction Therapy, University of Dundee). The blots were washed and then incubated with the appropriate secondary antibody used at 1:5,000 dilution: Alexa 680-labeled goat anti-rabbit, Alexa 680-labeled donkey anti-mouse (Molecular Probes, Invitrogen), IRDye 800-labeled goat anti-rabbit, and 800-labeled goat anti-rabbit (LI-COR).

Signals were detected and quantitated with the Odyssey Infrared Imaging System (LI-COR), and accompanying LI-COR software. Linearity of the detection system was verified for each protein by comparing the signal from the samples loaded at 1 versus ½ volume and, if not linear, sample volume readjusted. In addition, parallel protein loading gels were run at 5 μg/lane, stained (not blotted), and random bands were quantitated to assess even protein loading of homogenates, PM, and ICM.

Assessing Protein Complexes With Blue Native-PAGE

Pooled sorbitol density gradient fractions (window I = fractions 3–5, window II = fractions 6–8) or PM and ICM fractions were used as the starting material to assess NCC and NCCp protein complex size. Sample preparation was adapted from a previous application (54). Specifically, membrane fractions were incubated with n-dodecyl-β-d-maltoside (DDM) at a final concentration of 1% on ice for 20 min then centrifuged at 100,000 g for 10 min to remove insoluble material (which was negligible). Blue Native (BN) electrophoresis was performed on the supernatant fraction using Native-PAGE Novex 3–12% Bis-Tris gels (Invitrogen) according to the manufacturer’s directions. A constant volume of each window was loaded onto the gel and run at 4°C for 1 h at 150 V and then for another hour at 250 V. The gels were transferred at 4°C for 2 h at 25 V to PVDF membranes in NuPAGE Transfer Buffer (Invitrogen). Membranes were incubated in 8% acetic acid for 15 min to fix the proteins, rinsed in deionized H2O, and destained in 100% methanol for 3 min. The membranes were then incubated in blocking buffer overnight at 4°C, rinsed in TBS, and probed with antibodies and quantitated as described under Immunoblot Detection and Antibodies.

To reassess proteins resolved by BN-PAGE on denaturing SDS-PAGE gels, BN-PAGE gel lanes were cut into four fractions, then each fraction was cut into small pieces and transferred to microfuge tubes, incubated with 2× Laemmli sample buffer for 5 min at 100°C to elute the proteins, then passed through a spin filter to remove gel pieces before running on routine SDS polyacrylamide denaturing gels and immunoblotted as described.

Quantitation and Statistical Analysis

For quantitation of immunoblots of homogenates, PM, and ICM, the normalized abundance of a protein was calculated by first determining the control group mean density. Each control and experimental value was divided by the control group mean density to provide a normalized value. The normalized control group mean value > 1.0, and variance was calculated based on the normalized (not arbitrary) density values for both control and experimental groups. The ratio of NCCp to total NCC was calculated by dividing the arbitrary density value of NCCp by the arbitrary density value of NCC total for each sample. Each control and experimental ratio was then divided by the mean of the control group ratios, and variance was calculated for both control and experimental group ratios.

In samples fractionated on density gradients, the distribution pattern of a protein was expressed as the percentage of the total signal in each fraction (the sum total signal in 12 fractions = 100%); thus, the density pattern is independent of the total amount of protein loaded on the gradient.

Two-tailed Student’s t-test assuming equal variance was used to establish whether there was a significant effect of treatment on physiologic parameters or protein expression within a set of animals. In density gradient assays, two-way ANOVA was employed to determine whether there was a significant difference in the overall density distribution pattern of a protein (between windows I, II, and III). The repeated factors were treatment (control, captopril, or angiotensin II + captopril) and window. If the interaction between treatment and window was found to be significant (P < 0.05), then it was concluded that the treatment had a significant effect. After significance was established, the location of the difference in the pattern was assessed by unpaired two-tailed Student’s t-test assuming unequal variance with Bonferroni adjustments for multiple comparisons. Data are expressed as mean ± SE. Differences were regarded significant at P < 0.05.

RESULTS

Subcellular Localization of Phosphorylated NCC

Immunoelectron microscope localization of NCC in our previous studies demonstrated redistribution of NCC from the apical plasma membrane (APM) to subapical cytoplasmic vesicles (SCV) in response to acute treatment with the ACE inhibitor captopril, and from these subapical stores to the apical membrane when ANG II was added to the captopril infusion (45). Using the same treatment strategies, we examined the distribution of NCC phosphorylated at threonine 58 in the rat (NCCpT58) which is equivalent to Thr60 in human NCC. This site is phosphorylated in vivo by SPAK (37, 60) and is critical for subsequent phosphorylation of other NCC threonine and serine residues as well as NCC activation by hypotonic milieu (35, 39). NCCpT58 was localized exclusively to the apical plasma membrane in DCT cells from untreated control rats (Fig. 1A). NCC phosphorylated at Thr53 was, likewise, localized exclusively to the APM (Fig. 1B). A higher magnification of the apical membrane demonstrates that the NCCpT58 epitope is located at the inner surface of the apical plasma membrane (Fig. 1A, inset). Presumably, this reflects the location of the Thr 58 on the intracellular NH2-terminal domain of NCC.

NCC localization in rats treated with 12 μg captopril/min for 20 min then detected with either anti-NCCpT58 or anti-NCCpT53 antibodies is shown in Fig. 2, A and B, respectively. Both antibodies detect NCCp in the APM, not in the SCV. Likewise, NCCpT58 localization in rats coinfused with a nonpressor dose of ANG II (20 ng·kg<sup>-1</sup>·min<sup>-1</sup>) along with captopril is restricted to the APM and not detected in the SCV. These findings provide evidence that NCCp distribution is restricted to the APM and does not redistribute from APM to subapical stores.
SCV in response to ACEI or from SCV to APM in response to ANG II as we have quantitated for total NCC (45). The distinct distribution patterns of NCC total versus NCCpT58 in rats coinfused with ANG II plus captopril is illustrated in Fig. 3: total NCC is detected with secondary antibodies tagged with 5 nm Au and NCCpT58 is detected with secondary antibodies tagged with 15 nm Au particles. NCC total is detected both in apical membranes and in subapical cytoplasmic vesicles as described previously (17, 44, 45), while NCCpT58 is detected only in apical membranes (24, 36). These results suggest that NCC phosphorylation occurs in or at the APM. However, from these immuno-EM findings we cannot rule out the possibility that the phosphorylated epitope is masked and that pools of NCCp in SCV are not detectable. This question is addressed subsequently in fractionated membranes.

Specificity of NCC Phosphospecific Antibodies

NCC and NKCC are homologous sodium coupled cotransporters that are both regulated by the kinases SPAK and OSR1 (29). Sequence alignment of the NH₂-terminal regions of NKCC2 and NCC reveal multiple conserved Ser/Thr residues that constitute regions of phosphorylation by both SPAK and OSR1 (40). For this reason, it was important to determine the NCC specificity of antibodies before using them as tools to assess specific NCCp regulation in renal cortical membranes that contain both DCT NCC and thick ascending limb NKCC2, likely phosphorylated at analogous sites. Figure 4A is a confocal microscope image of renal cortex from a control rat colabeled with an antibody generated against total NCC (detected in green), and an antibody generated against NCCpT58 (detected in red) and

![Image](image-url)
Fig. 3. NCC total and NCCpT58 detection in ANG II + captopril-treated rats. Total NCC is detected with secondary antibodies tagged with 5 nm Au and NCCpT58 is tagged with 15 nm Au particles. NCC total is detected in both the apical membranes (arrows pointing down) and in subapical cytoplasmic vesicles (arrows pointing up) as described previously (44, 45), while NCCpT58 is detected in apical membrane alone (24, 36). Scale bars: 0.5 μm.

Neither Acute ACE inhibition nor acute ANG II Infusion Alter Abundance of NCC or NCCp

To determine whether acute changes in ANG II alter the phosphorylation state of NCC, we compared animals infused with the ACEI captopril (12 μg/min, to reduce endogenous production of ANG II) to animals coinfused with this level of captopril plus ANG II (20 ng·kg⁻¹·min⁻¹), an “ANG II clamp.” Previous studies (41, 45) demonstrated that these doses of captopril and ANG II did not change mean arterial pressure or glomerular filtration rate and that urine output increased during captopril treatment in one study (45) but not significantly in another (41), as in the current analysis (Fig. 5).

In the current study, we assessed renal cortex homogenates (2,000 g supernatants) by immunoblotting for NCC phosphorylated at T53 and at S71 as well as NCC total assayed in cortical membranes by immunoblot because of high specificity for NCC over NKCC.

ALTER ABUNDANCE OF NCC OR NCCp

To explore whether the significant animal to animal variation in NCC was due to differences in the length of the DCT/animal, we probed blots with an antibody directed against parvalbumin (PV), a cytoplasmic protein expressed in DCT1 (22). No direct relationship was evident between NCC and PV expression; in fact, an inverse relationship was suggested. To assess whether the variability was secondary to uneven protein loading or blotting, we reprobed the blots with an antibody against the actin bundling protein villin, chosen because it is expressed at high levels in renal cortex, its detection is in the cortical membranes by immunoblot because of high specificity for NCC over NKCC.
with anti-NCC, anti-NKCC, and phosphospecific antisera. As a positive
control, 3.5 µg/lane of renal cortical membranes from control rats (102
± 4 vs. 85 ± 11 mmHg, not significant) and mean arterial pressure was
significantly increased in ANG II-infused rats versus controls (137 ± 1.2 mmHg). In addition, urine output rate tended to fall with lower blood pressure (not significant) and doubled with ANG II infusion, evidence of a pressure natriuresis response (Fig. 6A).

Neither treatment altered the NCCpT53/NCC total ratio or the NCCpS71/NCC total ratio. SPAK and SPAKpS373 were also assayed in these same renal cortical homogenates to determine whether there was any relationship between this kinase and the abundance of total NCC or phosphorylated NCC across the samples. As recently reported, three main isoforms are evident between 50 and 60 kDa (26): full-length SPAK (FL-SPAK ~60 kDa), the faster migrating SPAK-2, and smaller kidney-specific SPAK (KS-SPAK), which lacks the kinase domain and inhibits the effects of FL-SPAK to phosphorylate NCC (26). In general, the pattern of all three isoforms varied similarly across samples, was unrelated to the pattern of variability of NCC and NCCp, and was unaffected by the 30-min treatments with losartan or ANG II. SPAK phosphorylation, detected on the same blot coincident with the SPAK-FL band and calculated as the SPAKpS373/SPAK-total ratio, was not significantly decreased during acute angiotensin receptor blocker treatment with losartan nor increased significantly with acute ANG II infusion (although there was a trend for an increase between losartan and ANG II-treated groups). In this sample set, uniformity of protein loading was verified by reprobing with an antibody to the clathrin adaptor AP2 (not shown).

The immuno-EM results (Figs. 1 and 2) indicated that phosphorylated NCC was localized to the apical plasma membrane alone. Because of the possibility that the phosphorylated NCC epitopes were masked in the tissue sections, we fractionated homogenates from control, losartan-treated, and ANG II-treated renal cortex into PM-enriched and intracellular membrane (ICM)-enriched pools, subjected the samples to denaturing SDS-PAGE and immunoblot, which would presumably expose epitopes, and examined total NCC and NCCp in each compartment (Fig. 6B). Confirming the immuno-EM results, NCCpT53 was restricted to the PM pool, and NCC total was present in both PM and ICM enriched pools. The percentages of total NCC in PM versus ICM, adjusting for the volume of protein recovered and loaded, was 90% in PM versus 10% in the ICM, respectively. Treatment with ANG II did not significantly increase NCCp/total ratio in enriched PM. Sample-to-sample variation precluded assessing NCC trafficking between PM and ICM.

To validate that our methods were consistent with detecting a significant change in NCC abundance and NCCp, we treated a set of rats with low-salt diet for 1 wk, a protocol previously demonstrated to increase NCC as well as NCCp/NCC total ratio in mice (5, 51). Cortical NCC total, NCCpT53, and NCCpS71 abundance increased to 1.48 ± 0.05-, 1.34 ± 0.05-, and 1.96 ± 0.13-fold over normal-salt-fed rats, respectively (all P < 0.05, Fig. 7). NCCpT53 increased in proportion to NCC total, while the NCCpS71/NCC total ratio increased 1.32 ± 0.05-fold (P < 0.05). The length of DCT1, assessed by measuring parvalbumin abundance (22), was not apparently increased during low-salt feeding, indicating an increase in the number of NCC per DCT cell.

![Image](http://ajpcell.physiology.org/)

Fig. 4. Assessing cross-reactivity of anti-NCC antibodies with NKCC and vice versa. A: renal cortex from a control rat probed with antibody generated against total NCC (detected in green), antibody generated against NCCpT58 (detected in red) and nuclear dye (blue). DCT were colabeled with both antibodies indicating appropriate detection of NCCpT58. Anti-NCCpT58 also labeled neighboring tubules where NCC total was not detected, presumably cross-reaction with cortical TAL-expressing NKCC that has a homologous NH₂-terminal phospho-threonine (40). B: cross-reaction of phosphospecific antibodies was evaluated by immunoprecipitation (IP) of total NCC and total NKCC from 35 µg renal cortical membranes followed by immunoblot (IB) probing with anti-NCC, anti-NKCC, and phosphospecific antisera. As a positive control, 3.5 µg of total membranes were directly probed (pre-IP membranes). The same linear range as NCC (10–20 µg/lane), and it is clearly separated from NCC on the blots. Villin abundance was uniform across samples (Fig. 5), indicating even loading and blotting.

The coinfusion of captopril along with ANG II could potentially blunt the actions of the infused ANG II, for example, by inhibiting the intrarenal production of ANG II (33). We implemented a second protocol to reexamine our hypothesis that ANG II infusion would increase NCC phosphorylation: rats were infused for 30 min either with the AT₁R blocker losartan (10 mg/kg) or with ANG II (50 ng·kg⁻¹·min⁻¹) and no added ACE inhibitor, or infused with vehicle. As evidence of the efficacy of the treatments, mean arterial pressure tended to fall in losartan-infused versus control-infused rats (102 ± 4 vs.
Acute ACE Inhibition and ANG II Stimulation Alter Density Gradient Distribution of NCCp and NCC

Figures 1–3 and 6 indicate that NCCp is located in the apical membranes, thus, unlike total NCC, there does not appear to be a recruitable pool of subapical NCCp. We previously provided evidence, using both subcellular fractionation on sorbitol density gradients as well as immuno-EM, that captopril treatment provokes net redistribution of NCC from low-density plasma membranes (PM) to higher-density subapical cytoplasmic vesicles (SCV), and that coinfusion of ANG II redistributes NCC from the higher density SCV back into the lower-density PM (45). Taken together, we predicted that the subcellular distribution of NCCpT53 on sorbitol density gradients would not change significantly during captopril or ANG II treatment. Figure 8 summarizes the sorbitol density distribution patterns of NCCpT53 (Fig. 8A) and NCC total (Fig. 8B) from renal cortex homogenates from 9–10 sets of rats treated as in protocol I: control, 20 min captopril, and 20 min ANG II + captopril after 20 min captopril. Unexpectedly, the results indicate that NCCpT53 redistributes to higher density membranes after 20 min of captopril treatment and returns to lower-density membranes after 20 min of ANG II treatment (Fig. 8A). NCCpS71, determined in a subset of samples, redistributed in an indistinguishable pattern to that of NCCpT53 (not shown). This redistribution, in the face of evidence for localization of all of the NCCp to plasma membranes (Figs. 1–3 and 6) and lack of change in NCCp/total ratio in total membranes (Figs. 5 and 6), suggests that NCCpT53 redistribution occurs between distinct domains within the apical plasma membranes. Our previously published redistribution of total NCC in response to captopril and ANG II (44) was reproduced in this sample set (Fig. 8B). The density distribution pattern of both NCCpT53 and NCC total in captopril-treated samples was significantly shifted to higher densities compared with control and ANG II treated samples (P < 0.05 by ANOVA).

Interestingly, the density distribution and redistribution patterns of NCC and NCCp are distinct, as predicted from their distinct subcellular distributions. Figure 8C summarizes the relative percentages of NCCpT53 and NCC total localized to pooled fractions of the density gradients: window I = fractions 3–5, window II = fractions 6–8, and window III = fractions 9–11. In control samples, NCCpT53 was preferentially enriched in window I (59 ± 5%) versus window II (36 ± 4%) with very little in window III (5 ± 1%). In contrast, NCC total (including both unphosphorylated and phosphorylated NCC) was equivalent in window I (46 ± 3%) and window II (42 ± 2%) with less in window III (11.5 ± 1%). Thus, window I has the highest ratio of %NCCp to %NCC total and window III the lowest (evident in Fig. 8C, bottom), consistent with the previous assignment of window I as enriched plasma membranes, window II as a pool of mixed PM and SCV, and window III as intracellular membranes (28, 44, 45). During ACE inhibition by captopril, 50% of the NCCpT53 in window I (30% of the total NCCpT53) redistributes to window II, increasing the amount in window II to 60 ± 3% of total NCCpT53 and decreasing the amount in window I to 30.5 ± 4%. In parallel, a smaller percentage of total NCC redistributes from window I to II and, unlike NCCpT53, also redistributes to window III, as reported previously (45). With acute ANG II infusion and stimulation, the percentage of NCCpT53 in window I increases >20% to 52 ± 3% of total at the expense of an equivalent fall in NCCpT53 in window II; NCC total in window I increases 15% and decreases in windows II and III as previously reported (45). Overall, the ratio of %NCCpT53 to %NCC total in window I decreased significantly during captopril treatment and increases significantly during ANG II stimulation (Fig. 8C, bottom); that is, ANG II increased the NCCpT53/NCC total ratio in low-density apical plasma membranes despite the fact that ANG II did not increase overall NCCpT53 to NCC ratio assessed in total homogenates. These unexpected findings provide evidence that captopril treatment as well as acute ANG II infusion provoke changes in the physical density of the NCCp containing plasma membranes independent of subcellular redistribution between PM and SCV. The results suggest
membranes (PM, 12 expressed as means 680-labeled donkey anti-sheep and IRDye 800-labeled goat anti-rabbit) and are using different secondary antibodies with distinct emission spectra (Alexa 20 densities of identical samples assayed in tandem and are expressed as means in the same order on each blot (n/H9262 g/lane) ratios. Each lane contains a sample from a different animal assayed at 45 g/lane or NCC total (assayed at 45 g/lane) and intracellular membranes (ICM, 45 g/lane) overlapped in mobility with full length NCCp in window III, a region of the gradient enriched in lysosomes that contained very low percentages of NCC and NCCp.

The results provide evidence for at least two physical states of NCC that differ as a function of phosphorylation state.

Analysis of Total NCC versus Phosphorylated NCC Multimeric Complexes on BN Gels

To determine whether NCC forms oligomeric structures that could account for redistribution patterns on the density gradients, membrane samples were analyzed by one-dimensional BN-PAGE (57) followed by immunoblotting. This approach uses mild neutral detergents to solubilize membrane proteins to preserve protein-protein interactions and uses the anionic dye Coomassie Blue G-250 in the sample buffer to provide negative charge to drive electrophoretic separation of the proteins. We pooled sorbitol density gradient fractions from control, captopril-infused, and ANG II + captopril-infused samples that the domain location of NCCp in the APM may be an additional important variable in regulating NCC activity.

Distinct Detergent Solubility of NCC versus NCCpT53

Given the evidence that NCCpT53 remains in the apical membrane during captopril and ANG II treatment, we aimed to determine whether NCC exists in multiple domains or conformations in the DCT that could account for the distribution pattern on density gradients. Lipid raft localization and cytoskeletal attachment of transporters in “detergent-resistant membranes” (3) are known to affect density distribution. Membrane proteins that are confined to lipid rafts or strongly associated with cytoskeleton have reduced solubility in nonionic detergents such as Triton X-100 (30). To assess detergent solubility of NCC versus NCCpT53 in control vehicle-infused samples,
Subjects were fractionated into plasma membranes and intracellular membranes (ICM). The results suggest that PM NCC is localized almost exclusively in PM and a 400 kDa complex predominates in ICM. The ratio of NCCpT53 and NCC total is shown in Fig. 10C: the sorbitol density distribution patterns of each sample for NCC total were mathematically pooled into three windows: Window I = sum of densities in fractions 3–5; Window II = sum of densities in fractions 6–8; Window III = sum of densities in fractions 9–11. Control (gray bars), captopril-infused (open bars), and ANG II + captopril-infused (black bars) densities are expressed as the percentage of the total signal in the 12 fractions (100%). Values are means ± SE, n = 9–10. The effect of treatments on distribution was analyzed by ANOVA. *P < 0.05 for captopril vs. control; #P < 0.05 for ANG II + captopril vs. captopril alone.

### Fig. 8. Effects of captopril and ANG II on density distribution of NCCpT53 and NCC total

A and B: density distributions of control samples (gray circles) versus acute captopril (12 μg/min × 20 min; ○), versus confusion of ANG II and captopril (20 ng·kg⁻¹·min⁻¹ ANG II along with 12 μg/min captopril × 20–30 min, ▲). Relative abundance, determined by immunoblotting of a constant volume of each fraction, is expressed as a percentage of total signal (% total) in all 12 fractions (defined as 100%). Values are means ± SE, n = 9–10. The density distribution patterns of captopril-treated samples were determined to be significantly different from their respective patterns in control and ANG II-treated samples for both NCCpT53 (A) and NCC total (B) (P < 0.05, by ANOVA as described in METHODS). The distribution patterns of ANG II + captopril versus control samples were not significantly different for either NCCpT53 or NCC total. Typical immunoblots of samples from each treatment group run on the same blot are shown under the graphs.

C: the sorbitol density distribution patterns of each sample for NCCpT53 and NCC total were mathematically pooled into three windows: Window I = sum of densities in fractions 3–5; Window II = sum of densities in fractions 6–8; Window III = sum of densities in fractions 9–11. Control (gray bars), captopril-infused (open bars), and ANG II + captopril-infused (black bars) densities are expressed as the percentage of the total signal in the 12 fractions (100%). Values are means ± SE, n = 9–10. The effect of treatments on distribution was analyzed by ANOVA. *P < 0.05 for captopril vs. control; #P < 0.05 for ANG II + captopril vs. captopril alone.

FRACTIONS: 3-5, 6-8, 9-11

### Graphs

#### A

- **% NCCpT53**
  - Control: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80
  - Captopril: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80
  - ANG II + Captopril: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80

#### B

- **% NCC total**
  - Control: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80
  - Captopril: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80
  - ANG II + Captopril: 20, 10, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80

#### C

- **NCCpT53**
  - Control: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60
  - Captopril: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60
  - ANG II + Captopril: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60

- **NCC total**
  - Control: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60
  - Captopril: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60
  - ANG II + Captopril: 60, 40, 20, 10, 5, 10, 15, 20, 30, 40, 50, 60

#### Graphs labels

- Window I
- Window II
- Window III

### Notes

- Fractions 3–5
- Fractions 6–8
- Fractions 9–11

### Analysis

To assess the subcellular distribution of the 400 and 700 kDa NCC oligomers, control samples of renal cortex from untreated rats were fractionated into plasma membranes and intracellular membranes (PM and ICM), as described in METHODS, and subjected to BN-PAGE followed by immunoblotting. A typical set is shown in Fig. 10C. NCCpT53 was detected primarily at 700 kDa almost exclusively in PM, consistent with Fig. 6B and the immuno-EM findings. For NCC total, a ~700 kDa complex predominates in PM and a 400 kDa complex predominates in ICM. The results suggest that 1) PM NCC is localized almost exclusively in a ~700 kDa complex that contains phosphory...
lated NCC (whether it also contains unphosphorylated NCC cannot be determined with the antibodies at hand) and 2) enriched ICM contains unphosphorylated NCC in a ~400 kDa complex not detected in PM.

To definitely establish that the complexes at 700 and 400 kDa contained NCC, they were further analyzed by SDS-PAGE. PM and ICM were resolved by BN-PAGE and gels were cut into four fractions as indicated on the right of Fig. 10C, denatured, resolved by SDS-PAGE, and immunoblotted (Fig. 10D). As predicted, both NCC and NCCp from the 700 kDa complex in fraction 3 of PM was detected at the expected size of 150 kDa and also just above 250 kDa, a size detected by other labs (9, 51); a smaller amount of NCC from ICM fraction 3 was also detected at 150 and 250 kDa. In ICM, NCC from the 400 kDa complex in fraction 2 was detected at 150 kDa, validating that this complex (seen in the WI, WII, and ICM fractions) contains NCC. NCC was not detected in fraction 1 (below 250 kDa) or fraction 4 (above ~900 kDa, not shown), which could have contained monomeric NCC or very large complexes of NCC, respectively.

Using pull-down assays, Dimke et al. (7) identified γ-adducin as a binding partner that bound to the NH2-terminal region of NCC and stimulated NCC transport activity in Xenopus oocytes. Based on this evidence for interaction and activation, we tested the hypothesis that the 700 kDa complex contained γ-adducin along with NCC and NCCp. Density gradient fraction membranes from rats treated with captopril versus ANG II and ANG II + captopril (WI and WII, as in Fig. 10A) were probed with the same anti-γ-adducin referenced by Dimke et al. Two complexes were detected: one at 700 kDa, coincident with the NCC complex and roughly proportional to the abundance of NCC, and another above the 720 kDa marker (Fig. 11A). γ-Adducin was not detected at 400 kDa. Coimmunoprecipitation (co-IP) was used to assess whether NCC and γ-adducin interact in a complex in vivo (Fig. 11C). In individual denaturing immunoprecipitations, γ-adducin was detected at ~125 kDa and NCC was detected at ~150 kDa. After nondenaturing IP of NCC, γ-adducin was detected at 125 kDa by immunoblotting, evidence of interaction in vivo and colocalization in the 700 kDa complex. Since the previous in vitro study indicated that adducin bound only to the nonphosphorylated form of NCC transporter (7), we next tested whether there was any interaction between phosphorylated NCC and γ-adducin in vivo. γ-Adducin was immunoprecipitated under non-denaturing conditions from WI and WII pooled fractions, blotted, and probed. Although anti-γ adducin detected bands in both windows at ~125 kDa (evidence of successful immunoprecipitation) and NCCpT58 was abundant in pre-IP WI and WII aliquots, anti-NCCpT58 did not detect phosphorylated NCC precipitating with γ-adducin (Fig. 11D). These in vivo results provide support for the in vitro finding (7) that γ-adducin interacts with nonphosphorylated, but not phosphorylated NCC.

DISCUSSION

In previous studies we provided evidence for regulated redistribution of NCC between apical plasma membranes and subapical cytoplasmic vesicles in response to acute treatment with the ACE inhibitor captopril, with ANG II, and in response to high-salt diet (44, 45, 59). Other labs have shown that NCC phosphorylation increases NCC transport activity (35, 39). In the current study we aimed to determine how NCC redistribution was related to NCC phosphorylation, specifically, if there was evidence for acute regulation of NCC phosphorylation by ANG II or captopril, regulation of NCCp subcellular distribution, and whether we could discern differences in the physical properties of NCCp versus NCC. We discovered that 1) NCCp is restricted to the APM and is not redistributed between APM and SCV in response to ACEI or ANG II treatment and 2) overall NCC phosphorylation, expressed as NCCp/NCC total ratio, did not increase in response to acute ACEI or ANG II infusion, however, 3) by density gradient fractionation we isolated a population of low density APM in which NCCp/total ratio decreased in response to acute ACEI or ANG II infusion and increased during ANG II infusion and another pool of higher-density APM that responded reciprocally, indicative of regulated redistribution between two APM pools, 4) NCCp and NCC total have distinct density gradient profiles, and 5) NCCp and NCC total exist in distinct multimeric complexes in distinct membrane domains.

Subcellular Localization of Phosphorylated NCC

We examined distribution of NCCp by immuno-EM in response to ACEI and ANG II and found that NCCp was detected only in the plasma membranes. From this we conclude that NCC is phosphorylated and dephosphorylated exclusively in the plasma membrane and that net trafficking between APM and SCV is unrelated to phosphorylation. However, from our results we cannot rule out the possibility that NCC is phosphorylated in the SCV and then moved so rapidly to the APM that an intracellular pool of NCCp is not detected by our methods. It will be interesting to determine whether phosphorylation increases the APM NCC pool size by stabilizing NCC and/or whether dephosphorylation increases the likelihood of internalization and degradation. The possibility that the epitopes for NCC phosphorylation were masked or unavailable in the intracellular membrane pools was addressed in our analysis of enriched pools of plasma membranes and intracellular membranes following denaturation and resolution by
SDS-PAGE and immunoblot (Fig. 6B): NCC total was detected in both PM and ICM while NCCp was almost completely in the PM, confirming the distribution observed in the APM by immuno-EM. This finding is in agreement with recent reports of NCCp distribution during vasopressin stimulation (31, 36). From the immuno-EM panels it is clear that there is a substantial pool of unphosphorylated NCC in the subapical compartment. In our previous study of NCC trafficking we calculated that this SCV pool contained ~30% of the total NCC (45); in comparison, after fractionation of PM versus ICM (Fig. 6B), we calculated the fraction of total NCC in ICM to be ~10% [based on relative immunodetection signals (Fig. 6B), the amount of protein loaded (12 µg for PM, 45 µg for ICM), and the total amount of protein recovered in PM versus ICM (90% in PM, 10% in ICM)]. The difference between these two estimates may be a result of incomplete separation of PM from ICM or incomplete recovery of NCC in low-density ICM (43). The direct counting of silver grains in PM versus subapical pools is likely to be more accurate. In summary, >70% of the NCC total and most all of the NCCp is localized to the apical plasma membrane while 30% or less of the NCC is localized to subapical stores and it is not phosphorylated at T53, T58, or S71.

Specificity of Phosphospecific Antibodies for NCC versus NKCC

In the renal cortex there are significant levels of thick ascending limb NKCC along with DCT NCC and even more NKCC in whole kidney due to the medullary thick ascending limbs. Thus, studies should, and often do, include assays of antibody specificity for NCCp versus NKCCp: assay of cortex versus medulla (no NCC in medulla), immunoprecipitation (Fig. 4), or assay of a NCC-knockout sample for studies in mice. This is less critical in immunohistochemistry assays, as nature separates expression of NKCC and NCC in cortex. Specificity is an important consideration because there is evidence for differential regulation of NCC versus NKCC phosphorylation by the kinases SPAK and OSR1, suggesting that SPAK primarily regulates NCC in cortex and OSR1 primarily regulates NKCC in medulla (21, 26, 60) and that the truncated form of SPAK inhibits SPAK and OSR1 kinase activity in both cortex and medulla (26). SPAK and OSR1 both phosphorylate rodent NCC at multiple conserved Ser and Thr residues in the cytoplasmic NH2-terminal domain. Phosphorylation at Thr58 appears to play a very critical role in the phosphorylation and activation of NCC as 1) experimental mutation of this site prevents the phosphorylation of the other NH2-terminal phos-
phorylation sites and 2) mutations at this site, observed in some Gitelman’s syndrome patients, cause a loss of function and a syndrome characterized by low blood pressure and salt wasting (29, 39). This phosphorylation site is homologous to a site in Gitelman’s syndrome characterized by low blood pressure and salt wasting (29, 39). This phosphorylation site is homologous to a site in renin-angiotensin system (31, 36). Chronic infusion of a non-pressor dose of ANG II over 4 days in mice increased pool size of NCCpT58 fourfold (4). The NCCpT58 antibody used in that study is the same as the anti-NCCpT58 used in Fig. 4 and may also detect NKCCp. However, we did measure an increase in NCCp total ratio in rats fed a low-salt diet for 1 wk (a protocol that stimulates the renin-angiotensin system), in agreement with analogous previous studies in mice (5, 51). Recent studies have reported acute and chronic regulation of NCC phosphorylation by a number of regulators. A study in mice reported a transient effect of ANG II after infusing 5 ng·g−1·min−1 for 5 min (equivalent to 5 μg·kg−1·min−1), a dose 100- to 250-fold higher than that used in this study, which may explain the discrepancy (50). Acute phosphorylation of NCC in rats by vasopressin has been reported (31, 36), as well as phosphorylation by acute insulin stimulation (47). Chronic infusion of a non-pressor dose of ANG II over 4 days in mice increased pool size of NCCpT58 fourfold (4). The NCCpT58 antibody used in that study is the same as the anti-NCCpT58 used in Fig. 4 and may also detect NKCCp. However, we recently confirmed the increase in NCCp after 14 days of ANG II infusion in mice using the phosphospecific NCCpT58 (Gonzalez-Villalobos RA and McDonough AA, unpublished observations). In adrenalectomized rats, ANG II infusion for 14 days at a non-pressor dose increased both NCC total and NCCp to the same extent; thus, ANG II increased NCCp abundance in the absence of aldosterone without changing the NCCp/NCC total ratio (52).

NCC and NCCp in renal homogenates exhibit significant animal to animal variability. This is also evident in other recent

ACE Inhibition, AT1R Blocking, and ANG II Infusion Do Not Change Pool Size of Renal Cortex Homogenate NCC or NCCp

We have established that acute infusion of the ACEI captopril or captopril plus ANG II provokes transporter trafficking and changes renal output (18, 19, 41, 45). In this study, using the same protocol, we assessed whether these responses were accompanied by a change in NCC phosphorylation, measured in renal cortical homogenates. We did not detect a decrease in NCCp with ACE inhibition or AT1 receptor inhibition or an increase in NCCp with ANG II infusion. However, we did measure an increase in NCCp total ratio in rats fed a low-salt diet for 1 wk (a protocol that stimulates the renin-angiotensin system), in agreement with analogous previous studies in mice (5, 51). Recent studies have reported acute and chronic regulation of NCC phosphorylation by a number of regulators. A study in mice reported a transient effect of ANG II after infusing 5 ng·g−1·min−1 for 5 min (equivalent to 5 μg·kg−1·min−1), a dose 100- to 250-fold higher than that used in this study, which may explain the discrepancy (50). Acute phosphorylation of NCC in rats by vasopressin has been reported (31, 36), as well as phosphorylation by acute insulin stimulation (47). Chronic infusion of a non-pressor dose of ANG II over 4 days in mice increased pool size of NCCpT58 fourfold (4). The NCCpT58 antibody used in that study is the same as the anti-NCCpT58 used in Fig. 4 and may also detect NKCCp. However, we recently confirmed the increase in NCCp after 14 days of ANG II infusion in mice using the phosphospecific NCCpT58 (Gonzalez-Villalobos RA and McDonough AA, unpublished observations). In adrenalectomized rats, ANG II infusion for 8 days at a non-pressor dose increased both NCC total and NCCp to the same extent; thus, ANG II increased NCCp abundance in the absence of aldosterone without changing the NCCp/NCC total ratio (52).
studies of NCC in rodent kidney (31, 36, 51, 52). The variability of NCC total and NCCp is generally proportional, indicating that the variability in NCCp is not due to loss of phosphorylation during sample isolation. Since the length of the DCT is subject to regulation (2), we probed the same samples with parvalbumin, expressed in DCT1, as a surrogate marker of DCT length. There was no positive correlation between the levels of NCC or NCCp and parvalbumin, indicating that the variability is more likely due to differences in the number of NCC per DCT cell rather than the number of DCT cells. The observation that acute ACE inhibition, AT1 receptor blocking, and/or ANG II infusion do not provoke changes in overall NCCp abundance or NCCp/NCC total ratio in renal cortex homogenates does not necessarily lead to the conclusion that this ratio is not important for ANG II-stimulated transport across the apical membrane of the renal DCT cell because reciprocal changes in subcellular pools of NCC and simultaneous increases in both phosphorylation and dephosphorylation (10) of this cotransporter could produce this outcome.

**Acute ACE Inhibition and ANG II Stimulation Alter Density Gradient Distribution of NCCp As Well As NCC Total**

McCormick et al. (27) recently generated an NCC transgenic mouse that had 1.7-fold higher NCC abundance in the renal cortex but none of the expected phenotypes of elevated NCC activity and no elevation in NCCp abundance, i.e., NCCp/total ratio decreased. This finding suggests that total NCC abundance is less important than NCC phosphorylation. The sorbitol density gradient studies in this study identified a pool of low-density membranes (*fractions 3–5, window I*, Fig. 8) that contained 60% of the NCCp which was reduced in half during acute captopril infusion and restored to baseline levels with ANG II infusion. The studies also identified a pool of NCCp at higher densities (*fractions 6–8, window II*) that contained 30% of the NCCp which nearly doubled with captopril and returned to baseline with ANG II infusion. Since the NCCp appears to be resident only in the APM (Figs. 1–3), the results suggest that NCCp acutely redistributes within domains of the APM in response to ACEI and ANG II. There are interpretations that do not involve redistribution, such as reciprocal phosphorylation and dephosphorylation within *window I* and *window II*, but whatever the cause, the analysis reveals distinct populations of membranes that exhibit pronounced changes in phosphorylation of NCC in response to inhibition of, or stimulation with, ANG II. In addition, the changes in NCCp were larger than the changes in NCC total redistribution such that the ratio of NCCpT53/NCC total in *window I* decreased during captopril and increased during ANG II infusion despite the fact that this ratio was unchanged in renal cortex homogenates.

These unexpected findings provide evidence that captopril and ANG II infusion provoke significant changes in the physical density of the NCCpT53 containing plasma membranes and suggest a new layer of regulation of cotransporter activity that complements regulation by phosphorylation and distribution between apical and subapical pools. The findings are not unprecedented: we previously established that renal proximal tubule Na⁺/H⁺ exchanger (NHE3) redistributes to higher-density membranes at the base of the microvilli during acute captopril treatment (where they have lower activity) and re-turns to low-density membranes in the body of the microvilli upon ANG II stimulation by moving within the membrane without ever leaving the APM (18, 28, 41).

The immuno-EM and subcellular fractionation results suggest distinct redistribution routes for NCC vs. NCCp in response to ACEI and ANG II. Certainly, this and previous studies demonstrate that NCCp remains resident in the APM while NCC total redistributes between the APM and SCV. What is currently unknowable (with the reagents available) is the density distribution pattern of unphosphorylated NCC and its redistribution in response to ACEI and ANG II, that is, whether there is significant redistribution of unphosphorylated NCC within the APM or only between the APM and SCV. With this caveat in mind, by combining these findings with our previous results, we postulate that 1) captopril (driving a drop in ANG II production) stimulates NCCp to redistribute within the APM to higher-density membranes (perhaps with lower transport activity) as well as net redistribution of unphosphorylated NCC from APM to SCV (or decreases NCC traffic from SCV to APM) and 2) ANG II stimulates NCCp to redistribute within the APM to lower density membranes (perhaps with higher transport activity) as well as net redistribution of unphosphorylated NCC from SCV to APM (or decreases NCC traffic from APM to SCV). These results provided the rationale for examining the physical properties of NCC and NCCp in apical and intracellular membranes.

**Distinct Detergent Solubility of NCC versus NCCpT53**

We previously assessed the fraction of NCC total in ordered lipid raft and nonraft domains on flotation gradients. Most of the NCC comigrated with flotillin, evidence of localization in ordered lipid domains (17). In the current study we performed a simpler assay of cold Triton X-100 solubility (without resolution on flotation gradients). Proteins resist Triton solubilization if they are in ordered lipid domains or attached to cytoskeletal elements. We found that ~30% of NCC total was Triton insoluble in all three windows of the density gradient (Fig. 9) and that within a window, the phosphorylated NCC was significantly more soluble in Triton. The unmeasured unphosphorylated NCC is likely to have an even higher percent detergent insolubility because the anti-total NCC recognizes both unphosphorylated and phosphorylated NCC (Figs. 3 and 4). The results imply that NCCp is less tethered to cytoskeletal elements and redistributes to more fluid lipid domains but we cannot conclude whether this is a consequence of NCC phosphorylation per se. The Triton solubility of NCCp was not significantly different between low-density *window I* and high-density *window II*, suggesting that redistribution of NCC in response to ACEI or ANG II does not involve changes in raft versus nonraft domain properties. Notwithstanding, there is a precedent for stimulus-provoked redistribution of renal TAL NKCC between membrane domains: Welker et al. (56) reported that 30% of the thick ascending limb NKCC was present in lipid rafts at baseline, assessed on flotation gradients, and that a short vasopressin treatment increased this percentage to 60%, interpreted as evidence for redistribution of NKCC into more ordered domains coincident with trafficking to the apical plasma membrane (NKCCp was not examined). It would be interesting to determine whether vasopressin also affects NCC distribution in raft versus nonraft domains. In comparison, in
the proximal tubule, ~80% of NHE3 and ~50% of the Na⁺ Pi transporter (NaPi2) are Triton insoluble, and in response to acute hypertension, NHE3 solubility remains very low as it retracts to the base of the microvilli while the fraction of insoluble NaPi2 increases to >70% as it is endocytosed (42), indicating quite distinct and variable sodium transporter membrane domain properties along the nephron.

**Multimeric Complexes of NCC and NCCp**

Determination of the number and sizes of native multimeric complexes of renal NCC and NCCp did not reveal an explanation for their acute redistribution on density gradients in response to captopril or ANG II infusion, but it did provide highly relevant information about the nature of the complex that undergoes the redistribution. First, virtually none of the NCC exists as simple NCC monomers or dimers in plasma membranes or intracellular membranes, but rather in discrete complexes that are around 400 kDa or 700 kDa. These sizes need to be viewed as apparent rather than absolute molecular weights because, like SDS-PAGE, the apparent mobility could be influenced by posttranslational modifications, and in addition, the complex mobility in the BN-PAGE can be influenced by bound lipids and Coomassie dye. On the positive side, for the case of the NKA α1, it turns out that the complex size is very close to the size in the membranes determined by radiation inactivation (15). In addition, the results demonstrate that BN-PAGE can be used to investigate native complexes of proteins that are expressed in only a small number of cells in an organ (e.g., the DCT cells of the kidney). Interestingly, the analysis suggests that the unphosphorylated NCC in intracellular membranes are found in a 400 kDa complex, while NCCp are found in a 700 kDa complex in the PM. Whether the 400 kDa complex is en route to or from the PM cannot be determined from these results. However, if this finding can be replicated in a cultured cell model, it will be possible to determine the biosynthetic relationship between the two complexes using pulse chase strategies.

The only candidate we tested for coexpression in the complex was γ-adducin based on the recent study of Dimke et al. (7) in heterologous expression systems that suggested γ-adducin binds to unphosphorylated NCC, where it can attract SPAK and OSR1 binding to the complex and phosphorylate NCC (6). The model predicts that γ-adducin would be released from NCC after phosphorylation. Our results indicate colocalization of NCC and γ-adducin in the same 700 kDa complex, as well as complex formation with unphosphorylated NCC but not NCCpT58, providing in vivo support for the previous in vitro findings (7). Clearly, it will be interesting to apply this technique to determine whether SPAK, OSR1, WNKs, or phosphatases are also 700 kDa complex members and whether proteins involved in targeting NCC to degradation pathways are present in the 400 kDa complex (49). Another attractive complex candidate is a myosin motor, which could drive NCC complexes between low- and high-density membranes in response to ANG II, analogous to myosin VI which drives NHE3 along the proximal tubule microvilli during ACE inhibition (28).

In summary, this study examined the molecular mechanisms responsible for the acute effects of ACE inhibition and ANG II on the renal NCC. The study does not provide evidence for acute overall NCC dephosphorylation or phosphorylation, but the study does provide evidence that all the NCCp is localized to the apical plasma membranes in multiple pools in detergent-soluble 700 kDa complexes containing γ-adducin and that these pools are subject to acute regulation: acute in vivo treatment with the ACEI captopril provokes redistribution of NCCp complexes within the apical membranes to a pool in higher-density membranes that have a lower ratio of NCCp/NCC total; subsequent acute in vivo treatment with ANG II provokes a return of NCC-containing complexes to the pool of lower-density membranes that have an overall higher NCCp/NCC ratio. Future studies can now be directed at determining the NCC complex constituents and how they participate in the bridge between signal and response.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


