The Drosophila NKCC Ncc69 is required for normal renal tubule function

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Rodan AR, Baum M, Huang CL. The Drosophila NKCC Ncc69 is required for normal renal tubule function. Am J Physiol Cell Physiol 303: C883–C894, 2012. First published August 22, 2012; doi:10.1152/ajpcell.00201.2012.—Epithelial ion transport is essential to renal homeostatic function, and it is dysregulated in several diseases, such as hypertension. An understanding of the insect renal (Malpighian) tubule yields insights into conserved epithelial ion transport processes in higher organisms and also has implications for the control of insect infectious disease vectors. Here, we examine the role of the Na+/K+2Cl− (NKCC) cotransporter Ncc69 in Drosophila tubule function. Ncc69 mutant tubules have decreased rates of fluid secretion and K+ flux, and these phenotypes were rescued by expression of wild-type Ncc69 in the principal cells of the tubule. Na+ flux was unaltered in Ncc69 mutants, suggesting Na+ recycling across the basolateral membrane. In unstimulated tubules, the principal role of the Na+/K+-ATPase is to generate a favorable electrochemical gradient for Ncc69 activity: while the Na+/K+-ATPase inhibitor ouabain decreased K+ flux in wild-type tubules, it had no effect in Ncc69 mutant tubules. However, in the presence of cAMP, which stimulates diuresis, additional Na+/K+-ATPase-dependent K+ transport pathways are recruited. In studying the effects of capa-1 on wild-type and Ncc69 mutant tubules, we found a novel antidiuretic role for this hormone that is dependent on intact Ncc69, as it was abolished in Ncc69 mutant tubules. Thus, Ncc69 plays an important role in transepithelial ion and fluid transport in the fly renal tubule and is a target for regulation in antidiuretic states.

Malpighian tubule; epithelial ion transport; capa-1; Na+/K+-ATPase; diuretic

Epithelial ion transport is central to the kidney’s role in maintaining internal homeostasis in the face of external challenges, such as fluid or solute ingestion. The insect renal epithelium, the Malpighian tubule, is a relatively simple system in which to study these processes, with implications for conserved processes in human kidney and for the eradication of insect disease vectors (17). The physiologic study of Drosophila melanogaster tubules, in which powerful genetics can be combined with pharmacologic techniques, was established in 1994 by Dow and colleagues (16).

The Drosophila renal excretory system consists of four Malpighian tubules, an anterior pair and a posterior pair, which empty into the gut at the midgut/hindgut junction (18). Because the tubules are blind-ended, all ion and water transport occurs by transepithelial flux. There are four segments and two cell types in the Drosophila renal tubule (73). In the main segment, which is K+- and water-secreting (53), cation flux occurs through principal cells, in which the apical H+/K+-ATPase drives proton secretion into the lumen (1, 10, 14, 16, 20, 52) (see Fig. 4B). It is thought that protons are then exchanged for Na+ and K+ through apical H+/cation exchangers, allowing cation secretion (13). Chloride flux occurs through stellate cells, probably through chloride channels, although the genes encoding these channels have not been identified (52, 55). Water follows the movement of KCl and NaCl, possibly through aquaporins, several of which are expressed in the fly tubule (39).

Work in several insect species, including Drosophila, has implicated Na+/K+-2Cl− (NKCC) cotransport in the uptake of cations from the hemolymph into the principal cell across the basolateral membrane (27, 30, 31, 33, 35, 37, 44, 54). These studies have relied on the use of the pharmacologic NKCC inhibitor bumetanide, as well as the determination of electrochemical gradients for Na+, K+, and Cl−. To date, the genes encoding insect tubule NKCCs have not been identified. Two genes with sequence homology to the mammalian sodium-chloride cotransporter (NCC)/NKCC family exist in Drosophila: CG31547 (also called Ncc83) and Ncc69 (42, 75). Of these, Ncc69 has been shown to encode a bona fide Na+/K+2Cl− cotransporter (42, 75). Ncc69 mutants have a glial defect, possibly due to impaired ion transport across the glial cell membrane (42), but tubule function has not previously been studied.

The apical H+/ATPase is thought to be the primary driver of transepithelial ion and water flux in the insect tubule, while the role of the Na+/K+2Cl−ATPase has been less clear. Bafilomycin, a H+/ATPase inhibitor, completely abolishes fluid secretion from the fly tubule, while ouabain, a Na+/K+2Cl−ATPase inhibitor, does not inhibit fluid secretion (16, 45). This led to the initial proposal that the Na+/K+2Cl−ATPase was not important in fly tubule function (16). However, Na+/K+2Cl−ATPase expression has been demonstrated in the Drosophila Malpighian tubule, including on the basolateral membrane of principal cells, by transgenic (74), enhancer trap (71), immunofluorescence (41, 72), and electrophysiologic (45) techniques, and more recent studies have suggested that the Na+/K+2Cl−ATPase does play a functional role in tubule physiology (35, 45, 78).

Insect tubules, like other renal epithelia, are under the regulatory control of peptide hormones. In Drosophila, three classes of hormones have been described: those that are cAMP-coupled (Drome-DH3 and Drome-DH4α) (8, 9); the Ca2+/nitric oxide (NO)/cGMP-coupled capability-1 (capa-1) (6, 7, 11, 12, 15, 40, 70); and Ca2+-coupled leucokinin (66, 77). All three classes of peptides have homologs in other insect species. In Drosophila, the cAMP and cGMP-coupled hormones stimulate the principal cell by increasing apical H+/ATPase activity, while leucokinin stimulates chloride flux through the stellate cells via Ca2+ signaling (9, 52, 55, 66, 77). Thus, these hormones all stimulate diuresis, allowing the fly to avoid retaining excess fluid and solute after food ingestion. However, between meals, insects, which have a high surface area-to-volume ratio, must avoid desiccation, and in other insects, antidiuretic hormones have been described. The best studied
example is the Chagas’ disease vector *Rhodnius prolixus*. The transport mechanisms of the *Rhodnius* tubule are similar to those found in the *Drosophila* principal cell, with an apical H⁺-ATPase and cation/H⁺ exchanger and basolateral Na⁺-K⁺-ATPase and NKCC. In *Rhodnius*, two peptides related to *Drosophila* capa-1, MasCAP2β and RhoprCAPα-2, have antidiuretic effects (38, 58–61, 64, 65). To date, however, no antidiuretic hormones have been described in *Drosophila*.

Here, we demonstrate that the NKCC Ncc69 is important for transepithelial fluid and K⁺ flux in *Drosophila* by acting in principal cells. A major role for the Na⁺-K⁺-ATPase in unstimulated tubules is to support Ncc69 cotransport and prevent excess urinary loss of Na⁺. In stimulated tubules, additional Na⁺-K⁺-ATPase-dependent pathways are also important. While Ncc69 is not required for the diuretic actions of cAMP and leucokinin, the transporter is required for a novel antidiuretic activity of capa-1. Thus, Ncc69 is important for an integrated response to the homeostatic challenges posed by a varying external milieu, in which periods of desiccation threat, characterized by the lack of food and water, are punctuated by bouts of fluid and solute intake.

**MATERIALS AND METHODS**

Chemicals and reagents. All chemicals and reagents were from Sigma (St. Louis, MO) unless otherwise specified.

**Fly stocks and genetics.** The following *Drosophila* melanogaster strains were used: w’*Berlin* (wild-type), obtained from Dr. Adrian Rothenfluh [Univ. of Texas (UT) Southwestern Medical Center, Dallas, TX]; w; Ncc69r2 (crossed to w’*Berlin* to obtain w; Ncc69r2/+ heterozygotes) and w; UAS-Ncc69-HA Ncc69r2, obtained from Dr. William Leiserson (Yale University, New Haven, CT) (42); w; *Df(3L)BSC380/TM6C Sb w*; UAS-Ncc69-HA Ncc69r2, obtained from Dr. Berlin (wild-type), obtained from Dr. Adrian Rothenfluh [Univ. of Texas (UT) Southwestern Medical Center, Dallas, TX]; w; w; Ncc69r2 (crossed to w’*Berlin* to obtain w; Ncc69r2/+ heterozygotes) and w; UAS-Ncc69-HA Ncc69r2, obtained from Dr. William Leiserson (Yale University, New Haven, CT) (42); w; *Df(3L)BSC380/TM6C Sb w*; UAS-Ncc69-HA Ncc69r2, obtained from Dr. Berlin (wild-type) unless otherwise specified.

**Drosophila** saline and bathing medium. A 1:1 mixture of saline and standard bathing medium, a 1:1 mixture of seawater and artificial seawater, was used as the starting fluid for all experiments. The artificial seawater was made by dissolving in 1 L of water the following (in mM): 117.5 NaCl, 20 KCl, 2 CaCl₂, 8.5 MgCl₂, 10.2 NaHCO₃, 2.38 L-serine, 2.94 L-threonine, 0.49 L-tryptophan, 2.76 l-tyrosine, 2.56 l-valine, 5.62 β-alanine, 5.41 CaCl₂, 15.06 MgSO₄, 21.33 KCl, 3.31 KH₂PO₄, 4.76 NaHCO₃, 36.21 NaCl, 4.94 Na₂HPO₄, 1.37 α-ketoglutaric acid, 11.11 t-glucose, 0.862 fumaric acid, 0.746 malic acid, 0.847 succinic acid, 5.85 trehalose, and 2,000 mg/l yeastolate. One tubule of the pair remained in the droplet, while the other tubule of the pair was wrapped around a Minutien pin (Fine Science Tools, Foster City, CA) as an anchor. Unless otherwise indicated, the secreted fluid droplet was examined at ~2 h and its diameter was measured using an ocular micrometer in a dissecting stereomicroscope (Nikon, Melville, NY) at >50 magnification. The volume of the droplet was calculated assuming spherical geometry (4/3 πr³). Secretion rate was calculated for each tubule by dividing volume by time.

Ion-specific and reference electrodes were prepared according to the method of Maddrell et al. (46). Unfilamented borosilicate glass capillaries with an outside diameter of 1.2 mm (Harvard Apparatus, Holliston, MA) were washed for 5 min with nitric acid, rinsed 3–5 times with deionized water, and dried on a hot plate set to 200°C for a minimum of 20 min. Pipettes were pulled to a tip size of 1–2 μm using a vertical pipette puller (Narishige, East Meadow, NY). They were then dried for at least 10 min on the hot plate and lightly silanized by application of a drop of dichloromethylsilane inside a 15-cm Pyrex dish, which was inverted over the pipettes on the hot plate for a minimum of 20 min. Silanized pipettes were stored over silica gel (Fisher) until use. For measuring K⁺ flux, backfill solution of 0.5 M KCl was added to the pipette, and a small amount of potassium ionophore I cocktail B was aspirated into the tip of the pipette by application of negative pressure. The reference electrode was prepared from filamented borosilicate glass capillaries with an outside diameter of 1.2 mm (Harvard Apparatus), pulled in a manner similar to the ion-specific electrode (ISE). The tip and shank were filled with 1 M sodium acetate and the electrode was backfilled with 3 M KCl. For measuring Na⁺ flux, sodium ionophore X was prepared in a cocktail containing 10% sodium ionophore X, 89.75% nitrophenyl octyl ether, and 0.25% sodium tetraphenyl borate (49, 58). The sodium ISE was backfilled with 150 mM NaCl, and the reference electrode was filled with 150 mM KCl. Selectivity of the potassium ISE for K⁺ compared with Na⁺ is >10⁻³, while selectivity of the sodium ISE for Na⁺ compared with K⁺ is >10⁻⁶ (49).

For K⁺ measurement, calibration drops consisting of 15, 75, 150, and 200 mM KCl were measured by immersing the reference and ion-specific electrodes into the fluid drop and recording the potential using a Digidata 1200 amplifier (Axon Instruments, Union City, CA) and an FD223a dual-channel electrometer (World Precision Instruments, Sarasota, FL). The ISE was calibrated before and after each set of experimental measurements. Slope/decile change in K⁺ concentration was calculated using the Nernst equation for the difference between 15 and 150 mM, 75 and 150 mM, and 150 and 200 mM, and the average slope was then calculated (Table 1). The mean ± SE slope/decile change in K⁺ concentration across all experiments was 53.67 ± 0.80 (n = 38). K⁺ activity in the experimental fluid was measured and the concentration was calculated according to the following equation:

\[
[K⁺] = \frac{[K⁺]_c}{10^{(V/3)}}
\]

where [K⁺]ₚ is the potassium concentration of the experimental drop, [K⁺]ₜ is the potassium concentration of the calibration drop, V is the change in potential (mV) between the experimental drop and the calibration drop, and s is the slope (mV) for a tenfold change in K⁺ concentration, determined by measurements from the calibration drops (Table 1). [K⁺]ₜ was determined by the mean of the two 200 mM calibration drops (pre- and postexperiment). In the example given in Table 1, [K⁺]ₜ = 31.25 (mean of 30.6 and 31.9). For Na⁺ measurement, 15 and 150 mM calibration drops were used, and Na⁺ concentration was calculated as for K⁺. The mean ± SE slope/decile change in Na⁺ concentration across all experiments was 49.34 ± 3.7 (n = 4). The K⁺ and Na⁺ flux of each tubule was calculated by multiplying K⁺ or Na⁺ concentration by the secretion rate.

**Pharmacology.** Ouabain was dissolved in hot H₂O at a concentration of 20 mM and was added to standard bathing medium to obtain a final concentration of 100 μM. Bumetanide was dissolved in ethanol at a 100 mM concentration and was added to standard bathing...
medium to obtain a final concentration of 100 μM, or it was dissolved in ethanol at a 10 mM concentration and added to standard bathing medium to obtain a final concentration of 10 μM. Ouabain and bumetanide stocks were prepared fresh each day. Dibutyryl (db)-cAMP was dissolved in H2O at a concentration of 200 mM and was added to standard bathing medium at a final concentration of 1 mM. Tubules were bathed in the drug-containing standard bathing medium for the entirety of the experiment (2 h). As controls, tubules were bathed in standard bathing medium containing vehicle (H2O or ethanol) alone at the same concentration.

The capa-1 (GANMGLYAFPRV-amide) and Drosophila leucokinin (NSVVLGKKQRFHSW-amide) peptides were synthesized by the UT Southwestern Protein Chemistry Core Facility (http://www.utsouthwestern.edu/research/core-facilities/protein-chemistry-technology-core/peptide-synthesis/index.html) and purified by reverse-phase HPLC to 98.6% (leucokinin) and 100% (capa-1) purity. Peptides were dissolved in H2O at a concentration of 10 M and added to standard bathing medium to achieve a final concentration of 10 μM.

**Statistics.** Results comprising two groups were compared using a two-sided unpaired t-test. For results with three or more groups, one-way ANOVA was used. Repeated-measures one-way ANOVA was used when the same tubule was analyzed at multiple time points. Bonferroni’s test was used for post hoc testing of one-way ANOVA results. Significance level was set at \( P < 0.05 \). Values greater than three standard deviations from the mean were considered outliers and were excluded. All statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, CA).

## RESULTS

A role for the Ncc69 NKCC cotransporter in fluid secretion and \( K^+ \) flux. To examine a potential role for the Ncc69 NKCC cotransporter in transepithelial fluid and ion transport in the fly renal tubule, we examined the physiology of Ncc69 mutant tubules using the Ramsay assay. This assay measures fluid secretion by the main segment of the renal tubule (16). By measuring \( K^+ \) and \( Na^+ \) concentrations in the secreted fluid using ion-specific electrodes, \( K^+ \) and \( Na^+ \) flux can also be calculated (45, 46). Values for the diameter and \( K^+ \) concentration of secreted fluid droplets from wild-type and Ncc69 mutant tubules are shown in Table 2. In wild-type tubules, rates of fluid secretion and \( K^+ \) flux were 0.55 ± 0.02 nl/min and 93 ± 3.5 pmol/min, respectively (Fig. 1, A and B), similar to previously published values for fluid secretion and \( K^+ \) flux in unstimulated tubules (16, 45). Fluid secretion and \( K^+ \) flux were decreased in Ncc69\(^2\) homozygous mutant tubules compared with controls, to 0.43 ± 0.03 nl/min and 62 ± 3.7 pmol/min, while Ncc69\(^2\)/+ heterozygotes had an intermediate phenotype of 0.46 ± 0.02 nl/min and 77 ± 3.5 pmol/min (Fig. 1, A and B). We also tested mutants transheterozygous for the Ncc69\(^2\) mutant allele and a chromosome carrying a deficiency resulting in the deletion of the entire Ncc69 locus. Tubules from Ncc69\(^2\)/Df mutant flies had lower rates of fluid secretion (Fig. 1C) and \( K^+ \) flux (Fig. 1D) compared with tubules from flies heterozygous for the deficiency, but did not have lower rates of fluid secretion (Fig. 1C) or \( K^+ \) flux (Fig. 1D) compared with Ncc69\(^2\)/Ncc69\(^2\) homozygotes. This indicates that Ncc69\(^2\) is an amorphic (null) allele for the fluid secretion and \( K^+ \) flux phenotypes, as is also the case for the axon-bulging phenotype observed by Leiserson et al. (42).

To confirm that the impairment in secretion and \( K^+ \) flux seen in Ncc69 mutants is due to the absence of the Ncc69 cotransporter, we expressed a wild-type Ncc69 transgene under the control of the c42-GAL4 driver using the GAL4-UAS system, which allows expression of transgenes in a spatially restricted manner (5). c42-GAL4 is expressed in principal cells of the main segment, but not in the stellate cells (70). Expression of Ncc69 in this pattern restored fluid secretion (Fig. 2A) and \( K^+ \) flux (Fig. 2B) of Ncc69\(^2\)/Ncc69\(^2\) mutant tubules to wild-type levels. This indicates that loss of Ncc69 in the principal cells of the main segment is responsible for the decreased fluid secretion and \( K^+ \) flux seen in the Ncc69 mutants.

Iwanowski and O’Donnell (35) have shown that the electrochemical gradients for \( K^+ \) and \( Cl^- \) favor movement of these two ions across the basolateral membrane of the principal cell from cell to bath (hemolymph). Therefore, a favorable electrochemical gradient for \( Na^+ \) movement must exist for Ncc69 to transport \( K^+ \), along with \( Na^+ \) and \( Cl^- \), from the hemolymph into the principal cell. Typically, this gradient is established by the \( Na^+\)-K\(^+-\)ATPase. To test this, we compared the effect of ouabain, an inhibitor of the \( Na^+\)-K\(^+-\)ATPase, on wild-type and Ncc69\(^2\) mutant tubules. Ouabain (100 μM) had no effect on fluid secretion in wild-type tubules (Fig. 3, A and D), a finding consistent with previously published data (16, 45), despite a decrease in luminal \( K^+ \) concentration and decreased \( K^+ \) flux (Fig. 3, B and C). This is likely due to an increase in luminal \( Na^+ \) concentration (Ref. 45 and Fig. 3E), which results in increased \( Na^+ \) flux (Ref. 45 and Fig. 3F). The increased \( Na^+ \) flux counterbalances the decreased \( K^+ \) flux (Fig. 3C), resulting in no net change in secretion. Of note, this does not imply that

## Table 1. *Calibration of K* \(^+\) ISE

<table>
<thead>
<tr>
<th>KCl concentration, mM</th>
<th>Potential, mV Pre</th>
<th>Potential, mV Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>-28.4</td>
<td>-29.4</td>
</tr>
<tr>
<td>75</td>
<td>9.1</td>
<td>9.4</td>
</tr>
<tr>
<td>150</td>
<td>24.4</td>
<td>25.0</td>
</tr>
<tr>
<td>200</td>
<td>30.6</td>
<td>31.9</td>
</tr>
</tbody>
</table>

**Slope/Decile**

<table>
<thead>
<tr>
<th>Interval, mM</th>
<th>Slope/Decile</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–150 (pre)</td>
<td>52.8</td>
</tr>
<tr>
<td>15–150 (post)</td>
<td>54.4</td>
</tr>
<tr>
<td>75–150 (pre)</td>
<td>51</td>
</tr>
<tr>
<td>75–150 (post)</td>
<td>52</td>
</tr>
<tr>
<td>150–200 (pre)</td>
<td>49.6</td>
</tr>
<tr>
<td>150–200 (post)</td>
<td>55.2</td>
</tr>
<tr>
<td>Mean</td>
<td>52.5</td>
</tr>
</tbody>
</table>

*For the 15–150 mV interval, slope/decile = mV for the 150 mM calibration drop–mV for the 15 mM calibration drop. For the 75–150 interval, the difference is divided by 0.125 [log (300/150)]. ISE, ion-specific electrode; Pre, before experiment; post, after experiment.

## Table 2. Droplet size and \( K^+ \) concentration for wild-type and Ncc69 mutant tubules

<table>
<thead>
<tr>
<th>Diameter, μm</th>
<th>([K^+]), mM</th>
<th>Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ncc69(^2)/+</td>
<td>499 ± 7</td>
<td>167 ± 4</td>
</tr>
<tr>
<td>Ncc69(^2)/Df</td>
<td>470 ± 7</td>
<td>160 ± 5</td>
</tr>
<tr>
<td>Ncc69(^2)/Df</td>
<td>452 ± 9</td>
<td>151 ± 4</td>
</tr>
<tr>
<td>Df/+</td>
<td>526 ± 9</td>
<td>151 ± 3</td>
</tr>
<tr>
<td>Ncc69(^2)/Df</td>
<td>476 ± 11</td>
<td>155 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE.
ion and water fluxes that occur in the presence of ouabain are passive, as there is ongoing activity of the apical H\(^+\)-ATPase. Indeed, inhibition of the H\(^+\)-ATPase with bafilomycin abolishes fluid secretion in *Drosophila* tubules (16).

In wild-type tubules, ouabain decreased K\(^+\) flux by 30% (Fig. 3C). This indicates that the Na\(^+-\)K\(^+\)-ATPase is required for ~30% of the normal K\(^+\) flux, with the remaining 70% of normal K\(^+\) flux mediated by non-Na\(^+-\)K\(^+\)-ATPase-dependent K\(^+\) uptake pathways. Although there may be residual Na\(^+-\)K\(^+\)-ATPase activity, Linton and O’Donnell (45) found no differences in the effects of 10 \mu M, 100 \mu M, or 1 mM ouabain on the depolarization of the basolateral membrane potential in *Drosophila* tubules, suggesting that the 100 \mu M dose is sufficient to inhibit most of the Na\(^+-\)K\(^+\)-ATPase activity. However, ouabain treatment resulted in depolarization of the basolateral membrane potential by only 8 to 10 mV (45), indicating that there are other mechanisms for maintenance of a cell-negative basolateral membrane potential. The ouabain-insensitive Na\(^+\)-ATPase may be one such mechanism, and it has been proposed to operate in the *Rhodnius* tubule (27).

Ouabain had no effect on secretion rate, K\(^+\) concentration, or K\(^+\) flux in *Ncc69* mutant tubules (Fig. 3, A–C). This indicates that non-Ncc69-mediated K\(^+\) transport pathways are not dependent on the Na\(^+-\)K\(^+\)-ATPase. Since there was no further inhibition of K\(^+\) flux in *Ncc69* mutant tubules, *Ncc69* is the principal Na\(^+-\)K\(^+\)-ATPase-dependent transporter in unstimulated tubules. Consistent with this, there was no difference in K\(^+\) flux between ouabain-treated wild-type tubules and untreated *Ncc69* mutant tubules (Fig. 3C).

In principle, an NKCC cotransporter could mediate transepithelial flux of Na\(^+\), K\(^+\), or both. It has previously been proposed (35) that Na\(^+\) transported across the basolateral membrane of the main segment principal cell is recycled through the Na\(^+-\)K\(^+\)-ATPase. To test this, we measured Na\(^+\) transport pathways are...
Together with previous work, these data are consistent with a model (Fig. 4B) in which the Na\(^+-\)K\(^+-\)ATPase, acting in the principal cell, generates a favorable electrochemical gradient for NKCC cotransport through Ncc69 by lowering the intracellular Na\(^+\) concentration and generating a cell-negative electrical potential. K\(^+\) and Cl\(^-\) are cotransported along with Na\(^+\) against their electrochemical gradients (secondary active transport). Na\(^+\), which is scarce in the fly diet, is recycled across the basolateral membrane through the Na\(^+\)-K\(^+\)-ATPase and thereby conserved, while K\(^+\) is secreted into the lumen through an H\(^+/\)K\(^+\) exchanger, driven by the active transport of H\(^+\) into the lumen through the H\(^+\)-ATPase. Cl\(^-\) may also be recycled across the basolateral membrane, as Ianowski and O’Donnell (35) have demonstrated a basolateral Cl\(^-\) conductance and loop current.

**Effect of bumetanide.** NKCC cotransporters are potently inhibited by “loop” diuretics, including furosemide and bumetanide. In heterologous expression systems, Ncc69 was inhibited by bumetanide with IC\(_{50}\) values of 49 nM (42) and 1.17 \(\mu\)M (75). We therefore tested the effects of 10 \(\mu\)M bumetanide on wild-type tubules, but found there was no effect on fluid secretion or K\(^+\) flux (Fig. 5, A and B). We then tested the effect of 100 \(\mu\)M bumetanide, which has previously been demonstrated to decrease fluid secretion and K\(^+\) flux in wild-type Drosophila tubules (45). As previously observed, bumetanide decreased fluid secretion and K\(^+\) flux in wild-type tubules, but fluid secretion and K\(^+\) flux were also decreased in Ncc69\(^{2+/+}\) homozygous mutant tubules (Fig. 5, C and D). This indicates that high-dose bumetanide is not a specific inhibitor of Ncc69.

**The response of Ncc69 mutant tubules to cAMP, leucokinin, and capa-1.** We next turned our attention to the physiology of stimulated tubules. As is the case for mammalian renal tubular function, insect renal tubule function is modulated by a number of hormones acting through a variety of signaling cascades. Drosophila tubules increase fluid secretion rates in response to cAMP signaling (8, 9, 16, 52), and the peptide hormones leucokinin (66, 77) and capa-1 (40). To determine whether Ncc69 plays a role in the response to any of these modulators, we examined fluid secretion and K\(^+\) flux in response to stimulation in wild-type and Ncc69 mutant tubules. First, we examined the effect of cAMP signaling. Both Ncc69\(^{2+/+}\) heterozygous and Ncc69\(^{2/2}\) homozygous mutant tubules exhibited a robust response to stimulation with 1 mM db-cAMP, with an increase in fluid secretion (Fig. 6A) and K\(^+\) flux (Fig. 6B). This indicates that there are non-Ncc69-mediated K\(^+\) transport pathways that are cAMP sensitive. Indeed, there may be compensatory upregulation of these pathways in Ncc69 mutants. This non-Ncc69-mediated, cAMP-sensitive fluid secretion and K\(^+\) transport was abolished by 100 \(\mu\)M ouabain (Fig. 6, C and D). Thus, while Ncc69 appears to be the principal Na\(^+\)-K\(^+\)-ATPase-dependent K\(^+\) flux pathway in unstimulated tubules (Fig. 3C), under cAMP-stimulated conditions, additional Na\(^+\)-K\(^+\)-ATPase-dependent pathways are recruited. This suggests either that the Na\(^+\)-K\(^+\)-ATPase itself is stimulated by cAMP, allowing increased K\(^+\) entry into the principal cell, or that additional secondary active transporters that are dependent on the electrochemical gradient established by the Na\(^+\)-K\(^+\)-ATPase are stimulated. Ouabain has also been shown to decrease fluid secretion in wild-type tubules stimulated with cAMP (45).

**Fig. 2.** Expression of wild-type Ncc69 in Malpighian tubule principal cells restores fluid secretion rate and K\(^+\) flux to wild-type levels. A: fluid secretion rate (nL/min per tubule) was measured in tubules from wild-type, Ncc69\(^{2/2}\) homozygous mutants, and Ncc69\(^{2/2}\) homozygous mutants in which wild-type Ncc69 was expressed under the control of c42-GAL4 (c42/UAS-Ncc69; Ncc69\(^{+/+}\); Ncc69\(^{2/2}\); n = 18 for all genotypes), c42-GAL4 drives expression in the principal cells of the main segment. Expression of wild-type Ncc69 in these cells normalizes secretion. *P < 0.05; **P < 0.01. B: K\(^+\) flux (pmol/min per tubule) was measured in tubules from wild-type, Ncc69\(^{2/2}\) homozygous mutants, and Ncc69\(^{2/2}\) homozygous mutants in which wild-type Ncc69 was expressed under the control of c42-GAL4 (n = 18 for all genotypes). Expression of wild-type Ncc69 in the principal cells normalizes K\(^+\) flux. **P < 0.01; ***P < 0.001.

**Fig. 2.** Expression of wild-type Ncc69 in Malpighian tubule principal cells restores fluid secretion rate and K\(^+\) flux to wild-type levels. A: fluid secretion rate (nL/min per tubule) was measured in tubules from wild-type, Ncc69\(^{2/2}\) homozygous mutants, and Ncc69\(^{2/2}\) homozygous mutants in which wild-type Ncc69 was expressed under the control of c42-GAL4 (c42/UAS-Ncc69; Ncc69\(^{+/+}\); Ncc69\(^{2/2}\); n = 18 for all genotypes), c42-GAL4 drives expression in the principal cells of the main segment. Expression of wild-type Ncc69 in these cells normalizes secretion. *P < 0.05; **P < 0.01. B: K\(^+\) flux (pmol/min per tubule) was measured in tubules from wild-type, Ncc69\(^{2/2}\) homozygous mutants, and Ncc69\(^{2/2}\) homozygous mutants in which wild-type Ncc69 was expressed under the control of c42-GAL4 (n = 18 for all genotypes). Expression of wild-type Ncc69 in the principal cells normalizes K\(^+\) flux. **P < 0.01; ***P < 0.001.
We next examined tubule response to the leucokinin and capa-1 peptides. In the absence of capa-1, leucokinin-stimulated fluid secretion and K⁺/H⁺ flux were similar in wild-type and Ncc69⁻² homozygous mutants in the absence or presence of 100 μM ouabain (n = 16–17 for each genotype/condition). Ouabain has no effect on fluid secretion. B: K⁺ concentration (mM) was measured in tubules from wild-type and Ncc69⁻² homozygous mutants in the absence or presence of 100 μM ouabain (n = 16–17 for each genotype/condition). Ouabain decreased K⁺ concentration in wild-type, but not Ncc69⁻² mutant tubules, *P < 0.05; ***P < 0.001. For the difference between ouabain-treated wild-type tubules and vehicle-treated Ncc69⁻² mutant tubules, P < 0.05. C: K⁺ flux (pmol/min per tubule) was measured in tubules from wild-type and Ncc69⁻² homozygous mutants in the absence or presence of 100 μM ouabain (n = 16–17 for each genotype/condition). Na⁺⁻K⁺⁻ATPase inhibition decreases K⁺ flux in wild-type tubules to the level seen in Ncc69⁻² mutant tubules, but has no further effect on mutant tubules, *P < 0.05; **P < 0.01. The difference between ouabain-treated wild-type tubules and vehicle-treated Ncc69⁻² mutant tubules was NS. D: in a separate experiment, secretion rate was measured in wild-type tubules in the absence (n = 13) or presence (n = 15) of 100 μM ouabain. As in A, Na⁺⁻K⁺⁻ATPase inhibition has no effect on fluid secretion. E: Na⁺ concentration (mM) was measured in wild-type tubules in the absence (n = 13) or presence (n = 15) of 100 μM ouabain. Na⁺ concentration increases in the presence of ouabain. ***P < 0.001. F: Na⁺ flux (pmol/min per tubule) was measured in wild-type tubules in the absence (n = 13) or presence (n = 15) of 100 μM ouabain. Na⁺⁻K⁺⁻ATPase inhibition increases Na⁺ flux. ***P < 0.001.

We next examined tubule response to the leucokinin and capa-1 peptides. In the absence of capa-1, leucokinin-stimulated fluid secretion and K⁺ flux were similar in wild-type and Ncc69⁻² homzygous mutant tubules (Fig. 7, A and B). This suggests that the stellate cells, which are the cells stimulated by leucokinin (52, 55, 66, 77), are not impaired by the loss of Ncc69 function, consistent with our results indicating that Ncc69 is functioning in the principal cell. These results also indicate that Ncc69 mutant tubules do not have general defects in epithelial function, as they are able to support the same rates of fluid secretion and K⁺ flux as wild-type tubules under these conditions.

Leucokinin and capa-1 have previously been shown to additively increase fluid secretion rates (40). We were therefore surprised to see that this combination of peptides resulted in the inhibition of both fluid secretion and K⁺ flux in wild-type tubules (Fig. 7, A and B). However, we assayed these processes over a period of 2 h, whereas the stimulatory effect of these peptides was previously observed over shorter time periods (30 min). Therefore, we tested fluid secretion rates in
stimulated wild-type tubules over three time periods: 0–30 min, 30–50 min, and 50–120 min. As seen in Fig. 7C, the antidiuretic effect was apparent only after 30 min, when secretion rates dropped to very low levels: 0.1 ± 0.05 nl/min for the 30–50 min time period, and 0.09 ± 0.02 nl/min for the 50–120 min time period. This is in contrast to unstimulated tubules, in which fluid secretion occurs at a constant rate over at least 4 h (16). Because rates of secretion are lower than those seen in unstimulated tubules (~0.5 nl/min), this appears to be an active antidiuretic effect, rather than a loss of hormone action over time, as might occur with receptor desensitization (76). Capa-1 alone also had antidiuretic and antikaliuretic effects on wild-type tubules (Fig. 7, D and E).

Ncc69R2 mutant tubules were resistant to the antidiuretic and antikaliuretic effects of capa-1 both in combination with leucokinin (Fig. 7, A and B) or on its own (Fig. 7, D and E). This
Fig. 6. cAMP stimulates fluid secretion and K⁺ flux in Ncc69 mutant tubules in a Na⁺-K⁺-ATPase-dependent manner. A: secretion rate (nl/min per tubule) was measured in tubules from wild-type, Ncc69² heterozygous, and Ncc69² homozygous mutants in the presence (n = 4–5 tubules/genotype) or absence (n = 11–12 tubules/genotype) of 1 mM dibutyryl (db)-cAMP. cAMP stimulates fluid secretion through a non-Ncc69-dependent pathway. *P < 0.05; ***P < 0.001. B: K⁺ flux (pmol/min per tubule) was measured in tubules from wild-type, Ncc69² heterozygous, and Ncc69² homozygous mutants in the presence (n = 11–12 tubules/genotype) or absence (n = 11–12 tubules/genotype) of 1 mM db-cAMP alone (n = 9), or in the presence of both 1 mM db-cAMP and 100 μM ouabain (n = 11). Non-Ncc69-mediated, cAMP-stimulated secretion is dependent on the Na⁺-K⁺-ATPase. ***P < 0.001. C: K⁺ flux (pmol/min per tubule) was measured in tubules from Ncc69² homozygous mutants in the absence of drugs (n = 9), in the presence of 1 mM db-cAMP alone (n = 9), or in the presence of both 1 mM db-cAMP and 100 μM ouabain (n = 11). The non-Ncc69-mediated, cAMP-stimulated K⁺ flux transport pathway is dependent on the Na⁺-K⁺-ATPase. ***P < 0.001.

indicates that Ncc69 is required for the antidiuretic and antikaliuretic effects of capa-1, and that Ncc69 may be an important target for regulation by capa-1 in antidiuretic states.

DISCUSSION

Cotransport of Na⁺, K⁺ and Cl⁻ across the basolateral membrane of insect renal tubule cells through NKCC co-transporters has been proposed in several species, including Drosophila melanogaster (27, 30, 31, 33, 35, 37, 44, 54). However, the genes encoding the involved co-transporters have not been identified. Here, we demonstrate a role for the Drosophila NKCC, encoded by the gene Ncc69, in fluid secretion and transepithelial K⁺ flux in the fly renal tubule. Ncc69 mutant flies have decreased rates of fluid secretion and K⁺ flux. Available evidence has indicated that cation flux occurs through principal cells of the fly tubule, while the stellate cells provide a pathway for chloride flux (18, 52, 55). Consistent with this model, expression of wild-type Ncc69 in principal cells only is sufficient to rescue impaired fluid secretion and K⁺ flux.

Although NKCCs generally transport equimolar Na⁺ and K⁺, Ncc69 mutant flies do not have impaired Na⁺ flux. The data presented here support the proposal by Janowski and O’Donnell (35) that Na⁺ is likely recycled across the basolateral membrane through the Na⁺-K⁺-ATPase back into the hemolymph, while K⁺ is secreted across the apical membrane into the tubule lumen, probably through an apical cation/H⁺ antiporter energized by the apical H⁺-ATPase (1, 10, 13, 14, 16, 20). Since the fly diet is rich in K⁺ but poor in Na⁺, this allows the fly to excrete a high-K⁺, low-Na⁺ urine. In contrast, in blood-sucking insects, such as mosquitoes and Rhodnius prolixus, in which a large Na⁺ load is ingested with the blood meal, NKCC inhibition in stimulated tubules results in decreased Na⁺ flux, suggesting that the Na⁺ transported across the basolateral membrane by NKCC is secreted across the apical membrane by the apical cation/H⁺ transporter (31, 33). Thus, NKCC function in the insect tubule is adapted to the dietary requirements of the organism. In fact, NKCC function is dispensable for Na⁺ flux in the Drosophila tubule, as robust fluid secretion and Na⁺ flux can occur in K⁺-free bathing saline (45).

Two groups have shown that Ncc69 is homologous to the mammalian NCC/NKCC family of SLC12 co-transporters, and both groups demonstrated that Ncc69 transports rubidium in a Na⁺- and Cl⁻-dependent manner, consistent with its function as an NKCC (42, 75). They also showed that when Ncc69 is expressed in cultured cells, bumetanide inhibits its activity with an IC50 of ~1 μM or less (42, 75). In this study, 10 μM bumetanide did not inhibit fluid secretion or K⁺ flux, but as seen by O’Donnell and coworkers (45), 100 μM bumetanide was inhibitory. It is unclear why the lower dose was ineffective. In perfused Rhodnius tubules, luminal applied bumetanide inhibited fluid secretion, presumably by inhibiting basolateral NKCC (30), suggesting a capacity for transepithelial bumetanide transport in insect tubules. Similarly, higher doses of ouabain than would be predicted by cellular studies are required to see effects on tubule function due to uptake of ouabain by organic anion transporters (78). Finally, there may
be differences in Ncc69 in its normally expressed environment compared with heterologous cells that affect sensitivity to bumetanide.

The high-dose bumetanide used here and previously (45) inhibits fluid secretion and K⁺ flux in both wild-type and Ncc69 mutant tubules, indicating sensitivity to Ncc69. One possibility is that bumetanide inhibits Ncc83. The Ncc83 gene sequence is homologous to the NCC/NKCC branch of SLC12 transporters (42, 75), but the putative cotransporter encoded by this gene has not been studied so far. Additional targets of high-dose bumetanide that have been demonstrated in other systems include potassium-chloride cotransporters (28, 29, 48), parallel Na⁺ exchange in cortical thick ascending limb (22–24), a K⁺−Na⁺−Cl⁻ exchange in cortical thick ascending limb (22–24), a K⁺−Na⁺−Cl⁻ exchange in cortical thick ascending limb (22–24), and the ouabain-insensitive Na⁺−H⁺−ATPase (69). Whether any of these mechanisms are operative in the fly renal tubule is remains to be determined.

Like the mammalian renal tubule, insect Malpighian tubules are under hormonal control. In Drosophila, a calcitonin-like peptide, Drome-DH31, stimulates fluid secretion and K⁺ flux through the cAMP signaling pathway by activating the apical vacuolar H⁺−ATPase. A corticotropin-releasing factor-like peptide, Drome-DH44, also increases tubule cAMP levels and stimulates fluid secretion (8). Whether these peptides have additional effects on basolateral membrane targets is unknown. Here, we demonstrate that cAMP robustly stimulates fluid secretion and K⁺ in Ncc69 mutant tubules, suggesting that either the Na⁺−K⁺−ATPase itself, or a second- order transporter dependent on its activity (such as Ncc83), allows K⁺ uptake across the basolateral membrane, and underscores the importance of coordinate regulation of apical and basolateral membrane transport.

The peptide capa-1 (or the related peptide MasCAP2b, which has similar effects on the Drosophila tubule) has also been shown to stimulate fluid secretion in Drosophila tubules, through a Ca²⁺/NO/cGMP signaling pathway (6, 7, 11, 12, 15, 40, 70). Surprisingly, we observed a novel antidiuretic and antikaliuretic effect of capa-1 that was apparent after 30 min of exposure of tubules to capa-1. This is the first observation of antidiuretic peptide activity in a wild-type Drosophila renal tubule, but it is consistent with the antidiuretic activity of capa-1-related peptides in other insects, such as Rhodnius and Tenebrio (38, 58–61, 64, 65, 81). This may serve as a mechanism to limit excess diuresis, preventing desiccation. Indeed, knockdown of the capa-1 receptor in the tubule results in resistance to starvation/desiccation stress, suggesting that excess capa-1 activity is deleterious under these conditions (76).

What is the mechanism by which capa-1 exerts its antidiuretic effect? MasCAP2b and cGMP modulate the transepithelial potential, indicating regulation of the apical H⁺−ATPase (11, 52). Our data suggest that regulation of basolateral trans-
port pathways is important as well, since Ncc69 mutant tubules escape the antidiuretic effect of capa-1. This is also reminiscent of the Rhodnius tubule, in which the Rhodnius capa homolog, RhoprCAPA-α2, can either stimulate the apical H+–ATPase or inhibit the basolateral NKCC, depending on the hormonal milieu (58). The inhibition of basolateral NKCC by RhoprCAPA-α2 results in antidiuresis, indicating similar regulatory mechanisms of capa signaling in Drosophila and Rhodnius.

The signaling pathways by which capa-1 inhibits diuresis in flies are unknown. The cGMP signaling pathway mediates both the early stimulatory effects of capa-1/MasCAP2b in flies as well as the antidiuretic effects of capa-1/MasCAP2b on Rhodnius tubules and Drosophila tubules overexpressing the cation/proton exchanger CG10806B (6, 11–13, 15, 40, 64, 65). Future studies are needed to determine whether the antidiuretic effect of capa-1 on Drosophila tubules is also mediated by cGMP signaling.

In fly, capa-1/MasCAP2b stimulates cGMP downstream of NO (7, 12, 15, 40, 62). Interestingly, a NO/cGMP signaling pathway inhibits NaCl reabsorption in the thick ascending limb (51, 56) through inhibition of NKCC2 (4, 57). Thus, although NKCC2 is on the apical membrane of the thick ascending limb and functions to absorb NaCl, while Ncc69 is presumably on the basolateral membrane of the fly tubule and is important for K+ secretion, an attractive speculation is that signaling through NO and cGMP may be an evolutionarily conserved mechanism to limit NKCC activity. Dysregulation of this pathway may also be clinically important: in an animal model of hypertension using the NO inhibitor N-nitro-l-arginine-methyl ester (l-NAME), NKCC2 is upregulated, resulting in excess NaCl reabsorption (80). Further understanding of fly Ncc69 regulation may thus lead to insights into human hypertension. Similarly, the three mammalian homologs of Ncc69—NKCC1, NKCC2, and the sodium-chloride cotransporter NCC—are all conserved, as Ncc69 is required for normal renal tubule function, given the low Na+ content of the fly diet, is not dependent on Ncc69. The inhibition of Ncc69 cotransporter activity may also be important in conditions where K+ and water need to be conserved, as Ncc69 is required for the antidiuretic and antikaliuretic effects of the regulatory hormone capa-1.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

A.R.R. conception and design of the research; A.R.R. performed the experiments; A.R.R. analyzed the data; A.R.R. interpreted the results of the experiments; A.R.R. prepared the figures; A.R.R. drafted the manuscript; A.R.R., M.B., and C.-L.H. edited and revised the manuscript; A.R.R., M.B., and C.-L.H. approved the final version of the manuscript.

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