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ORIGINAL ARTICLE

Dietary Na^+ depletion up-regulates NKCC1 expression and enhances electrogenic Cl^- secretion in rat proximal colon

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Abstract

The corticosteroid hormone, aldosterone, markedly enhances K^+ secretion throughout the colon, a mechanism critical to its role in maintaining overall K^+ balance. Previous studies demonstrated that basolateral NKCC1 was up-regulated by aldosterone in the distal colon specifically to support K^+ secretion—which is distinct from the more well-established role of NKCC1 in supporting luminal Cl^- secretion. However, considerable segmental variability exists between proximal and distal colonic ion transport processes, especially concerning their regulation by aldosterone. Furthermore, delineating such region-specific effects has important implications for the management of various gastrointestinal pathologies. Experiments were therefore designed to determine whether aldosterone similarly up-regulates NKCC1 in the proximal colon to support K^+ secretion. Using dietary Na^+ depletion as a model of secondary hyperaldosteronism in rats, we found that proximal colon NKCC1 expression was indeed enhanced in Na^+-depleted (i.e., hyperaldosteronemic) rats. Surprisingly, electrogenic K^+ secretion was not detectable by short-circuit current (I_{SC}) measurements in response to either basolateral bumetanide (NKCC1 inhibitor) or luminal Ba^{2+} (non-selective K^+ channel blocker), despite enhanced K^+ secretion in Na^+-depleted rats, as measured by $^{86}Rb^+$ fluxes. Expression of BK and IK channels was also found to be unaltered by dietary Na^+ depletion. However, bumetanide-sensitive basal and agonist-stimulated Cl^- secretion (I_{SC}) were significantly enhanced by Na^+ depletion, as was CFTR Cl^- channel expression. These data suggest that NKCC1-dependent secretory pathways are differentially regulated by aldosterone in proximal and distal colon. Development of therapeutic strategies in treating pathologies related to aberrant colonic K^+/Cl^- transport—such as pseudo-obstruction or ulcerative colitis—may benefit from these findings.

Keywords Na^+ depletion · Aldosterone · CFTR · Cl^- secretion · Ussing chamber

Abbreviations

ENaC	Epithelial Na^+ channel
NKCC1	$Na^+/K^+/2Cl^-$ co-transporter 1
BK	Large conductance K^+ channel
IK	Intermediate conductance K^+ channel
CFTR	Cystic fibrosis transmembrane conductance regulator
EDTA	5 Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
PMSF	Phenylmethylsulphonyl fluoride
CCH	Carbachol
FSK	Forskolin
I_{SC}	Short-circuit current
G.I	Gastrointestinal

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Introduction

Aldosterone is a primary regulator of colonic epithelial ion transport, serving to enhance Na^+ and water absorption and simultaneously induce K^+ secretion—two functions which are critical to overall fluid and electrolyte homeostasis [1–3]. A key feature of aldosterone is that its effects on colonic transport processes are known to vary substantially by anatomical region within the colon. For example, aldosterone inhibits electroneutral NaCl absorption and stimulates electrogenic Na^+ absorption through the epithelial Na^+ channel (ENaC) in the distal colon [4–6], while enhancing electroneutral (Na^+/H^+ exchanger 3; NHE-3)-dependent Na^+ absorption in the proximal colon [7, 8]. With respect to K^+ transport, aldosterone enhances K^+ secretion in both distal and proximal regions of the colon [1, 9, 10], although there may be differences in the secretory pathways and channels/transporters involved [11, 12].

In the distal colon, expression of the apical membrane large conductance K^+ (BK) channel is increased by aldosterone [13–15], correlating to enhanced BK-mediated K^+ secretion as measured by isotopic flux assays [16] or electrophysiological recordings [11, 14, 17]. Aldosterone-induced apical K^+ secretion is supported by basolateral K^+ uptake via Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$ co-transporter 1 (NKCC1) [15]. $^{86}Rb^+$ (K^+ surrogate) flux experiments have established that net K^+ secretion is also enhanced by aldosterone in the proximal colon [9, 18]. Still, the mechanism of aldosterone-induced K^+ secretion in the proximal colon is not known, apart from recent functional and immunohistochemical evidence against a role for aldosterone in the regulation of BK channels or BK-mediated K^+ currents in rat [11].

Recently, NKCC1, which facilitates basolateral K^+ uptake, was shown to be functionally up-regulated by aldosterone via the prevention of its proteasomal degradation in colonic epithelial cells in vitro [19, 20]. Beyond this, studies from our group have demonstrated that aldosterone transcriptionally enhances NKCC1 expression both in vivo and ex vivo, specifically to support K^+ secretion through the apical membrane BK channels in rat distal colon [21]. However, it is still unknown whether aldosterone controls NKCC1 expression and/or function within the proximal region of the tissue. The present study was therefore designed to test the hypothesis that aldosterone similarly up-regulates NKCC1 to support enhanced K^+ secretion in rat proximal colon. To this end, we employed the well-established model of dietary Na^+ depletion to induce secondary hyperaldosteronism in rats [2, 5, 7, 8]. Our results indicate that NKCC1 expression is indeed up-regulated in the proximal colons of Na^+-depleted rats, but that this does not support electrogenic K^+ secretion. Rather, basal and agonist-stimulated Cl^- secretion are enhanced, concurrent with an increase in NKCC1, as well as cystic fibrosis transmembrane conductance regulator (CFTR) protein abundance. Thus, the involvement of NKCC1 in aldosterone-regulated K^+ and Cl^- secretory pathways appears to be specific to the distal and proximal colon, respectively.

Methods

Animals

Male Sprague–Dawley rats (126–150 g; Charles River, Wilmington, MA) were housed 2 per cage and maintained in 12-h light/dark cycles in temperature-controlled housing. Rats were randomly assigned to groups receiving either standard rodent chow or Na^+-deficient diet (MP Biomedicals #02960364) for 7 days to induce secondary hyperaldosteronism, as has been described previously [2, 5, 7, 8, 22, 23]. All rats were allowed free access to water throughout. On the day of experiments, rats were rapidly anesthetized with 5% isoflurane and maintained under a deep plane of anesthesia with 3% isoflurane delivered via nose cone. Colons were removed by cutting away mesenteric attachments and excising tissue from the caecum to the rectum and were subsequently flushed with ice cold saline prior to use in downstream experiments. After tissue removal, rats were euthanized by cutting the diaphragm. All procedures used in this study were approved by the West Virginia University Institutional Animal Care and Use Committee prior to the start of the project.

Plasma aldosterone measurements

Plasma aldosterone levels from normal and Na^+-depleted rats were determined using an ELISA kit (IBL-America #IB79134). Aldosterone concentrations for each sample were calculated by fitting sample absorbance values to a standard curve using nonlinear regression in GraphPad Prism 9.0 software, as outlined in the kit's instructions.

Ussing chamber experiments

After flushing, the colon was placed on a dissection stage and submerged in ice cold HCO_3^- Ringer's solution containing the following, in mM: 140 Na^+, 119.8 Cl^-, 25 $HCO3^-$, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 2.4 HPO_4^{2-}, 0.4 $H_2PO_4^-$, and 10 glucose (pH: 7.4). The solution was continuously bubbled with 5% CO_2 balanced with O_2 to maintain pH at 7.4. The colon was opened with surgical scissors along the mesenteric border and placed mucosal side facing downward. A lateral incision was made with a razor blade just beneath the longitudinal and circular smooth muscle layers, which were peeled away to leave a submucosa/muscularis mucosae/mucosa preparation.

Segments of proximal colon, identified by striated folds in the mucosa, were then mounted on 1.1 cm^2 plastic sliders for use in an Ussing-style recording chamber (Physiologic Instruments, San Diego, CA). The recording chambers were equipped with one pair of voltage-sensing AgCl pellet electrodes and one pair of current-injecting AgCl wire electrodes, each connected to the chamber bath via agar salt bride (3.5% agar in 3 M KCl). The chamber bath itself was temperature controlled and contained 5 mL of bubbled HCO_3^- Ringer's solution in each chamber half. A multichannel voltage clamp (VCC MC8, Physiologic Instruments, San Diego, CA) was operated automatically by computer software to measure short-circuit current (I_{SC}), a function of electrogenic ion transport. By convention, positive I_{SC} values correspond to anion secretion/cation absorption and negative values correspond to cation secretion/anion absorption. Intermittent bi-polar 200 ms, 5 mV pulses were applied at 30 s intervals to monitor trans-epithelial conductance (G_{TE}).

For $^{86}Rb^+$ (99% Radionuclidic purity; PerkinElmer, Billerica, MA) flux experiments, 2 µCi of isotope was added to either the mucosal or serosal chamber bath. Unidirectional fluxes were performed using matched tissue pairs exhibiting conductance values within 20% of each other [24]. Following a 20-min equilibration period, 500 µL samples were taken from the chamber bath opposite to where the isotope was added (i.e., the "sink" side), as well as 10 µL from the isotope bath ("hot" side) to be used in the calculation of basal K^+ transport. Samples were mixed with scintillation cocktail solution (Sigma-Aldrich, #03999-5L) and counted using a Tri-carb 4910TR liquid scintillation counter (PerkinElmer, Waltham MA). Unidirectional $^{86}Rb^+$ flux rates were calculated from the measured counts per minute (cpm) values using the following equation:

$$K^+\text{flux(J)}\left(\mu\text{Eq/cm}^2\cdot\text{hr}\right) = (5\times B-[A\times 0.9])/\left(\left[S/5.2\right]\times 1.1\times 0.25\right)$$

"A" and "B" represent cpm/mL values measured from the beginning and end of the flux period, respectively. 5 is the total volume (in mL) of the chamber bath, and 0.9 is dilution factor accounting for removal of 500 µL of bath volume upon sample collection. "S" represents the cpm/µEq (in 1 mL) measured from the standard obtained from the "hot" bath of the chamber. 5.2 is the K^+ concentration in the bath (mM = mMol/L = µEq/mL), 1.1 corresponds to the slider aperture (in cm^2), and 0.25 is the time (in hours) for the flux period (26). Net K^+ fluxes were calculated by subtracting the serosal to mucosal (S-M) from the mucosal to serosal (M-S) fluxes from paired tissues.

Mucosal scraping

Proximal colon sacs were made by filling with ice cold buffer containing (in mM): 15.4 NaCl, 8 HEPES/Tris (pH 7.5), 5 ethylenediaminetetraacetic acid (EDTA), 0.5 dithiothreitol (DTT), and 0.5 phenylmethylsulphonyl fluoride (PMSF) and incubated in the same solution on ice for 45 min. The drained sacs were opened along the mesenteric border. The proximal mucosal layer was scraped off using glass slides and frozen immediately in liquid nitrogen until use. Scraped mucosae were used for RNA isolation and protein extraction.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from frozen colonic mucosa (50 mg) using the TRIzol method and quantitated with NanoDrop spectrometer (Thermo Sci. ND2000C). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using a One-Step reaction kit (NEB #E3005) per the manufacturer's protocol. Custom gene-specific primers were designed using published DNA sequences (NCBI database) and synthesized by Thermo Scientific (sequences listed in Table 1). qRT-PCR reactions performed on a 96-well plate were read using a CFX96 real time PCR machine (BioRad). mRNA abundance was determined using the $^{\Delta\Delta}$Ct method, with β-actin as a reference housekeeping gene, and represented in the figures as fold

Table 1 Primer sequences used in qRT-PCR analysis

Gene target and accession number	Primer sequence	Amplicon length
NKCC1 (Slc12a2) NM_001270617.1	F: 5′-TGGCAAGACTTCAACTCAGC-3′ R: 5′-GGTATCAACAAGGTCAAACCTCC-3′	177
BK (Kcnma1) NM_001393699.1	F: 5′-ATGTCTACAGTGGGTTACGG-3′ R: 5′-TGGGTGGTAGTTCTTTATGG-3′	464
IK (Kcnn4) NM_001270701.1	F: 5′-TGGCTGAGCACCAAGAGC-3′ R: 5′-TACAGCACCCACTTGCAACC-3′	197
CFTR (Cftr) NM_031506.1	F: 5′-TTCTTCAGCTGGACCACACC-3′ R: 5′-TGGAAGCTTGTTCCCTGTCC-3′	106
TMEM16A (Ano1) NM_001107564.1	F: 5′-TTATGGCCCTCTGGGCTCG-3′ R: 5′-CACCTCCTCTTCCTCGAAGC-3′	102
β-actin (Actb) NM_031144.3	F: 5′-AGATCAAGATCATTGCTCCTCC-3′ R: 5′-AGTAACAGTCCGCCTAGAAGC-3′	165

change in mRNA transcript expression in the experimental (Na^+-depleted) relative to the control (normal diet) animals.

Western blot analysis

Proteins were extracted from frozen colonic mucosae (50 mg) by homogenizing in ice cold lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% Triton-X100) plus protease inhibitor cocktail (Thermo #A32965), followed by a brief sonication (Fisher Sonic Dismembrator Model 100). Samples were centrifuged for 20 min at 12,000 × *g*, and the supernatant was mixed with 4 × Laemmli buffer (Life Technologies #NP0007) and heated to 90 °C for 5 min. Samples were then chilled on ice before adding β-mercaptoethanol (5% w/v), and the aliquoted samples were flash frozen and stored at − 80 °C. Proteins (~ 20 µg) were resolved on 8% Tris–glycine polyacrylamide gels and transferred on to PVDF membranes. Membranes were incubated in blocking buffer (3% BSA in tris-buffered saline plus 0.1% Tween-20 (TBST) for 1 h at room temperature, followed by overnight incubation at 4 °C in primary antibody solution. Table 2 provides the source and dilutions used for all primary antibodies. The membranes were then washed in TBST (5 min × 5) and incubated in TBST + 3% BSA containing goat anti-rabbit HRP-conjugated secondary antibody (Thermo #31,462; 1:20,000 dilution) for 1 h at room temperature before being washed again in TBST (5 min × 5). Immune complexes were detected using enhanced chemiluminescence (West Dura, Thermo #34075). Images were captured using a G:BOX (Syngene), and band intensities were quantified by densitometry using Image J software and normalized to β-actin as a loading control.

Immunofluorescence microscopy

Segments of whole proximal colon were fixed overnight at 4 °C in 4% paraformaldehyde. The next day, tissues were washed in PBS, and flash frozen in isopentane cooled with liquid nitrogen and embedded in tissue freezing medium. 10 µm sections were cut with a cryostat and mounted on charged glass slides. 40 min of heated antigen retrieval were performed by placing the slides in 10 mM citrate + 0.05% Tween-20 buffer and microwaving on very low power for 45 min before allowing gradual cooling to room temperature. Sections were then permeabilized in PBS + 5% goat serum + 0.1% Triton-X for 15 min. Afterward, primary antibody solution containing PBS + 0.01% Triton-X (PBST) + 5% goat serum with a 1:500 dilution of NKCC1 primary antibody (Cell Signaling #14581) [25] was placed on the sections overnight at 4 °C. The next day, tissues were washed 5 times for 5 min each in PBST and subsequently incubated in PBST + 5% goat serum containing goat anti-rabbit Alexa Fluor 488 secondary antibody (Thermo #11008; 1:2,00 dilution) for 1 h at room temperature. After washing an additional 5 times for 5 min each, slides were mounted with SlowFade Diamond antifade mountant with DAPI (Thermo #36971) and sealed with clear fingernail polish. IF images were captured using a Zeiss 710 confocal microscope at 20 × magnification (Carl Zeiss, Oberkochen, Germany).

Results

Dietary Na^+ depletion induces secondary hyperaldosteronism

Dietary Na^+ depletion is a well-established model of secondary hyperaldosteronism that has been extensively characterized [22]. Previous experiments from our laboratory using this model have demonstrated that plasma aldosterone levels increase ~ ten-fold after 7 days of Na^+-deficient diet administration [21]. To confirm that dietary Na^+ depletion-induced

Table 2 Primary antibody product and usage information

Target	Host species	Supplier	Dilution (application)	Predicted molecular weight (kDa)
NKCC1	Rabbit	Cell Signaling	1:500 (WB) 1:500 (IF)	160–200
BK	Rabbit	Alomone	1:250 (WB)	137
IK	Rabbit	Gift from Dr. Michael Kashgarian	1:3,000 (WB)	48
CFTR	Rabbit	Alomone	1:250 (WB)	120–170
TMEM16A	Rabbit	Alomone	1:250 (WB)	110
β-actin	Mouse (HRP-conjugated)	SCBT	1: 1,000 (WB)	42

secondary hyperaldosteronism in the present study, plasma aldosterone levels were measured via ELISA. Na^+ depleted rats exhibited a robust increase in aldosterone compared to normal diet-fed controls (4154 + 966 vs 497 + 151 pg/mL; $p < 0.05$) (Supplementary Fig. 1).

Dietary Na^+ depletion enhances NKCC1 protein expression in proximal colon

Initial experiments were aimed at evaluating the effect of dietary Na^+ depletion-induced hyperaldosteronism on the expression of NKCC1 in the proximal colon. Western blot analysis of normal and Na^+-depleted rat proximal colonic mucosae revealed a ~ two-fold increase in NKCC1 protein abundance (Fig. 1A, B) ($p < 0.05$). However, no significant change in mRNA expression was detected by qRT-PCR (Fig. 1C) ($p = 0.32$). Immunofluorescent labeling of NKCC1 was congruent with our Western blot analysis, as the signal was more prominent in Na^+-depleted tissues, particularly in the crypt region (Fig. 2B_I, B_{III}). No visible signal was detected when primary antibody was omitted from the incubation protocol (data not shown). The observed ~ two-fold increase in NKCC1 protein abundance mirrors the effect previously described in the distal colon using the same dietary model system [21]. However, the present discrepancy between changes in NKCC1 mRNA transcript and protein abundance in the proximal colon suggests that the up-regulation of NKCC1 in proximal colon may not be the result of a transcriptional mechanism, as was previously demonstrated in the distal colon. While the nature of this phenomenon was not further explored here, a similar pattern has been observed in cultured epithelial cells in response to aldosterone [19, 20]. This was attributable to changes in protein turnover, which may give insight as to a possible explanation for our current observations, as discussed in more depth below.

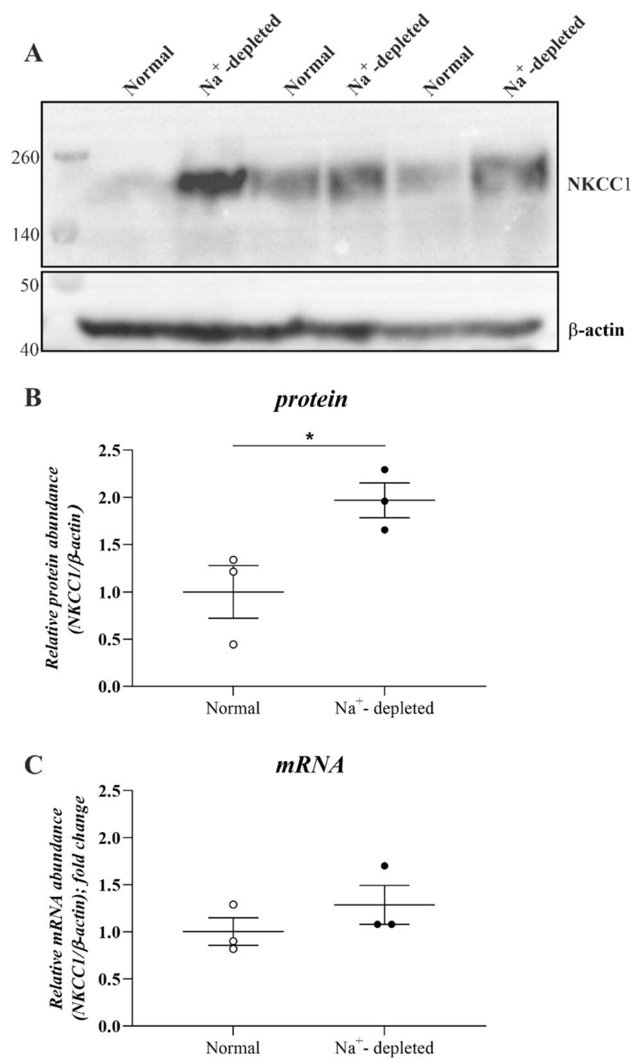

Fig. 1 Dietary Na^+ depletion enhances NKCC1 protein, but not mRNA expression in rat proximal colon. **A** Western blot performed using mucosal homogenates from normal and Na^+-depleted rat proximal colon. Standard molecular weights and primary antibody targets are indicated to the left and right of the blot images, respectively. **B** NKCC1 band intensity was quantified using densitometry analysis in ImageJ and normalized to actin band intensity as a loading control for each sample. **C** qRT-PCR analysis of NKCC1-specific mRNA transcript abundance between normal and Na^+-depleted rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean ± SEM; $n = 3$ animals per group. *$p < 0.05$ compared with control (Normal) using unpaired Student's t test

NKCC1 does not support electrogenic bumetanide- or Ba^{2+}-sensitive K^+ secretion in normal or Na^+-depleted rat proximal colon

Experiments were next designed to assess the role of NKCC1 in supporting electrogenic K^+ secretion in normal and Na^+-depleted rat proximal colon, measured as short-circuit current (I_{SC}). Recording chambers were configured such that luminal cation (K^+) and anion (Cl^-) secretion are represented by negative and positive I_{SC} values, respectively [14, 26]. Baseline I_{SC} was higher in proximal colon segments from Na^+-depleted rats compared to controls (128.6 ± 16.8 vs. 89.2 ± 8.7 µA/cm2; $p < 0.05$) (Fig. 3C), as previously reported [7]. Surprisingly, we were unable to detect electrogenic K^+ secretion that was sensitive to either serosal bumetanide (NKCC1 inhibitor) or mucosal Ba^{2+} (non-selective K^+ channel blocker). In fact, bumetanide substantially reduced the I_{SC}, likely corresponding to inhibition of NKCC1-dependent Cl^- secretion (Fig. 3B, D). However, we could not determine that bumetanide-sensitive I_{SC} was greater in Na^+-depleted animals as compared to control (ΔI_{SC}(BUMET) = − 51.4 ± 8.3 vs. -28.4 ± 7.8 µA/cm^2; $p = 0.07$) (Fig. 3D), likely due to large variability in starting I_{SC} values. Although a significant difference was not

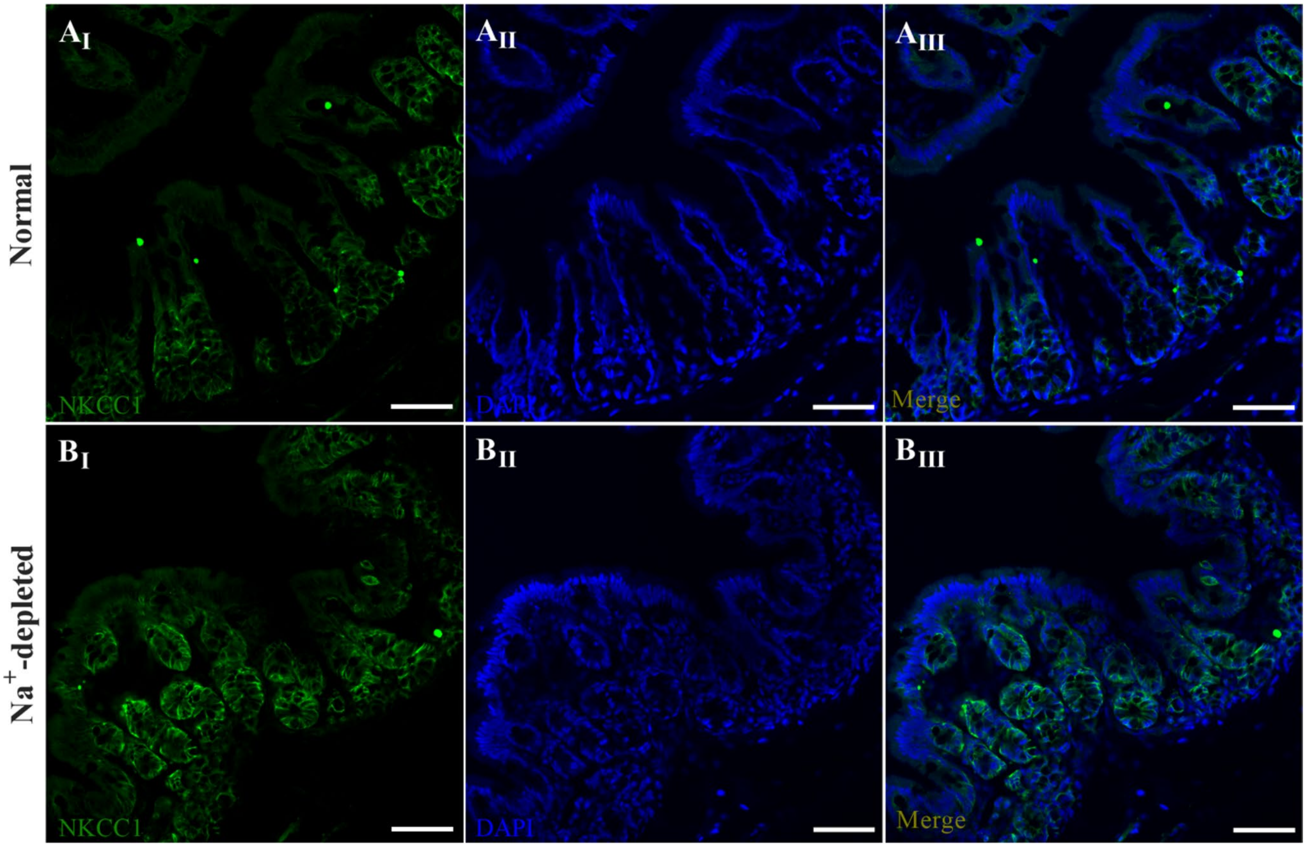

Fig. 2 Immunofluorescent labeling of NKCC1 is enhanced by dietary Na^+ depletion in rat proximal colon. NKCC1-specific labeling (green; left panels) in normal (top) and Na^+-depleted (bottom) rat proximal colon sections. Nuclei are labeled with DAPI (blue; middle panels). Merged images are shown in the far-right panels. All images were captured at 20 × magnification using a laser-powered confocal microscope. Scale bar = 50 µm

observed here, the data generally suggest that Na^+-depletion (aldosterone) enhances NKCC1 expression to support Cl^- secretion, rather than K^+ secretion, in proximal colon.

Mucosal Ba^{2+} also induced a slight negative deflection in I_{SC} in Na^+-depleted, but not in control proximal colon ($\Delta I_{SC(Ba2+)} = -12.3 \pm 3.1$ vs. -2.9 ± 2.6 µA/cm^2; $p < 0.05$) (Fig. 3E), although this effect was subtle. The absence of measurable Ba^{2+}-sensitive K^+ secretion was unexpected, given that $^{86}Rb^+$ flux studies have established that net luminal K^+ secretion is present in the proximal colon and is markedly enhanced by dietary Na^+ depletion [10] or by aldosterone directly [18]. Indeed, the rate of serosal to mucosal, as well as net K^+ flux, was increased by Na^+ depletion in our own experiments using $^{86}Rb^+$ as a K^+ tracer ($p < 0.05$ for both; Fig. 4). Our electrophysiological data therefore imply that aldosterone-induced K^+ secretion in the proximal colon is either not electrogenic or may proceed via some Ba^{2+}-insensitive pathway. Unfortunately, $^{86}Rb^+$ was taken off market during the course of this study, and thus, a thorough interrogation of a potentially yet unexplored K^+ secretory pathway could not be performed. Nonetheless, our data suggest that if NKCC1 does support luminal K^+ secretion in the proximal colon to some extent, it appears to be overshadowed by a more prominent role in supporting Cl^- secretion.

Dietary Na^+ depletion does not enhance mucosal BK and IK channel expression in proximal colon

We next sought to determine whether the expression of mucosal membrane K^+ channels in proximal colon was altered by Na^+-depletion. Perry et al. reported the presence of large conductance K^+ (BK) channels in patch clamp recording from proximal colonocytes [11]. In their study, dietary K^+ loading—another maneuver that induces secondary hyperaldosteronism—did not increase the number of functional K^+ channels in the proximal colon detectable by patch clamp, Western blot analysis or immunohistochemistry. This was in sharp contrast to effects observed in the distal colon. Expression of intermediate conductance K^+ (IK) channels, which are also present in the proximal colon in rat, was not quantified in that study. Further,

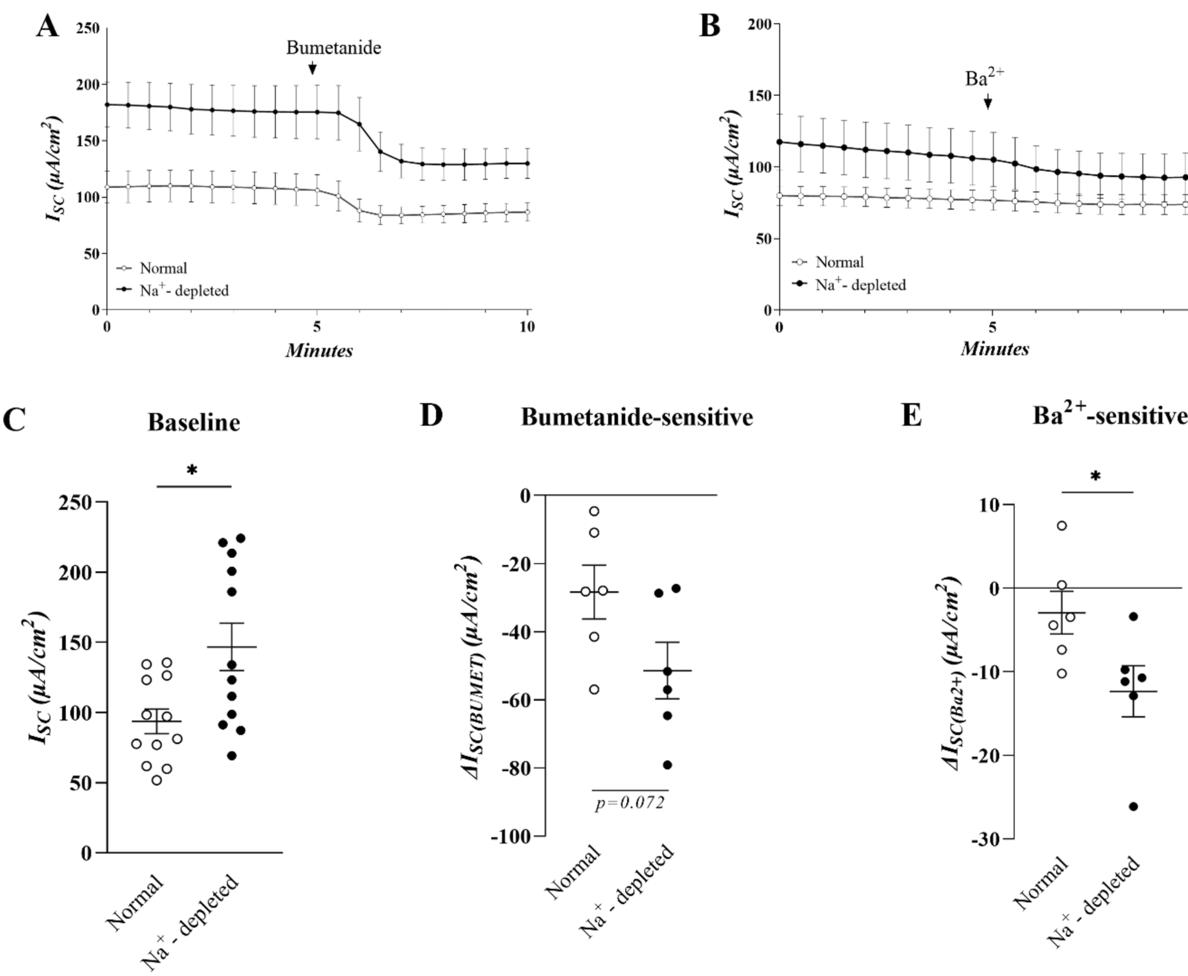

Fig. 3 Dietary Na^+ depletion does not enhance Ba^{2+}- or bumetanide-sensitive electrogenic K^+ secretion in rat proximal colon. **A**, **B** Short-circuit current (I_{SC}) recordings from normal (open circles) and Na^+-depleted (closed circles) proximal colon treated with serosal 200 µM bumetanide (**A**), or mucosal 3 mM Ba^{2+} (**B**). C-E: Basal (**C**), bumetanide-sensitive (**D**), and Ba^{2+} sensitive (**E**) I_{SC} from normal and Na^+-depleted proximal colon. Ba^{2+}-sensitive and bumetanide-sensitive I_{SC} values were calculated as the change in I_{SC} (ΔI_{SC}) from before and after the addition of drug to the chamber bath. Lines and error bars represent mean ± SEM; $n = 6$ animals per group. $^*p < 0.05$ compared with control (Normal) using unpaired Student's t test

because the models of dietary Na^+ depletion and K^+ loading are not equivalent, quantification of both BK and IK channel expression was performed here.

Neither BK nor IK abundance was found to be altered in Na^+-depleted animals, as detected by Western blot (Fig. 5A–C) or qRT-PCR (Fig. 5D, E), corroborating the findings of Perry et al. Interestingly, although electrogenic, Ba^{2+}-sensitive K^+ secretion was not observed in either normal or Na^+-depleted rat proximal colon via I_{SC} recordings, both BK and IK channels were detected at both the mRNA and protein level. Thus, it is possible that BK and IK channels may not facilitate luminal K^+ secretion in the proximal colon, but rather serve only non-vectorial transport function(s), such as participating in K^+ recycling at the basolateral membrane.

Dietary Na^+ depletion enhances NKCC1-dependent, Ca^{2+}- and cAMP-stimulated Cl^- secretion in proximal colon

NKCC1 is known to support aldosterone-induced K^+ secretion in the distal colon [15, 21, 27] and other tissues [20, 28]. In the proximal colon, basal I_{SC} is elevated by aldosterone (Fig. 3, as well as Foster et al. [7]), suggesting enhanced anion secretion. Indeed, the most well-defined role of NKCC1 throughout the gastrointestinal tract is that of supporting basal and stimulated Cl^- secretion to the lumen in

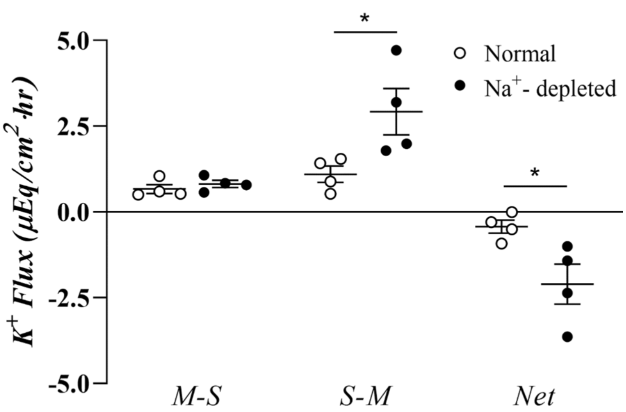

Fig. 4 Overall luminal K^+ secretion is enhanced by dietary Na^+ depletion in rat proximal colon. Unidirectional K^+ fluxes were measured via $^{86}Rb^+$ isotope tracer experiments with normal (open circles) and Na^+-depleted (closed circles) rat proximal colon (see methods for details). Net fluxes were calculated by subtracting serosal to mucosal (S-M) from mucosal to serosal (M-S) fluxes. Lines and error bars represent mean ± SEM; $n = 4$ animals per group. *$p < 0.05$ compared with control (Normal) using unpaired Student's t test

response to various stimuli [29–32]. We therefore aimed to expand upon our earlier observations by assessing whether pharmacologically stimulated Cl^- secretion, in addition to basal secretion, was also augmented in Na^+-depleted animals.

Carbamoylcholine (carbachol; muscarinic agonist) and forskolin (adenylate cyclase activator) were chosen because they induce Ca^{2+}- and cAMP-dependent Cl^- secretion, respectively, representing the two predominant secretory pathways in intestinal epithelia. The responses to both carbachol [ΔI_{SC} (CCH): 239.6 ± 5.6 vs 105.7 ± 33.5 μA/cm^2; $p < 0.05$] and forskolin [ΔI_{SC} (FSK): 154.3 ± 8.7 vs. 115.3 ± 8.7 μA/cm^2; $p < 0.05$] were enhanced in Na^+-depleted rat proximal colon compared to controls (Fig. 6A, B). Responses to both carbachol and forskolin were also attenuated by the presence of bumetanide in the serosal bath (Fig. 6A, B). These data suggest that increased NKCC1 expression correlates to an increased capacity for both Ca^{2+}- and cAMP-stimulated Cl^- secretion. In addition, cAMP-induced Cl^- secretion was significantly blunted by bumetanide in normal animals (ΔI_{SC} (FSK): 154.3 ± 8.7 vs. 61.8 ± 2.5 μA/cm2; $p < 0.01$) (Fig. 6B), whereas Ca^{2+}-induced Cl^- secretion was not (ΔI_{SC} (CCH): 105.7 ± 33.5 vs. vs 71.0 ± 21.9 μA/cm2; $p = 0.81$) (Fig. 6A). This discrepancy may reflect a greater contribution of HCO_3^- secretion in the response to Ca^{2+} versus cAMP signaling.

Dietary Na^+ depletion enhances CFTR channel protein expression in proximal colon

Luminal Cl^- secretion in the colon is primarily mediated by apical membrane-localized CFTR Cl^- channels [33–36] with evidence of some involvement of TMEM16A as well [37–40]. Because basolateral NKCC1 protein expression and basal/stimulated Cl^- secretion were all enhanced by Na^+ depletion, we sought to determine if there was a parallel increase in the expression of either of these two Cl^- channel proteins. Western blot analysis revealed that CFTR protein, and in particular the mature, glycosylated form ("c" band) [39] was increased ~ 1.5 fold in Na^+-depleted rat proximal colonic mucosae (Fig. 7A, B; $p < 0.05$). CFTR mRNA transcript abundance was not significantly increased (Fig. 7C). Interestingly, TMEM16A protein was not detected in these samples (Supplementary Fig. 2A). The TMEM16A antibody used here has been validated previously by our laboratory and others [40]. As a positive control, distal colonic mucosae tissue lysates were probed alongside proximal colon samples (Supplementary Fig. 2B). TMEM16A was detected only in the distal colon (Supplementary Fig. 2B), suggesting possible regional variability in the expression and/or functional role of TMEM16A in colonic epithelia. Therefore, CFTR is likely the apical membrane channel that mediates the basal and agonist-stimulated Cl^- secretion that is enhanced by dietary Na^+ depletion in the proximal colon.

Discussion

Our findings indicate that the expression of basolateral NKCC1 in rat proximal colon was enhanced by dietary Na^+ depletion, a model of hyperaldosteronism. Further, the increase in NKCC1 protein abundance was correlated with an augmented capacity for electrogenic Cl^- secretion, rather than K^+ secretion. This is in stark contrast to the effects of dietary Na^+ depletion on K^+ and Cl^- transport processes in the distal colon—where K^+, but not Cl^- secretion is enhanced—despite a similar increase in NKCC1 expression. Given that the basolateral ion transport processes within proximal and distal colon are similarly enhanced under these experimental conditions, the distinct effects of Na^+ depletion/hyperaldosteronemia on K^+ or Cl^- secretion likely arise from differences in apical membrane transport. Accordingly, a parallel increase in the apical membrane CFTR Cl^- channel protein was observed, whereas changes in BK and IK channel expression were not found. This also differs from the effects of Na^+ depletion on the distal colon. Segmental variability within the two regions of the colon has been well-documented in terms of the effects of corticosteroid hormones, including their influence of Na^+, K^+, and Cl^- transport. Here, we report a novel example of

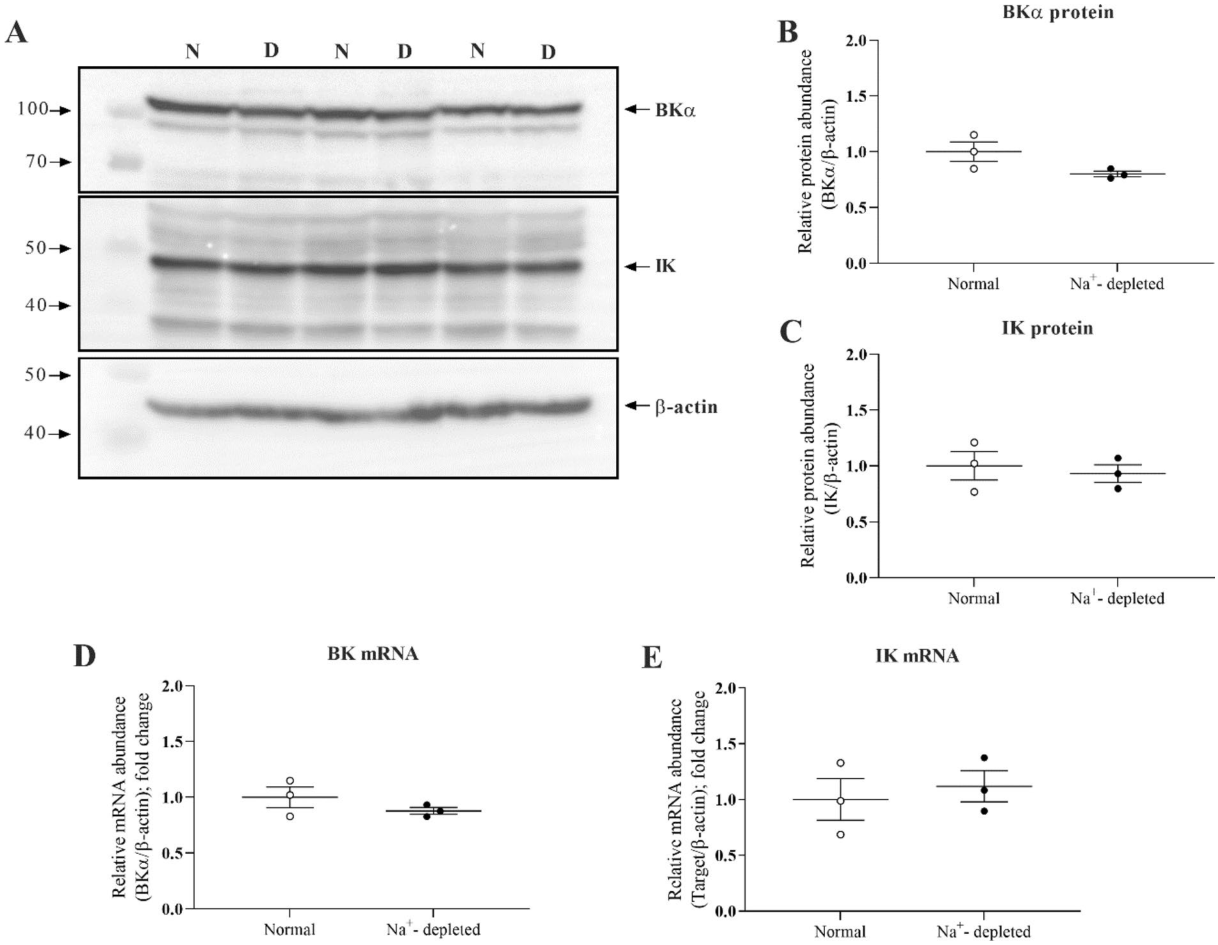

Fig. 5 Proximal colon BK and IK channel expression is unaffected by dietary Na^+ depletion. **A** Western blots performed using mucosal homogenates from normal and Na^+-depleted rat proximal colon. Standard molecular weights and primary antibody targets are indicated to the left and right of the blot images, respectively. B-C: BKa (**B**) and IK (**C**) band intensities were quantified using densitometry analysis in ImageJ and normalized to actin band intensity as a loading control for each sample. D-E: qRT-PCR analysis of BK-specific (**D**) and IK-specific (**E**) mRNA transcript abundance between normal and Na^+-depleted rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean ± SEM; $n = 3$ animals per group

differential regulation of proximal and distal colonic ion transport systems by hyperaldosteronemia induced by dietary Na^+ depletion.

Aldosterone controls the expression of target genes either by directly influencing their transcription (e.g., ENaC subunits) [41], or through the action of intermediate kinases (e.g., SGK-1) [42] or ubiquitin ligases (e.g., Nedd4-2) [43]. In many cases, these mechanisms overlap. We found that Na^+ depletion enhanced NKCC1 protein expression in the proximal colon despite no apparent increase in mRNA abundance. This suggests that aldosterone may control NKCC1 expression through post-translational effects, such as reducing the rate of protein degradation. NKCC1 is known to be ubiquitinylated by the E3 ubiquitin ligase, Nedd4-2, which is also suppressed by aldosterone signaling. Jiang et al. demonstrated that Nedd4-2 deletion enhanced both ENaC and NKCC1 activity in the distal colon [27]. This raises the possibility that aldosterone signaling similarly increases NKCC1 in the proximal colon by reducing Nedd4-2-dependent ubiquitinylation. Future studies should examine the tissue-specific translational and post-translation effects of aldosterone/Na^+ depletion on NKCC1 expression.

The results of this study have clinical relevance because colonic K^+ and Cl^- transport is a critical aspect of both gastrointestinal (G.I.) and non-G.I. pathologies. For example, in patients with end-stage renal disease (ESRD), the colon provides an alternative outlet for K^+ which can prevent life-threatening hyperkalemia [44, 45]. On the other hand, colonic pseudo-obstruction [46] and ulcerative colitis (UC) [12, 47, 48] are characterized by excessive fecal K^+ losses that contribute to the manifestation of diarrhea. A mineralocorticoid receptor antagonist has even been used clinically in at least one case of pseudo-obstruction (Ogilvie's syndrome)

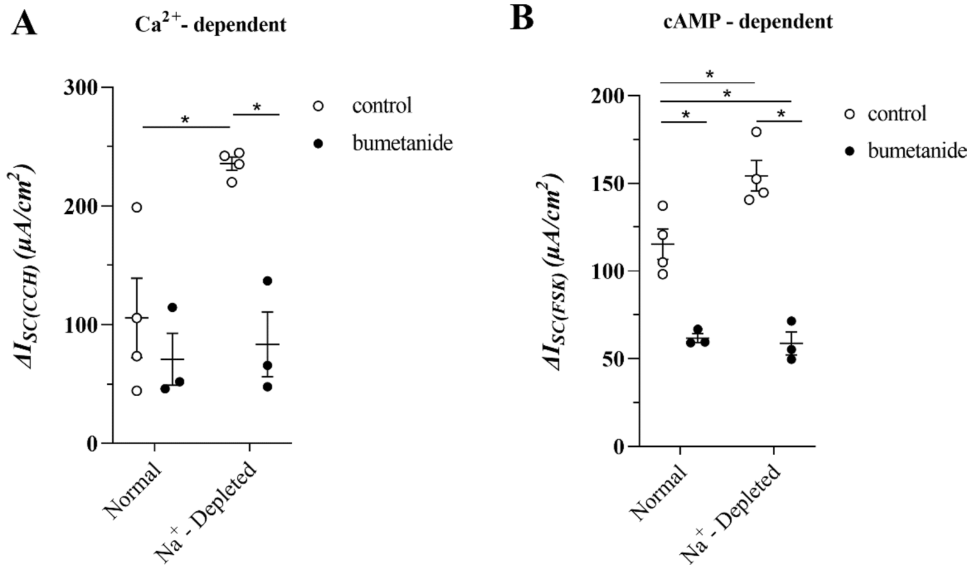

Fig. 6 Dietary Na^+ depletion enhances NKCC1-dependent, Ca^{2+}- and cAMP-stimulated Cl^- secretion in rat proximal colon. Short-circuit current responses to serosal 100 µM carbachol (CCH; **A**) or serosal 10 µM forskolin (FSK; **B**) from normal and Na^+-depleted rat proximal colon in the presence (closed circles) or absence (open circles) of 200 µM serosal bumetanide. ΔI_{SC} values were calculated as the change from baseline to the maximal response after carbachol or forskolin application. Lines and error bars represent mean ± SEM; $n = 3$–4 animals per group. $^*p < 0.05$ using an ordinary two-way ANOVA with Šidák post hoc

to relieve diarrheal symptoms associated with excessive stool K^+ output [49]. Other diarrheal pathologies such as enteroviral or enterobacterial infection primarily affect Cl^- secretion independently of K^+ secretion [50, 51]. Given the high degree of variability between colonic regions in terms of aldosterone-regulated K^+ and Cl^- transport, results from this and related studies may help with management of such pathologies.

The overall function of aldosterone signaling in the colon is to promote salt and fluid retention, while also driving the removal of excess K^+ from the body [52]. Increased NKCC1 expression that may be tied only to Cl^- secretion appears counterproductive because up-regulation of this pathway would hinder fluid absorption, in theory. However, Cl^- absorption in the proximal colon is known to be electroneutral (mediated primarily by DRA; "down-regulated in adenoma") and occurs mostly in surface absorptive cells [7]. On the other hand, NKCC1 and CFTR are known to be expressed primarily within the crypt region [32, 36]. Cl^- flux studies in the past have shown an increase in net Cl^- absorption within the proximal colon of Na^+-depleted rats compared to controls. However, the magnitude of Cl^- flux did not match that of Na^+, [7, 9] even though the transport of these two ions is functionally coupled through Na^+/H^+ and Cl^-/HCO_3^- exchangers. The increase in basal I_{SC} measured in those studies as well as ours, along with the augmented response to secretagogues (e.g., those derived from basal neurogenic input), may account for the discrepancy in net Na^+ versus Cl^- fluxes. In other words, a portion of the Cl^- absorbed via a couple electroneutral transport in surface epithelial cells may ultimately be "recycled" back to the lumen via secretion occurring within the crypt. Despite this possible explanation, the physiological significance of having an enhanced capacity for Cl^- secretion under hyperaldosteronemia conditions remains unclear.

Another puzzling observation is that although the net secretion of K^+ is enhanced by aldosterone in the proximal colon, as indicated by $^{86}Rb^+$ flux experiments (Fig. 4) [10, 18], the nature of this luminally-directed K^+ transit pathway has yet to be identified. Our data indicate that at least electrogenic K^+ secretion in both normal and Na^+-depleted rat proximal colon is insensitive to Ba^{2+} (a non-selective K^+ channel blocker) because no effect on I_{SC} was observed. Other experiments from our laboratory indicate that basal and cAMP-stimulated K^+ secretion in normal proximal colon is also unaffected by other K^+ channel inhibitors (e.g., iberiotoxin, charybdotoxin, Tram-34, paxilline) as measured by I_{SC} and $^{86}Rb^+$ fluxes (unpublished observations). The apparent insensitivity to K^+ channel inhibitors/blockers, especially Ba^{2+}, raises the possibility that luminal K^+ secretion in the proximal colon is not facilitated by an electrogenic process, but rather through some other secretory mechanism.

Perry et al. examined BK channel activity in normal and hyperaldosteronemia (K^+-loaded) rat proximal colonocytes by whole-cell and single channel patch clamp recording [11]. Although this approach allows for fine resolution of

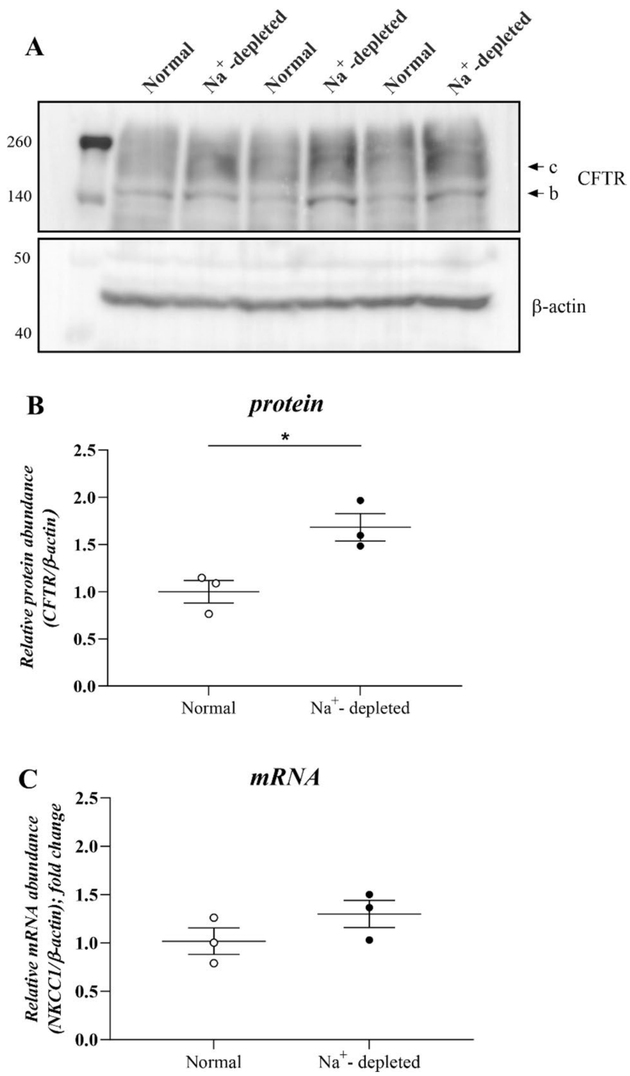

Fig. 7 Dietary Na^+ depletion enhances CFTR Cl^- channel expression in rat proximal colon. **A** Western blot performed using mucosal homogenates from normal and Na^+-depleted rat proximal colon. Standard molecular weights and primary antibody targets are indicated to the left and right of the blot images, respectively. **B** CFTR band intensity was quantified using densitometry analysis in ImageJ and normalized to actin band intensity as a loading control for each sample. **C** qRT-PCR analysis of CFTR-specific mRNA transcript abundance between normal and Na^+-depleted rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean ± SEM; $n = 3$ animals per group. *$p < 0.05$ compared with control (Normal) using unpaired Student's t test

the biophysical properties of K^+ channels for the purpose of identifying specific channels, vectorial transport functions cannot be measured using this technique. Nonetheless, our data are congruent with the findings from that study, as BK (and IK) channels were detectable, but aldosterone had no apparent effect on the expression of either channel type. It is possible that the function of these K^+ channels in the proximal colon is unrelated to luminal electrogenic K^+ secretion.

In conclusion, we have presented novel evidence of the up-regulation of NKCC1 and Cl^- secretory capacity by aldosterone in the rat proximal colon. This effect appears to be unrelated to electrogenic K^+ secretion, which differs from the effects of aldosterone on K^+ and Cl^- transport in the distal colon. Segmental differences should thus be accounted for when investigating or managing aldosterone-induced changes to colonic K^+ and Cl^- transport processes in research or clinical settings.

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Author contributions AJN conceived the idea, performed experiments, wrote manuscript. VMR conceived the idea and got funding. Both AJN and VMR discussed and edited the manuscript.

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Availability of data and material Data will be available on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval and consent to participate All animal experimental procedures used in this study were approved by the West Virginia University Institutional Animal Care and Use Committee prior to the start of the project.

Consent for publication Not applicable.

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