Regulation of Epithelial Na\(^+\) Transport by Soluble Adenylyl Cyclase in Kidney Collecting Duct Cells* 

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Alkalosis impairs the natriuretic response to diuretics, but the underlying mechanisms are unclear. The soluble adenylyl cyclase (sAC) is a chemosensor that mediates bicarbonate-dependent elevation of cAMP in intracellular microdomains. We hypothesized that sAC may be an important regulator of Na\(^+\) transport in the kidney. Confocal images of rat kidney revealed specific immunolocalization of sAC in collecting duct cells, and immunoblots confirmed sAC expression in mouse cortical collecting duct (mpkCCDc14) cells. These cells exhibit aldosterone-stimulated transepithelial Na\(^+\) currents that depend on both the apical epithelial Na\(^+\) channel (ENaC) and basolateral Na\(^+\),K\(^+\)-ATPase. RNA interference-mediated 60–70% knockdown of sAC expression comparably inhibited basal transepithelial short circuit currents (I\(_{sc}\)) in mpkCCDc14 cells. Moreover, the sAC inhibitors KH7 and 2-hydroxyestradiol reduced I\(_{sc}\) in these cells by 50–60% within 30 min. 8-Bromoadenosine-3',5'-cyclic-monophosphate substantially rescued the KH7 inhibition of transepithelial Na\(^+\) current. Aldosterone doubled ENaC-dependent I\(_{sc}\) over 4 h, an effect that was abolished in the presence of KH7. The sAC contribution to I\(_{sc}\) was unaffected with apical membrane nystatin-mediated permeabilization, whereas the sAC-dependent Na\(^+\) current was fully inhibited by basolateral ouabain treatment, suggesting that the Na\(^+\),K\(^+\)-ATPase, rather than ENaC, is the relevant transporter target of sAC. Indeed, neither overexpression of sAC nor treatment with KH7 modulated ENaC currents in Xenopus oocytes. ATPase and biotinylaton assays in mpkCCDc14 cells demonstrated that sAC inhibition decreases catalytic activity rather than surface expression of the Na\(^+\),K\(^+\)-ATPase. In summary, these results suggest that sAC regulates both basal and agonist-stimulated Na\(^+\) reabsorption in the kidney collecting duct, acting to enhance Na\(^+\),K\(^+\)-ATPase activity.

Maintenance of intracellular pH depends in part on the extracellular to intracellular Na\(^+\) gradient, and elevation of intracellular [Na\(^+\)] can lead to acidification of the cytoplasm. It has been shown that acidification of the cytoplasm of cells from frog skin and toad bladder by increased partial pressure of CO\(_2\) reduces Na\(^+\) transport and permeability (1, 2). Conversely, the rise in plasma bicarbonate caused by metabolic alkalosis with chronic diuretic use has been shown to increase net renal Na\(^+\) reabsorption independently of volume status, electrolyte depletion, and/or increased aldosterone secretion (3, 4). However, the underlying mechanisms involved in these phenomena remain unclear.

The soluble adenylyl cyclase (sAC) is a chemosensor that mediates the elevation of cAMP in intracellular microdomains (5–7). Unlike transmembrane adenylyl cyclases (tmACs), sAC is insensitive to regulation by forskolin or heterotrimeric G proteins (8) and is directly activated by elevations of intracellular calcium (9, 10) and/or bicarbonate ions (11). Thus, sAC mediates localized intracellular increases in cAMP in response to variations in bicarbonate levels or its closely related parameters, partial pressure of CO\(_2\) and pH. Mammalian sAC is more similar to bicarbonate-regulated cyanobacterial adenylyl cyclases than to other mammalian nucleotidyl cyclases, which may indicate that there is a unifying mechanism for the regulation of cAMP signaling by bicarbonate across biological systems. Although sAC appears to be encoded by a single gene, there is significant isoform diversity for this ubiquitously expressed enzyme (11, 12) generated by alternative splicing (reviewed in Ref. 13). sAC has been shown to regulate the subcellular localization and/or activity of membrane transport proteins such as the vacuolar H\(^+\)-ATPase (V-ATPase) and cystic fibrosis transmembrane conductance regulator in epithelial cells (14, 15). Functional activity of sAC has been reported in the kidney (16), and sAC has been localized to epithelial cells in the distal nephron (14, 17).

Given that natriuresis is decreased during metabolic alkalosis, when bicarbonate is elevated, and Na\(^+\) reabsorption is impaired by high partial pressure of CO\(_2\), we hypothesized that bicarbonate-regulated sAC may play a key role in the regulation

2 The abbreviations used are: sAC, soluble adenylyl cyclase; ENaC, epithelial Na\(^+\) channel; tmAC, transmembrane adenylyl cyclase; V-ATPase, vacuolar H\(^+\)-ATPase; PKA, protein kinase A; 8-Bromo-CAMP, 8-bromo-adenosine-3',5'-cyclic monophosphate; PBS, phosphate-buffered saline; HA, hemagglutinin; sACT, truncated sAC; cRNA, complementary RNA; CE, catechol estrogen; siRNA, small interfering RNA.

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of transepithelial Na\(^+\) transport in the distal nephron. Reabsorption of Na\(^+\) in the kidney and other epithelial tissues is mediated by the parallel operation of apical ENaC and basolateral Na\(^+\),K\(^+\)-ATPase, and both transport proteins can be stimulated by cAMP via the cAMP-dependent protein kinase (PKA) (18, 53). The aims of this study were to investigate the role of sAC in the regulation of transepithelial Na\(^+\) transport in the kidney through the use of specific sAC inhibitors and electrophysiological measurements. We found that sAC inhibition blocks transepithelial Na\(^+\) reabsorption in polarized mpkCCD\(_{c14}\) cells under both basal and hormone-stimulated conditions. Selective membrane permeabilization studies revealed that although ENaC activity appears to be unaffected by sAC inhibition, flux through the Na\(^+\),K\(^+\)-ATPase is sensitive to sAC modulation. Inhibiting sAC decreases ATPase activity without affecting plasma membrane expression of the pump; thus, tonic sAC activity appears to be required for Na\(^+\) reabsorption in kidney collecting duct.

**EXPERIMENTAL PROCEDURES**

Reagents and Chemicals—All of the chemicals were obtained from Sigma-Aldrich unless otherwise stated. The specific sAC inhibitor KH\(_7\) was synthesized, purified, and characterized as previously described (20). Estradiol and the catechol estrogen (CE) 2-hydroxyestradiol were obtained from Steraloids, Inc. 8-Bromoadenosine-3',5'-cyclic monophosphate (8-Bromo-cAMP) was obtained from Biomol.

Tissue Preparation, Immunofluorescence Labeling, and Confocal Microscopy—All of the animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague-Dawley rats were anesthetized using sodium pentobarbital (65 mg/kg of body weight, intraperitoneally), and their kidneys were perfused via the left ventricle with phosphate-buffered saline (PBS, pH 7.4) and then cryoprotected in 30% sucrose for 12 h at 4 °C. The sections were collected (Sakura Finetek), mounted on a cutting block, and then frozen (Sakura Finetek) 2-hydroxyestradiol were obtained from Steraloids, Inc. preincubated at a concentration of 50 μg/ml with the R21 antibody, and this peptide-antibody mixture was then used as the primary antibody. After primary antibody exposure the slides were washed twice for 5 min in PBS with 2.7% NaCl and then once with PBS. The secondary antibodies applied were goat anti-mouse conjugated to fluorescein isothiocyanate (1:100) or donkey anti-chicken conjugated to CY5 (1:100; secondary antibodies from Jackson Immunologicals). After washing off the secondary antibody as above, the slides were mounted in Vectashield (Vector Labs) and ovarized with an argon laser, as well as a green and red helium-neon lasers. The images obtained with the CY5 fluorophore were pseudo-colored red. The confocal laser acquisition settings were identical for both the peptide inhibition and the R21 antibody-stained slides, and the contrast levels of the two images were adjusted simultaneously in Photoshop (Adobe).

RNA Interference Knockdown of sAC in mpkCCD\(_{c14}\) Cells—mpkCCD\(_{c14}\) cells at ~70% confluency in a 6-well plate were transected as per the manufacturer’s recommendations using 10 μl of Lipofectamine 2000 (Invitrogen)/well along with 10 μl (200 pmol) of either siRNA directed against mouse sAC (5’-UCGGAGCUGAUUGAAUCGUU-3’), or control siGENOME® NonTargeting siRNA (mixtures of sequences 5’-UGCGACUAAACACACUCAA-3’, 5’-UAA-GCCUAUGAAGAGAUC-3’, 5’-AUGUAUUGCCUG-UUUAG-3’, and 5’-AUGAAGCUAUCGCUAC-3’). The following day the cells were trypsinned and seeded onto 0.33-cm² Costar Transwell filters (~2.5 × 10⁵ cells/filter; two filters/transfection). Transwell filters were then used for electrophysiological and biochemical measurements after an additional 3 days when transepithelial resistance values exceeded 3 kΩ·cm².

Transepithelial I\(_{sc}\) Measurements—mpkCCD\(_{c14}\) cells were cultured as previously described (24). The cells grown on Transwell filter supports (Costar) were mounted in modified Costar Ussing chambers, and the cultures were continuously short-circuited with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance was measured by periodically applying a 2.5-mV bipolar pulse and calculated by Ohm’s law. The bathing Ringer’s solution composition, gassing, and washing techniques have been described previously (25). In some experiments equivalent I\(_{sc}\) was measured using a portable epithelial volt ohmmeter (World Precision Instruments), as previously described (26).

To isolate apical membranes electrically, 100 μM nystatin was added to the basolateral side to permeabilize the membrane, and an apical (140 mM) to basolateral (25 mM) Na\(^+\) gradient was established as described previously (25). For apical membrane permeabilization with nystatin, bathing Ringer’s solutions were kept identical on both sides of the chamber. In some experiments where the apical membrane was permeabilized, the same modified Ussing chambers were used to record
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simultaneous \( I_{sc} \) and total capacitance (\( C_t \)) traces, as described in detail previously (25, 27, 28).

**Oocyte Two-electrode Voltage Clamp Measurements**—NH\textsubscript{2}-terminal glutathione S-transferase-tagged truncated mouse sAC (sACt) was subcloned into the dual mammalian oocyte expression vector pMO (29) using the BamHI and EcoRI restriction sites in pMO and PCR amplification of template sACt (8). An NH\textsubscript{2}-terminal hemagglutinin (HA) tag was also added. The following primers were used for PCR amplification: 5’-TCATCATGGATCCCCATGTACACGATGTTCAGATTACGCTTTCTCATT-3’ (sense) and 5’-TCATCATCGAATTCTAACAAGTCACTTTCTCATT-3’ (antisense). The resulting pMO-HA-sACt plasmid was verified by DNA sequencing. Complementary RNAs (cRNAs) of sACt used for Xenopus oocyte expression were synthesized using the mMessage mMachine kit (Ambion) according to the manufacturer’s instructions after linearizing template pMO-HA-sACt plasmid DNA with HpaI. ENaC cRNAs were similarly synthesized, and oocytes were harvested, collagenase-treated, and maintained as described previously (29), and injected with cRNAs as indicated in the Fig. 7 legend. Two-electrode voltage clamp measurements of amiloride-sensitive ENaC currents in oocytes were performed 1–2 days after cRNA injection as described previously (29) after microinjection with KH\textsubscript{7} or vehicle (Me\textsubscript{2}SO).

**Immunoblotting and Cell Surface Biotinylation Assays**—R21 primary antibody against sAC was used at 1:1000 for immunoblotting. For peptide competition controls, immunizing peptide was incubated with R21 antibody diluted in Tris-buffered saline Tween 20 + 5% milk for 30 min prior to blotting. The final concentration of immunizing peptide used was 25 \( \mu \)g/ml.

Surface biotinylation studies were performed on mpkCCD\textsubscript{c14} cells based on a previously described protocol (30). Cells grown on Transwells (Costar) were washed three times for 5 min with ice-cold PBS containing Mg\textsuperscript{2+} and Ca\textsuperscript{2+} with agitation on ice to remove media. The basolateral membrane was biotinylated using 1.6 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS for 20 min. The apical surface was incubated in medium containing 10% fetal bovine serum to prevent biotinylation of apical proteins. The biotinylation reaction was then quenched by adding 10% fetal bovine serum-containing medium to the basolateral surface. The monolayers were washed three times with ice-cold PBS with agitation on ice prior to lysing cells in cell lysis buffer (0.4% deoxycholic acid, 1% Nonidet P-40, 50 mm EGTA, 10 mm Tris-Cl, pH 7.4) plus protease inhibitors at room temperature for 10 min. Protein concentration of the postnuclear supernatant was determined, and 250 \( \mu \)g of protein was combined with streptavidin-Sepharose beads (Pierce) and incubated overnight at 4 °C. Samples from the streptavidin beads were washed three times in radioimmunoprecipitation assay buffer and collected in 2 \( X \) sample buffer containing 10% \( \beta \)-mercaptoethanol and incubated for 20 min at room temperature. The proteins were heated to 95°C for 3 min, separated by SDS-PAGE, and subjected to Western blot analysis using anti-Na\textsuperscript{+},K\textsuperscript{+}-ATPase \( \alpha \) subunit antibody (Santa Cruz Biotechnology).

Na\textsuperscript{+},K\textsuperscript{+}-ATPase Activity Assays—ATPase assays were performed essentially as described previously (31), based on the method of Forbush (32). mpkCCD\textsubscript{c14} cells grown on 6-well Transwells were washed three times for 5 min in ice-cold PBS containing Mg\textsuperscript{2+} and Ca\textsuperscript{2+}. The cells were scraped in PBS and pelleted. The cells were then resuspended in lysis buffer (150 mm NaCl, 10 mm Tris-Cl, 2 mm EDTA, pH 7.0) containing protease inhibitors and sonicated for 5 s. The lysate was centrifuged at 13,000 \( \times \) g for 3 min, and the supernatant was transferred to a high speed microcentrifuge tube. The supernatant was centrifuged at 100,000 \( \times \) g for 1 h. The pellet containing Na\textsuperscript{+},K\textsuperscript{+}-ATPase was resuspended in 50 mm imidazole, 2 mm EDTA, pH 7.0, and protein concentration was determined. To activate latent Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, 50 \( \mu \)g of total protein was then added to a final volume of 150 \( \mu \)l in buffer containing 0.065% deoxycholic acid in 50 mm imidazole, 2 mm EDTA, pH 7.0, and incubated for 30 min at room temperature. Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity was measured by incubating 25 \( \mu \)l of the activated sample for 10 min at 37°C with 1 ml of assay buffer (120 mm NaCl, 25 mm KCl, 4 mm ATP, 4 mm MgCl\textsubscript{2}, 60 mm Tris-Cl, 1 mm EDTA, pH 7.5) in the presence or absence of 1 mm ouabain and 100 \( \mu \)g/ml strophanthidin. The reactions were stopped with 1 ml of ice-cold 0.5 M HCl containing 30 mg of ascorbic acid, 5 mg of ammonium hematolysate, and 10 mg of SDS. The tubes were then transferred to an ice bath for 10 min prior to adding 1.5 ml of color development solution containing 30 mg of sodium meta-arsenite, 30 mg of sodium citrate, and 30 \( \mu \)l of acetic acid. The tubes were finally heated for 10 min at 37°C, and absorbance was read at 850 nm. The difference in absorbance between untreated samples and samples treated with Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitors is reported.

**Statistics**—Statistical analyses were performed using either StatView (SAS) or SigmaPlot (Jandel Scientific) software. Unpaired Student’s \( t \) tests were performed to compare relevant data samples from \( I_{sc} \), biotinylation, and ATPase measurements. Analysis of variance was used to compare data obtained from different batches of oocytes for two-electrode voltage clamp experiments. In all cases \( p \) values < 0.05 were considered significant.

**RESULTS**

sAC Expression in Kidney and mpkCCD\textsubscript{c14} Cells—We and others have previously shown sAC expression in rat kidney using immunofluorescence labeling and confocal microscopy in distal tubular epithelial cells (especially thick ascending limb of Henle’s loop and collecting duct) (14, 17). We confirmed sAC localization in the thick ascending limb of Henle’s loop and demonstrated its presence in both collecting duct principal cells, which are the cells that express ENaC, and intercalated cells, which express apical membrane V-ATPase (Fig. 1A, right panel) (33). Immunofluorescence labeling of sAC was fully competed off using the immunizing peptide for this antibody (Fig. 1A, left and middle panels), demonstrating the specificity of this staining for sAC. To confirm that sAC is expressed in immortalized mouse collecting duct cells, we immunoblotted mpkCCD\textsubscript{c14} cell lysates using the R21 antibody (Fig. 1B). Several bands are apparent, including major bands at ~50–53 kDa, consistent with the predicted mobility of somatic sAC isoforms previously identified in whole kidney (34). All of the bands were competed off with the immunizing peptide, suggesting that the
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FIGURE 1. Expression of sAC in rat kidney sections and in mpkCCD$_{c14}$ cells. A, confocal immunofluorescence staining of sAC in rat kidney (left panel) was completely abolished by preincubating the primary R21 antibody with sAC immunizing peptide (middle panel). Right panel, immunofluorescence localization of sAC in the thick ascending limb of Henle's loop (*) and in a principal cell (inset, arrowhead; sAC, green) and in an intercalated cell, which expresses the V-ATPase (inset, arrow; V-ATPase, red) in kidney collecting duct segments (inset). Scale bar, 25 μm. B, Western blot of mpkCCD$_{c14}$ cell lysates for sAC expression using the R21 antibody preincubated in the absence (left lane) or presence of the immunizing peptide (right lane).

other bands represent additional, as yet uncharacterized sAC isoforms or degradation bands of the known full-length or sACt splice variants. Thus, sAC is present in kidney epithelial cells, both intercalated cells and principal cells, and in the sodium reabsorbing, principal cell-like mpkCCD$_{c14}$ cell line.

Knockdown of sAC Inhibits Transepithelial Na$^+$ Current in mpkCCD$_{c14}$ Cells—To test whether sAC plays a role in regulating transepithelial Na$^+$ transport under basal conditions, we knocked down sAC protein expression in polarized mpkCCD$_{c14}$ cells using siRNA oligonucleotides whose sequences are analogous to siRNAs previously shown to be effective in rat cell lines (35, 36) and primary cells (37). We then mounted the polarized monolayers in Ussing chambers to measure the effect of sAC knockdown on $I_{sc}$ (Fig. 2). As compared with control (nontargeting) siRNA, transfection of siRNA directed against mouse sAC yielded ~60% knockdown of the predominant somatic sAC isoforms of ~50–53 kDa (Fig. 2, A and B). In association with this sAC knockdown, amiloride-sensitive $I_{sc}$ was inhibited to a comparable extent (~70%; Fig. 2C). This finding in kidney-derived collecting duct cells suggests that sAC is required for Na$^+$ reabsorption under base-line conditions. However, this experimental approach cannot address the potential role of acute changes in sAC activity on Na$^+$ transport.

sAC Inhibitors Block Basal and Stimulated Transepithelial Na$^+$ Reabsorption in mpkCCD$_{c14}$ Cells—To test how quickly and to what extent Na$^+$ current responds to sAC modulation, we treated polarized mpkCCD$_{c14}$ cells mounted in Ussing chambers with the recently characterized sAC-specific inhibitor KH7 (20, 36–39). Apical KH7 treatment (60 μM) caused a rapid reduction of $I_{sc}$ to 40–50% of control levels by 30 min (Fig. 3A). We observed a similar time- and concentration-dependent inhibition of $I_{sc}$ by an alternative sAC-selective inhibitor (40), the CE compound 2-hydroxyestradiol (20–120 μM; Fig. 3B). The structurally similar compound 17β estradiol (120 μM), which does not inhibit sAC, had no effect on $I_{sc}$ (Fig. 3B). Monitoring of transepithelial resistance, as measured by periodic voltage pulses during voltage-clamp recordings of $I_{sc}$ (see “Experimental Procedures”), demonstrated that these sAC inhibitors did not have discernable effects on tight junction

FIGURE 2. RNA interference-mediated sAC knockdown inhibits transepithelial Na$^+$ currents in mpkCCD$_{c14}$ cells. A, representative immunoblots of mpkCCD$_{c14}$ cell lysates of two filters per condition derived from a single transfection with either control (Con), nontargeting siRNA (left panels) or siRNA directed against sAC (right panels) and probed for either sAC (upper panels) or β-actin (lower panels). B, mean (± S.E.) relative sAC protein expression levels corrected for β-actin expression in control siRNA- and sAC siRNA-transfected cells (*, p = 0.003; unpaired t tests relative to controls, n = 5 filters for each condition from three separate transfections). C, mean (± S.E.) relative amiloride-sensitive $I_{sc}$ values (differences in $I_{sc}$ values measured in Ussing chambers before versus after addition of 10 μM amiloride to the apical bath) in control and sAC siRNA-transfected cells. (#, p < 0.001; unpaired t tests relative to controls, n = 5 filters for each condition from three separate transfections).
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**FIGURE 3.** sAC activity is required in the short term to sustain transepithelial Na\(^+\) currents. Continuous measurements of transepithelial \(I_{sc}\) in polarized mpkCCD\(_{14}\) cells were performed in Ussing chambers as described under “Experimental Procedures.” The mean current at time 0 was 11.6 \(\mu A/cm^2\). The data shown for each time point are \(I_{sc}\) values (±S.E.) normalized to the corresponding \(I_{sc}\) values at time 0 from 4–9 replicate filters. A, filters were treated starting at time 0 with either 60 \(\mu M\) KH7 (open circles) or vehicle control (Me\(\text{SO}_2\), closed circles). KH7-treated filters had significantly reduced \(I_{sc}\) compared with controls at all time points after time 0 (\(p < 0.01\)). B, filters were treated starting at time 0 with either 120 \(\mu M\) estradiol (control, closed circles) or graded concentrations of the CE 2-hydroxyestradiol: 20 \(\mu M\) (open circles), 40 \(\mu M\) (closed triangles), 80 \(\mu M\) (open triangles), or 120 \(\mu M\) (closed squares). All of the filters treated with CE at concentrations ≥40 \(\mu M\) had significantly reduced \(I_{sc}\) compared with estradiol-treated filters at the same time point (\(p < 0.02\)).

Consistent with our earlier results, 10 \(\mu M\) forskolin induced a rapid increase in Na\(^+\) current to ∼300% of control levels over 60 min following pretreatment with vehicle control (Fig. 4). Interestingly, however, forskolin had no stimulatory effect on the measured \(I_{sc}\) following a 30-min pretreatment with KH7. Because forskolin stimulation of tmAC is not directly inhibited by KH7 (20, 36, 37, 39), these results suggest that the cAMP generated by forskolin-stimulated tmACs is not sufficient to overcome sAC inhibition and that sAC activity may be required for the increased transepithelial \(I_{sc}\) caused by stimulation of tmACs as well as basal Na\(^+\) transport.

Because the enzymatic product of sAC is cAMP, we next tested whether treating polarized mpkCCD\(_{14}\) cells with the cell-permeant cAMP analog 8-Bromo-cAMP could override the inhibition of Na\(^+\) current by the sAC inhibitor KH7. Treatment for 15 min with 1 mM 8-Bromo-cAMP approximately doubled the amiloride-sensitive equivalent \(I_{sc}\) under control conditions (Fig. 5, left bars). KH7 treatment for 30 min inhibited \(I_{sc}\) by ∼50% relative to untreated controls (gray bars). However, the addition of 8-Bromo-cAMP during the last 15 min of KH7 exposure substantially blunted this KH7 inhibition, approximately doubling the current relative to cells treated with KH7 alone (Fig. 5, right bars). These results suggest that treating these cells with membrane-permeable cAMP analogs can at least partially overcome the effects of sAC inhibition.

We next examined the effects of the sAC inhibitor KH7 on stimulation of ENaC-dependent Na\(^+\) transport by the mineralocorticoid aldosterone, an agonist that acts by fundamentally different mechanisms. Aldosterone stimulates Na\(^+\) transport primarily through the synthesis of new proteins, although non-genomic pathways have been described in some cell systems and tissues (43). Aldosterone stimulates the synthesis of proteins that enhance ENaC abundance (\(N\)) at the apical membrane by inhibiting Nedd4–2 dependent retrieval of the channel (44) and may also affect open probability (\(P_o\)) of the channel by either phosphatidylinositol 1,4,5-trisphosphate or direct methylation of the channel (45, 46). In addition, aldosterone...
induces increased synthesis of both ENaC subunits and the basolateral Na\(^{+}\),K\(^{+}\)-ATPase (47, 48). It was thus of interest to determine whether sAC activity is required for the aldosterone-dependent stimulation of Na\(^{+}\) transport in polarized mpkCCD\(_{14}\) cells. Cells treated with 1 \(\mu\)M aldosterone exhibited a doubling of amiloride-sensitive Na\(^{+}\) current over 4 h relative to untreated controls (Fig. 6, closed symbols). However, pretreatment with KH7 for 30 min prior to aldosterone addition caused a substantial reduction in current that was not significantly modulated by subsequent aldosterone treatment (Fig. 6, open symbols). These results suggest that the aldosterone-de-
meabilizes the plasma membrane to small univalent ions (49, 50). To isolate and examine potential effects on ENaC conductance at the apical membrane, nystatin was added basolaterally, followed by KH7 treatment and then amiloride (Fig. 8A). Under these conditions with basolateral conductance shunted, all current should reflect the activity of the apical Na\(^+\)/H\(^+\) channel ENaC. Application of KH7 had no effect on the Na\(^+\)/H\(^+\) current under these conditions, whereas subsequent amiloride addition fully inhibited \(I_{sc}\), confirming the presence of a robust apical membrane ENaC conductance (Fig. 8A). Specifically, the treatment-associated change in \(I_{sc}\) was not significantly different from 0 in either KH7- or vehicle-treated cell monolayers (0.9 ± 2.3 versus 0.4 ± 2.0 μA/cm\(^2\), respectively; \(p = 0.86\)), nor was there any difference in amiloride-sensitive current between KH7 or vehicle treatment (17.5 ± 2.9 versus 15.5 ± 1.8 μA/cm\(^2\), respectively; \(p = 0.57\); \(n = 7\) filters for both conditions).

B. KH7 inhibits \(I_{sc}\) (solid line) following apical nystatin permeabilization, and ouabain addition rapidly blocks remaining \(I_{sc}\). The large increase in \(C_T\) (dashed line) within 10 min of apical nystatin treatment indicates effective apical membrane permeabilization (25). C. Acute activation of the Na\(^+\)/K\(^+\)-ATPase through cellular Na\(^+\) loading triggered by apical nystatin permeabilization was performed to measure pump capacity with prior treatment of vehicle (solid line) or KH7 (dotted line) to inhibit sAC. Ouabain-sensitive \(I_{sc}\) was defined as the difference in steady-state \(I_{sc}\) following nystatin permeabilization before and after ouabain treatment (indicated for vehicle-treated cells by dashed lines). D. Summary of mean (± S.E.) ouabain-sensitive currents with or without 60 μM KH7 treatment from experiments shown in C (*, \(p < 0.001\), \(n = 3–7\) filters for each condition).

To determine whether sAC inhibition affected basolateral membrane conductance, we added KH7 following nystatin permeabilization of the apical membrane. This caused a substantial inhibition of \(I_{sc}\) that was further inhibited by subsequent ouabain treatment, thus implicating the basolateral membrane Na\(^+\)/K\(^+\)-ATPase as the target for sAC inhibition (Fig. 8B). The large increase in \(C_T\) (Fig. 8B, dashed line) within 10 min following nystatin treatment indicates effective apical membrane permeabilization. To test the effects of
sAC inhibition on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, specifically pump capacity in the face of intracellular Na\textsuperscript{+} loading, we compared changes in $I_{sc}$ after initially treating with KH7 or vehicle for 30 min, followed by acute apical membrane permeabilization, and then subsequent ouabain treatment (Fig. 8C). Following a reproducible, transient spike in $I_{sc}$ as nystatin permeabilizes the apical membrane (see also Fig. 8B), the steady-state ouabain-sensitive $I_{sc}$ was significantly reduced in the KH7-treated cells by a mean of $\sim 60\%$ (Fig. 8D), indicating that sAC activity is required for Na\textsuperscript{+} pump activity.

\textbf{sAC Inhibition Blocks ATPase Activity without Affecting Surface Expression of the Na\textsuperscript{+} Pump—Regulation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase by sAC could conceivably occur via effects on basolateral membrane expression of the Na\textsuperscript{+} pump or on its ATPase activity. To test whether decreased membrane expression could account for the inhibition of Na\textsuperscript{+} pump-mediated conductance associated with sAC inhibition, surface biotinylation labeling was performed to measure expression of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase catalytic $\alpha$ subunit at the basolateral membrane in polarized mpkCCD\textsubscript{14} cells 0–30 min after treatment with KH7 (Fig. 9). Consistent with previous observations in this cell line and \textit{in vivo} that the Na\textsuperscript{+} pump is localized largely in the basolateral membrane by immunofluorescence staining (51, 52), virtually all of the $\alpha$ subunit appeared in the biotinylated fraction (Fig. 9A). Of note, the relative proportion of biotinylated $\alpha$ subunit did not change significantly at 15 or 30 min after the addition of 60 $\mu$M KH7 (Fig. 9, A and B). Parallel measurements at the 30-min time point demonstrated a reduced equivalent $I_{sc}$ of $5.83 \pm 0.24 \mu A/cm^2$ in KH7-treated cells (versus $11.43 \pm 1.45 \mu A/cm^2$ in vehicle-treated cells; $p = 0.02, n = 3$ filters for each condition). These findings suggest that the KH7-dependent inhibition of current does not result from a reduction in Na\textsuperscript{+} pump expression at the basolateral membrane.

\textbf{DISCUSSION}

Intracellular coupling between pH changes and transepithelial salt transport has long been recognized, although the mechanisms for such coupling remain unclear. This is the first study to recognize and investigate sAC-dependent regulation of Na\textsuperscript{+} transport across epithelia. Our results confirm that sAC is specifically expressed in distal nephron renal epithelial cells (Fig. 1) and demonstrate that its activity is required for Na\textsuperscript{+} transport under basal conditions both acutely and chronically (Figs. 2 and 3) and after cAMP or aldosterone stimulation (Figs. 4–6). The addition of the cell-permeant cAMP analog 8-Bromo-cAMP partially restored the KH7-inhibited Na\textsuperscript{+} current, demonstrating that the cells exposed to KH7 retained their ability to respond to cAMP (Fig. 5). The finding that 8-Bromo-cAMP was not able to activate $I_{sc}$ fully in the presence of KH7 to a level which is significantly different.
similar to that achieved in the absence of KH7 raises the possibility that sAC effects on the Na\(^+\) pump in these cells may be compartmentalized or sequestered in microdomains not readily accessible to 8-Bromo-cAMP. Alternatively or additionally, the Na\(^+\) transport inhibition caused by the sAC inhibition may not be rapidly reversible. Because ENaC-dependent Na\(^+\) transport at the apical membrane is rate-limiting in mpkCCD14 cells under normal conditions (24), we initially suspected that ENaC was the relevant target for sAC regulation. However, direct co-expression of ENaC and sAC in oocytes (Fig. 7) and selective permeabilization of the basolateral membrane in CCD cells to isolate ENaC conductance (Fig. 8) did not support the hypothesis that ENaC is modulated by sAC or by sAC inhibition. On the other hand, selective apical permeabilization studies to isolate pump current strongly suggest that the basolateral pump is the target of sAC inhibition (Fig. 8). Direct measures of ATPase activity confirmed this hypothesis and demonstrated that sAC-dependent regulation of the Na\(^+\) pump occurs via effects on ATPase activity (Fig. 10) rather than basolateral membrane expression (Fig. 9). This result is not unexpected given the rapidity of the current modulation in the face of sAC inhibitors (i.e. within minutes). Moreover, it has been widely recognized that, unlike in the case of ENaC, a very high proportion of total cellular Na\(^+\) pump expression exists at the basolateral membrane rather than in intracellular or cytoplasmic compartments in epithelial cells. (cf. Fig. 9 and Refs. 51 and 52).

The mechanism of sAC-dependent regulation of the Na\(^+\),K\(^+\)-ATPase is currently unclear. Because sAC activity generates cAMP, PKA is an attractive candidate mediator. Indeed, PKA-dependent phosphorylation of \(\alpha\) and \(\gamma\) (FXYD) subunits has been reported to regulate Na\(^+\) pump catalytic activity (53–55). In addition, PKA phosphorylation may regulate association of the \(\alpha\) and \(\gamma\) subunits, which may also be an important mechanism of pump activity regulation (56). Although CAMP and PKA have been implicated in the regulation of trafficking of membrane transport proteins (e.g. V-ATPase, aquaporin 2, and cystic fibrosis transmembrane conductance regulator) (14, 19, 57, 58), our results demonstrate that surface expression of the \(\alpha\) subunit of the pump was not affected by the sAC inhibitor KH7 (Fig. 9). However, we cannot exclude the possibility that translocation of either the \(\beta\) or \(\gamma\) subunits may be affected. Nevertheless, the reduction in ATPase activity with sAC inhibition appears to be sufficient to account for the current inhibition given the reductions in both current and ATPase activity observed following KH7 treatment (Fig. 10). Thus, a direct effect on catalytic activity appears to be the simplest explanation to account for our results. Other candidate mediators of the sAC effect are Epacs, which are small guanine nucleotide exchange factors that are activated by cAMP and function independently of PKA (59). These unresolved mechanistic issues are questions for future investigation.

Potential future approaches to confirm our in vitro data could involve knock-out or tissue-specific and temporal knock-down approaches in vivo. Importantly, however, there is specificity to our data because both RNA silencing and treatment with two different classes of sAC inhibitors (KH7 and CEIs) yielded similar dramatic effects on Na\(^+\) transport. Transepithelial resistance was also well preserved following these treatments, confirming the integrity and viability of the cell monolayers. Moreover, ENaC activity was not affected in any detectable manner, arguing against a generalized effect of sAC on ion transport proteins or cellular processes.

In summary, our results demonstrate that sAC activity is an important regulator of Na\(^+\) transport in collecting duct epithelial cells via regulation of the Na\(^+\),K\(^+\)-ATPase at the basolateral membrane. Further characterization of the sAC-dependent regulation of transepithelial Na\(^+\) transport is warranted and could identify novel targets for the treatment of hypertension and diuretic resistance. Moreover, because the Na\(^+\) pump is expressed ubiquitously in cells of all organs, its potential regulation by sAC could be important in other tissues such as cardiac muscle and neurons. Indeed, it would be of interest to determine whether sAC-dependent regulation of the Na\(^+\) pump plays a role in the pathogenesis of certain cardiac and nervous system disorders, such as heart failure, arrhythmias, and seizures.

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REFERENCES
1. Palmer, L. G. (1985) J. Membr. Biol. 83, 57–69
2. Ussing, H. H., and Zerahn, K. (1951) Acta Physiol. Scand. 23, 110–127
3. Bosch, J. P., Goldstein, M. H., Levitt, M. F., and Kahn, T. (1977) Am. J. Physiol. 232, F397–F404
4. Loon, N. R., and Wilcox, C. S. (1998) Clin. Sci. (Lond.) 94, 287–292
5. Zippin, J. H., Chen, Y., Nahirny, P., Kamenetsky, M., Wuttke, M. S., Fischman, D. A., Levin, L. R., and Buck, J. (2003) FEBS J. 17, 82–84
6. Zippin, J. H., Farrell, J., Huron, D., Kamenetsky, M., Hess, K. C., Fischman, D. A., Levin, L. R., and Buck, J. (2004) J. Cell Biol. 164, 527–534
7. Sill, R. A., and Insel, P. A. (2004) Sci. STKE 2004, pe19
8. Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J., and Levin, L. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 79–84
9. Litvin, T. N., Kamenetsky, M., Zarifyan, A., Buck, J., and Levin, L. R. (2003) J. Biol. Chem. 278, 15922–15926
10. Jaiswal, B. S., and Conti, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10676–10681
11. Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Buck, J. (2000) Science 289, 625–628
12. Geng, W., Wang, Z., Zhang, J., Reed, B. Y., Pak, C. Y., and Moe, O. W. (2005) Am. J. Physiol. 298, C1305–C1316
13. Kamenetsky, M., Middelhaufe, S., Bank, E. M., Levin, L. R., Buck, J., and Steegborn, C. (2006) J. Mol. Biol. 362, 623–639
14. Pastor-Soler, N., Beaulieu, V., Litvin, T. N., Da Silva, N., Chen, Y., Brown, D., Buck, J., Levin, L. R., and Breton, S. (2003) J. Biol. Chem. 278, 49523–49529
15. Wang, Y., Lam, C. S., Wu, F., Wang, W., Duan, Y., and Huang, P. (2005) Am. J. Physiol. 289, C1415–C1151
16. Wittig, T. W., Guo, W. B., and Kobayashi, K. (1993) Am. J. Physiol. 264, F1060–F1064
17. Paunesku, T. G., Da Silva, N., Russo, L. M., McKee, M., Lu, H. A., Breton, S., and Brown, D. (2008) Am. J. Physiol. 294, F130–F138
18. Snyder, P. M., Olson, D. R., Kabara, R., Zhou, R., and Steines, J. C. (2004) J. Biol. Chem. 279, 45753–45758
19. Pastor-Soler, N. M., Hallows, K. R., Smolak, C., Gong, F., Brown, D., and Breton, S. (2008) Am. J. Physiol. 294, C488–C494
20. Hess, K. C., Jones, B. H., Marquez, B., Chen, Y., Ord, T. S., Kamenetsky, M., Miyamoto, C., Zippin, J. H., Kopf, G. S., Suarez, S. S., Levin, L. R., Williams, C. J., Buck, J., and Moss, S. B. (2005) Dev. Cell 9, 249–259
21. Breton, S., Hammar, K., Smith, P. J., and Brown, D. (1998) Am. J. Physiol.
| Reference                                                                 | Page |
|--------------------------------------------------------------------------|------|
| Breton, S., Nsumu, N. N., Galli, T., Sabolic, I., Smith, P. J., and Brown, D. (2000) | 278, F717–F725 |
| Brown, D., Lydon, J., McLaughlin, M., Stuart-Tilley, A., Tyszkowski, R., and Alper, S. (1996) | 105, 261–267 |
| Bens, M., Vallet, V., Cluzeaud, F., Pascual-Letallec, L., Kahn, A., Rafestin-Oblin, M. E., Rossier, B. C., and Vandewalle, A. (1999) | Am. Soc. Nephrol. 10, 923–934 |
| Butterworth, M. B., Edinger, R. S., Johnson, J. P., and Frizzell, R. A. (2005) | J. Gen. Physiol. 125, 81–101 |
| Mohan, S., Bruns, J. R., Weixel, K. M., Edinger, R. S., Bruns, J. B., Kleyman, T. R., Johnson, J. P., and Weisz, O. A. (2004) | J. Biol. Chem. 279, 32071–32078 |
| Carattino, M. D., Edinger, R. S., Grieser, H. J., Wise, R., Neumann, D., Schlattner, U., Johnson, J. P., Kleyman, T. R., and Hallows, K. R. (2005) | J. Biol. Chem. 280, 17608–17616 |
| Gonin, S., Deschenes, G., Roger, F., Bens, M., Martin, P. Y., Carpentier, J. L., Vandewalle, A., Doucet, A., and Feraille, E. (2001) | Mol. Biol. Cell 12, 255–264 |
| Rokaw, M. D., West, M., and Johnson, J. P. (1996) | J. Biol. Chem. 271, 32468–32473 |
| Forbush, B., Ill (1983) | Anal. Biochem. 128, 159–163 |
| Nelson, R. D., Guo, X. L., Masood, K., Brown, D., Kalkbrenner, M., and Gluck, S. (1992) | Proc. Natl. Acad. Sci. U. S. A. 89, 3541–3545 |
| Farrell, J., Ramos, L., Tresguerres, M., Kamenetsky, M., Levit, L. R., and Buck, J. (2008) | PLoS ONE 3, e3251 |
| Ramos, L. S., Zippen, J. H., Kamenetsky, M., Buck, J., and Levin, L. R. (2008) | J. Gen. Physiol. 132, 329–338 |
| Stessin, A. M., Zippen, J. H., Kamenetsky, M., Hess, K. C., Buck, J., and Levin, L. R. (2006) | J. Biol. Chem. 281, 17253–17258 |
| Wu, K. Y., Zippen, J. H., Huron, D. R., Kamenetsky, M., Hengst, U., Buck, J., Levin, L. R., and Jaffrey, S. R. (2006) | Nat. Neurosci. 9, 1257–1264 |
| Schmid, A., Sutto, Z., Nlend, M. C., Horvath, G., Schmid, N., Buck, J., Levin, L. R., Conner, G. E., Fregien, N., and Salathe, M. (2007) | J. Gen. Physiol. 130, 99–109 |
| Young, J. J., Mehdhi, A., Stohl, L. L., Levin, L. R., Buck, J., Wagner, J. A., and Stessin, A. M. (2008) | J. Neurosci. Res. 86, 118–124 |
| Steegborn, C., Litvin, T. N., Hess, K. C., Capper, A. B., Taussig, R., Buck, J., Levin, L. R., and Wu, H. (2005) | J. Biol. Chem. 280, 31754–31759 |
| Butterworth, M. B., Edinger, R. S., Frizzell, R. A., and Johnson, J. P. (2009) | Am. J. Physiol. 296, F10–F24 |
| Kleyman, T. R., Ernst, S. A., and Coupaye-Gerard, B. (1994) | Am. J. Physiol. 266, F506–F511 |
| Harvey, B. J., Alzamora, R., Stubbs, A. K., Irenaten, M., McEneaney, V., and Thomas, W. (2008) | J. Steroid Biochem. Mol. Biol. 108, 310–317 |
| Bhatta, V., Soundararajan, R., Pao, A. C., Li, H., and Pearce, D. (2006) | Am. J. Physiol. 291, F714–F721 |
| Pochynyuk, O., Tong, Q., Staruschenko, A., Ma, H. P., and Stockand, J. D. (2006) | Am. J. Physiol. 290, F949–F957 |
| Stockand, J. D., Edinger, R. S., Eaton, D. C., and Johnson, J. P. (2000) | News Physiol. Sci. 15, 161–165 |
| Masilamani, S., Kim, G. H., Mitchell, C., Wade, J. B., and Knepper, M. A. (1999) | J. Clin. Investig. 104, 19–23 |
| Welling, P. A., Caplan, M., Sutters, M., and Giebish, G. (1993) | J. Biol. Chem. 268, 23469–23476 |
| Cass, A., Finkelstein, A., and Krepsi, V. (1970) | J. Gen. Physiol. 56, 100–124 |
| Lewis, S. A., Eaton, D. C., Clausen, C., and Diamond, J. M. (1977) | J. Gen. Physiol. 70, 427–440 |
| Summa, V., Camargo, S. M., Bauch, C., Zeecevic, M., and Verrey, F. (2004) | J. Physiol. 555, 355–364 |
| Wetzel, R. K., and Sweadner, K. J. (2001) | Am. J. Physiol. 281, F531–F545 |
| Cheng, X. J., Fisone, G., Aizman, O., Aizman, R., Levenson, R., Greengard, P., and Aperia, A. (1997) | Am. J. Physiol. 273, C893–C901 |
| Therien, A. G., and Blostein, R. (2000) | Am. J. Physiol. 279, C541–C566 |
| Cortes, V. F., Veiga-Lopes, F. E., Barrabin, H., Alves-Ferreira, M., and Fontes, C. F. (2006) | Int. J. Biochem. Cell Biol. 38, 1901–1913 |
| Franzin, C. M., Gong, X. M., Teriete, P., and Marassi, F. M. (2007) | J. Bioenerg. Biomembr. 39, 379–383 |
| Brown, D. (2003) | Am. J. Physiol. 284, F893–F901 |
| Kleizen, B., Braakman, I., and de Jonge, H. R. (2000) | Eur J Cell Biol. 79, 544–556 |
| de Rooij, I., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A., and Bos, J. L. (1998) | Nature 396, 474–477 |