Localization and role of inward rectifier K⁺ channels in Malpighian tubules of the yellow fever mosquito *Aedes aegypti*.

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Abstract

Malpighian tubules of adult female yellow fever mosquitoes *Aedes aegypti* express three inward rectifier K^+ (Kir) channel subunits: *AeKir1*, *AeKir2B* and *AeKir3*. Here we 1) elucidate the cellular and membrane localization of these three channels in the Malpighian tubules, and 2) characterize the effects of small molecule inhibitors of *AeKir1* and *AeKir2B* channels (VU compounds) on the transepithelial secretion of fluid and electrolytes and the electrophysiology of isolated Malpighian tubules. Using subunit-specific antibodies, we found that *AeKir1* and *AeKir2B* localize exclusively to the basolateral membranes of stellate cells and principal cells, respectively; *AeKir3* localizes within intracellular compartments of both principal and stellate cells. In isolated tubules bathed in a Ringer solution containing 34 mM K^+ , the peritubular application of VU590 (10 μ M), a selective inhibitor of *AeKir1*, inhibited transepithelial fluid secretion 120 min later. The inhibition brings rates of transepithelial KCl and fluid secretion to 54% of the control without a change in transepithelial NaCl secretion. VU590 had no effect on the basolateral membrane voltage (V_{bl}) of principal cells, but it significantly reduced the cell input conductance (g_{in}) to values 63% of the control within ~90 min. In contrast, the peritubular application of VU625 (10 μ M), an inhibitor of both *AeKir1* and *AeKir2B*, started to inhibit transepithelial fluid secretion as early as 60 min later. At 120 min after treatment, VU625 was more efficacious than VU590, inhibiting transepithelial KCl and fluid secretion to ~35% of the control without a change in transepithelial NaCl secretion. Moreover, VU625 caused the V_{bl} and g_{in} of principal cells to respectively drop to values 62% and 56% of the control values within only ~30 min. Comparing the effects of VU590 with those of VU625 allowed us to estimate that *AeKir1* and *AeKir2B* respectively contribute to 46% and 20% of the transepithelial K^+ secretion when the tubules are bathed in a Ringer solution containing 34 mM K^+ . Thus, we uncover an important role of *AeKir1* and stellate cells in transepithelial K^+ transport under conditions of peritubular K^+ challenge. The physiological role of *AeKir3* in intracellular membranes of both stellate and principal cells remains to be determined.

64 **Keywords:** potassium channels, pharmacology, small molecules, immunolocalization,
65 electrophysiology, transepithelial fluid secretion

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Introduction

Adult female mosquitoes produce urine via the transepithelial secretion of fluid by the blind-ended distal segments of the Malpighian tubules. The epithelium of the distal segment consists of large, thick, fusiform principal cells that are intercalated by small, thin, star-like (stellate) cells. In general, principal cells contribute to the active transcellular transport of K^+ and Na^+ from the hemolymph to the tubule lumen. The transport of cations into the lumen provides a favorable electrochemical gradient for the accompanying transcellular and paracellular transport of anions (primarily Cl^-) through stellate cells and septate junctions, respectively. The resulting accumulation of KCl and NaCl in the lumen provides an osmotic gradient for water to follow, thereby forming urine that is isosmotic to the hemolymph (Beyenbach and Piermarini, 2009; Beyenbach and Piermarini, 2011; Beyenbach et al., 2010).

An important cellular mechanism of both fluid and K^+ secretion in the Malpighian tubules of the yellow fever mosquito *Aedes aegypti* and other insects is a barium-sensitive K^+ -conductance in the basolateral membrane of principal cells (Beyenbach and Masia, 2002; Blumenthal, 2001; Leyssens et al., 1993; O'Donnell et al., 1996; Scott et al., 2004; Weltens et al., 1992; Wiehart et al., 2003). In principal cells of *A. aegypti*, as much as 64% of the total membrane conductance is blocked by barium (Beyenbach and Masia, 2002; Scott et al., 2004). Recent molecular, physiological and pharmacological studies by our group suggest that inward rectifier K^+ (Kir) channels mediate this K^+ conductance in Malpighian tubules of *A. aegypti*. Namely, we have cloned 3 cDNAs encoding Kir channel subunits (*AeKir1*, *AeKir2B*, *AeKir3*) from adult female Malpighian tubules (Piermarini et al., 2013). When expressed heterologously in *Xenopus* oocytes, the *AeKir1* and *AeKir2B* subunits each form barium-sensitive homomeric Kir channels in the plasma membrane, but the *AeKir3* subunits do not (Piermarini et al., 2013). Moreover, the cation permeability sequence of the principal cell basolateral membrane is consistent with the presence of Kir channels (Piermarini et al., 2013), and a small molecule modulator of *AeKir1* and *AeKir2B* channels (VU573) alters the transepithelial secretion of K^+ , Na^+ , Cl^- , and fluid in isolated Malpighian tubules (Raphemot et al., 2013; Rouhier et al., 2014a).

Despite these advances, the physiological contributions of specific Kir subunits in mosquito Malpighian tubules remain unresolved, because 1) their cellular and membrane localizations in the tubule epithelium are unknown, and 2) VU573 is an inhibitor of *AeKir1* channels and an activator of *AeKir2B* channels (Raphemot et al., 2013; Rouhier et al., 2014a). Thus, the dual effects of VU573 complicate interpretations of which Kir subunits are responsible for the observed alterations to tubule physiology. The goals of the present study are to 1) localize the expression of *AeKir1*, *AeKir2B*, and *AeKir3* subunits in Malpighian tubules using subunit-specific antibodies, and 2) evaluate the contributions of *AeKir1* and *AeKir2B* in tubule physiology using small molecules VU590 and VU625. VU590 is an inhibitor of *AeKir1* that does not affect the activity of *AeKir2B* (Rouhier et al., 2014b), whereas VU625 is an inhibitor of both *AeKir1* and *AeKir2B*, with a preference for *AeKir1* (Raphemot et al., 2014b). Both of these compounds decrease the capacity of adult female mosquitoes to excrete urine as assessed with an in vivo diuresis assay (Raphemot et al., 2014b; Rouhier et al., 2014b), but their effects on isolated Malpighian tubules are unstudied.

We hypothesized that the Kir subunit immunoreactivity would be expressed primarily on the basolateral membrane of principal cells given the results of our previous physiological studies on mosquito Malpighian tubules (Beyenbach and Masia, 2002; Masia et al., 2000; Piermarini et al., 2013; Scott et al., 2004). Moreover, a previous in situ hybridization study in Malpighian tubules of *Drosophila melanogaster* by the Dow laboratory found the expression of Kir subunit mRNAs to occur only in principal cells (Evans et al., 2005). We also hypothesized that VU625 would elicit more prominent inhibitory effects on tubule fluid secretion and electrophysiology than VU590 given that VU625 inhibits both *AeKir1* and *AeKir2B* channels. We find evidence for cell-specific expression of *AeKir1* and *AeKir2B* subunits along the basolateral membranes of stellate and principal cells, respectively, whereas *AeKir3* subunits occur within intracellular compartments of both cell types. Consistent with our hypothesis, in isolated Malpighian tubules, VU625 is more efficacious than VU590 at inhibiting 1) transepithelial fluid and K^+ secretion, and 2) the basolateral membrane voltage and input conductance of principal cells. Estimates of *AeKir1*- and *AeKir2B*-mediated K^+ secretion when the tubules are

118 challenged to secrete K^+ at high rates indicate that *AeKir1* expressed in stellate cells, the minority cell
119 type in *Aedes* Malpighian tubules, transports twice as much K^+ than *AeKir2B* expressed in principal cells,
120 the majority cell type. Altogether, our results reveal an unexpected important role of stellate cells in renal
121 K^+ homeostasis in the yellow fever mosquito.

Materials and Methods

Mosquitoes and isolation of Malpighian tubules

Mosquitoes (*Aedes aegypti*) were raised in the laboratory as described previously (Hine et al., 2014; Piermarini et al., 2011). Only Malpighian tubules from adult females (3-10 days old) were used in the present study. In brief, the females were immobilized on ice and decapitated with fine forceps (Dumont #5; Fine Science Tools, Inc.; Foster City, CA). The carcass was then submerged in low K⁺ Ringer solution. The alimentary tract of the mosquito was removed by pulling on the rectum. Malpighian tubules were removed from the alimentary tract at the junction of the midgut and hindgut.

Ringer solutions

All Ringer solutions contained 25 mM HEPES, 1.8 mM NaHCO₃, 1.7 mM CaCl₂, 1 mM MgCl₂ and 5 mM glucose. The low K⁺ Ringer solution contained 3.4 mM KCl and 150 mM NaCl, and the high K⁺ Ringer solution contained 34 mM KCl and 119.4 mM NaCl. Ringer solutions were prepared fresh on the day of the experiment and adjusted to pH 7.1 using 2 M NH₄OH or 1 M NaOH.

Small molecules

VU625, VU688 (a.k.a. VU0477688), and VU608 were synthesized by the Vanderbilt Institute of Chemical Biology as described previously (Raphemot et al., 2014b; Rouhier et al., 2014b). VU590 was purchased from Sigma Aldrich (St. Louis, MO). All small molecules were dissolved in 100% DMSO to yield 100 mM stock solutions and were added to the peritubular Ringer solution immediately before use at a concentration of 10 μ M. Given that VU625 is a substrate of ATP-binding cassette (ABC) and/or organic anion transporters (Raphemot et al., 2014b), probenecid (VWR Intern., Germany) was also added to the peritubular Ringer solution at a concentration of 100 μ M in all experiments with small molecules to limit their depletion from the peritubular bath. When present, barium nitrate was used at concentrations of 10 μ M or 5 mM.

Antibodies

We hired Genscript USA (Piscataway, NJ) to raise and affinity-purify polyclonal rabbit antibodies against synthetic peptides corresponding to fragments of *AeKir1* and *AeKir3*. We hired 21st Century Biochemicals (Marlborough, MA) to raise and affinity-purify a polyclonal guinea pig antibody against a synthetic peptide corresponding to a fragment of *AeKir2B*. The peptide sequences for *AeKir1*, *AeKir2B*, and *AeKir3* are ‘SRRIRKRVIFKQGDC’, ‘VEKLEQYHVENEQNHQRPLC’, and ‘LRRDPEHQGRRRAIRC’, respectively; the corresponding locations of these peptides on their respective proteins are shown in Figure S1. We have previously used the Kir1 antibody to confirm knockdown of Kir1 protein expression in *Anopheles gambiae* mosquitoes injected with Kir1 dsRNA (Raphemot et al., 2014a).

Heterologous expression of *AeKir* subunits in *Xenopus* oocytes

We purchased defolliculated oocytes of *Xenopus laevis* from a commercial supplier (Ecocyte Bioscience, Austin, TX) and injected them with capped RNA (cRNA) encoding *AeKir1*, *AeKir2B*, or *AeKir3* subunits, as described in a previous study (Piermarini et al., 2013). Control oocytes were injected with nuclease-free H₂O. The oocytes were cultured for 4-6 days in OR3 media (Piermarini et al., 2009) before freezing them at -80°C.

Heterologous expression of the *AeKir* subunits was assessed via Western blotting of total membrane-protein fractions, as described previously (Piermarini et al., 2010; Piermarini et al., 2009). In brief, cleared oocyte lysates were centrifuged at 100,000 g for 60 min at 4°C to isolate the membrane proteins, which were then separated on 12% polyacrylamide gels (10 µg per lane) and transferred to polyvinylidene difluoride (PVDF) membranes (Piermarini et al., 2010; Piermarini et al., 2009). The Kir1, Kir2B, and Kir3 antibodies were dissolved in blocking buffer at concentrations of 0.16 µg/ml, 1.74 µg/ml, and 0.345 µg/ml, respectively. The blocking buffer consisted of 5% nonfat dry milk dissolved in a Tween-Tris-

buffered saline (TTBS). The TTBS consisted of 10 mM Tris-HCl, 150 mM NaCl, and 0.01% Tween 20 (pH 7.4).

After an overnight incubation with the Kir1 or Kir3 antibody at 4°C, the PVDF membrane was washed in TBST and incubated at room temperature for 2 h in a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Thermo Scientific) diluted in blocking buffer at 1:20,000. A similar procedure was followed for PVDF membranes incubated with the Kir2B antibody except for the use of a HRP-conjugated goat anti-guinea pig IgG secondary antibody (Thermo Scientific) diluted in blocking buffer. The PVDF was washed again in TTBS and incubated for 5 min with SuperSignal West Dura ECL substrate (Thermo Scientific) before imaging it on a MyECL imager (Model 62236X, Thermo Scientific).

Western blots of Malpighian tubules

Crude lysates of Malpighian tubules isolated from ~30 adult females (~150 tubules) were prepared as described in previous studies (Piermarini et al., 2010; Piermarini et al., 2011). The samples were boiled for 5 min before separating them on 12% polyacrylamide gels (~50 tubule equivalents per lane). Western blots were performed and developed exactly as described above for *Xenopus* oocytes, except the Kir1, Kir2B, and Kir3 antibodies were used at concentrations of 0.78 µg/ml, 1.74 µg/ml, and 1.725 µg/ml, respectively.

Immunolabeling of Malpighian tubules

Intact, isolated Malpighian tubules were fixed with 4% paraformaldehyde, washed with a phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 (Thermo Scientific), and blocked with 10% normal goat serum (Thermo Scientific) supplemented with casein (Vector Laboratories, Burlingame, CA), exactly as described previously (Calkins et al., 2015). The PBS was diluted from a 10x commercially-supplied concentrate (Thermo Scientific) and consisted of 9.5 mM phosphate, 2.7 mM KCl, and 137 mM NaCl (pH 7.4). The tubules were then incubated overnight at 4°C with the Kir1, Kir2B, or Kir3 antibody

dissolved in incubation buffer at 0.78 $\mu\text{g/ml}$, 87 $\mu\text{g/ml}$, and 3.45 $\mu\text{g/ml}$, respectively. The incubation buffer consisted of Tween-PBS (TPBS; 0.10% Tween 20) supplemented with 1x casein solution (Vector Laboratories).

After the incubation with the Kir1 or Kir3 antibody, the tubules were washed in TPBS and incubated at room temperature for 2 h in a fluorescently-labelled goat anti-rabbit IgG secondary antibody (Dylight 488 or Dylight 633; Thermo Scientific) diluted in incubation buffer at 1:400. A similar procedure was followed for tubules incubated with the Kir2B antibody, except for the use of a fluorescently-labelled goat anti-guinea pig IgG secondary antibody (Dylight 488; Thermo Scientific) diluted in blocking buffer at 1:100. The tubules were washed again in TPBS and incubated with DAPI according to the manufacturer's protocol (Thermo Scientific) to label nuclei. After additional washes in TPBS, the tubules were visualized as whole mounts on a Leica TCS SPS II confocal microscopy system with Leica Microsystems LAS AF software (Leica Microsystems, Buffalo Grove, IL) at the Molecular and Cellular Imaging Center of the Ohio Agricultural Research and Development Center (Wooster, OH).

Ramsay fluid secretion assay

Ramsay assays of fluid secretion were performed using the 'sister tubule' approach developed by us previously (Hine et al., 2014). In brief, two Malpighian tubules isolated from the same adult female mosquito were transferred to separate 50 μl droplets of high K^+ Ringer solution under oil. The use of the high K^+ Ringer stimulates a spontaneous kaliuresis that magnifies the contributions of Kir channels to transepithelial K^+ secretion (Hine et al., 2014). The open ends of the Malpighian tubules were pulled with a glass hook into mineral oil, while the blind, distal end of the tubule remained in the Ringer bath. The tubules were nicked with a fine needle near the glass hook so that secreted fluid could exit into the light mineral oil. Once both tubules were confirmed to secrete fluid, one served as the sham tubule and the other served as the experimental tubule. Each tubule was studied first for a 30 min control period (P1) in high K^+ Ringer solution. Thereafter, the experimental tubule was treated with 10 μM of a small molecule, whereas the sham tubule was treated with the vehicle (0.05% DMSO). Both sham and experimental

tubules were then studied in parallel for periods P2 to P5 (30 min each). At the end of each period, the diameter of the secreted droplet was measured with an ocular micrometer (to determine its volume) and removed from the proximal end with a glass picoliter pipette. The fluid droplet secreted at the end of P5 was collected for the measurement of Na^+ , K^+ and Cl^- concentrations by the method of electron probe, as previously described (Hine et al., 2014).

Electrophysiological studies

We used the method of two-electrode voltage clamping of single principal cells in Malpighian tubules, as described previously (Masia et al., 2000). In brief, a principal cell of a Malpighian tubule was impaled with two microelectrodes. One electrode served to record the basolateral membrane voltage (V_{bl}) of a principal cell continuously (AxoClamp, Axon Instruments). The other electrode served to periodically inject current into the principal cell for the measurement of the cell input conductance (g_{in}) from current/voltage (I/V) plots. Both electrodes were filled with 3 M KCl. I/V plots were obtained by voltage-clamping V_{bl} for 300 ms at 6 incremental voltage steps of 5 or 10 mV.

The microelectrode resistance was on average $60.7 \pm 1.9 \text{ M}\Omega$ ($n = 77$ electrodes). The experiment was started with the tubule bathed in low K^+ Ringer solution. The bath was then changed to high K^+ Ringer solution, then to high K^+ Ringer solution containing a small molecule, and finally to high K^+ Ringer solution containing barium.

Perfusion chamber

The isolated Malpighian tubules were superfused with Ringer solutions in a bath with a volume that ranged from 500 to 850 μl . The flow of Ringer solution through the bath was 3 ml/min when the effect of rapid bath changes on V_{bl} and g_{in} were of interest as in the wash-in and wash-out of K^+ or Ba^{2+} . To economize on the use of small molecules, bath flow was stopped in high K^+ Ringer solution. The Ringer solution was subsequently aspirated to a level that barely covered the tubules and replaced with 500 μl of high K^+ Ringer containing the small molecule.

250 *Statistical analysis*

251 For Ramsay assays, statistical significance was evaluated by comparing the data from sister sham
252 (control) and experimental tubules using a paired Student's t-test (for rationale see Rouhier et al., 2014a).
253 For electrophysiological assays, each tubule was used as its own control, which permitted the statistical
254 analysis of the data by the paired Student's t-test.

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Results

Immunochemical Expression of Kir subunits in Malpighian tubules

To confirm the selectivity of the Kir1, Kir2B, and Kir3 antibodies, we tested them in Western blots of isolated total-membrane fractions (i.e., plasma, vesicular, and non-nuclear organellar membranes) from *Xenopus* oocytes injected with *AeKir1*, *AeKir2B*, or *AeKir3* cRNAs. Total-membrane fractions from H₂O-injected oocytes served as controls. As shown in Figure 1A, the Kir1 antibody detects a band of ~58 kDa in *AeKir1* oocytes, but not in the H₂O-injected, *AeKir2B*, or *AeKir3* oocytes. The immunoreactive band is slightly higher than the expected size of the encoded *AeKir1* protein (55.2 kDa) (Piermarini et al., 2013). The Kir2B antibody detects bands of ~55 kDa, ~116 kDa, and > 130 kDa in the *AeKir2B* oocytes, but not in H₂O-injected, *AeKir1*, or *AeKir3* oocytes (Figure 1B). The strongest band (~55 kDa) is at the expected size of the encoded *AeKir2B* protein (55.2 kDa) (Piermarini et al., 2013). The Kir3 antibody detects a strong band of > 130 kDa and a weak band of ~126 kDa in the *AeKir3* oocytes, but not in H₂O-injected, *AeKir1*, or *AeKir2B* oocytes (Figure 1C). These two bands are larger than the expected size of the encoded *AeKir3* protein (47.8 kDa) (Piermarini et al., 2013). The anti-Kir3 antibody also detects a band of ~52 kDa in all oocytes, including the H₂O-injected controls (Figure 1C), which likely indicates cross-reactivity with an endogenous membrane protein in *Xenopus* oocytes, as we have observed in a prior study with an antibody to a mosquito Na/H exchanger (Piermarini et al., 2009). The above data indicate that each antibody is selective for detecting its intended *AeKir* subunit target, and that when expressed in *Xenopus* oocytes, portions of the *AeKir2B* and *AeKir3* subunits are likely post-translationally modified (e.g., glycosylated) and/or possess SDS-resistant secondary structures that cause them to run anomalously of their expected size on SDS-PAGE (Piermarini et al., 2010; Piermarini et al., 2011; Piermarini et al., 2009).

We next tested the Kir antibodies in Western blots of crude lysates of Malpighian tubules isolated from adult female mosquitoes. As shown in Figure 2A, the Kir1 antibody primarily detects strong bands of ~52 and ~31 kDa, along with several weak bands of >100 kDa, ~74 kDa, ~40 kDa, and ~25 kDa. The Kir2B antibody detects a strong band of ~70 kDa along with a set of weak bands between ~56-61 kDa

(Figure 2B). The Kir3 antibody detects a strong band of ~62 kDa along with weak bands of ~55 kDa, ~36 kDa, and ~15 kDa (Figure 2C). Thus, each antibody detects a novel set of proteins, consistent with their ability to distinguish the different Kir subunits expressed in *Xenopus* oocytes (Figure 1). Although the immunoreactive bands do not match the expected sizes of the proteins encoded by the cDNAs cloned from Malpighian tubules (Piermarini et al., 2013) or those detected in *Xenopus* oocytes (Figure 1), the intensities of the detected bands are diminished when the antibodies are preadsorbed with their respective immunogenic peptides (data not shown). Furthermore, we have observed similar discrepancies in the sizes of membrane-bound proteins detected in mosquito Malpighian tubules vs. those expressed in *Xenopus* oocytes, which can be attributed to 1) differential post-translational modifications, 2) differential degrees of protein degradation, and/or 3) stubborn secondary structures/higher-ordered oligomeric forms that are resistant to denaturation (Piermarini et al., 2010; Piermarini et al., 2011; Piermarini et al., 2009). In summary, the data of Figure 2 indicate that all three Kir subunit proteins are expressed in Malpighian tubules, which is consistent with results of our previous RT-PCR studies of Kir subunit mRNA expression in mosquito Malpighian tubules (Piermarini et al., 2013; Raphemot et al., 2014a).

Immunochemical localization of Kir subunits in Malpighian tubules

As shown in Figure 3, Kir1 immunoreactivity (magenta) localizes to stellate cells; principal cells (diamonds) contain no detectable labeling. An optical section through the ‘arm’ of a stellate cell—in the plane indicated by the dashed line in Figure 3C—shows that the labeling occurs along the basolateral surface of the cell (Figure 3D). As shown in Figure 4, the immunolabeling of Kir2B (green) appears to be the inverse of that for Kir1. That is, Kir2B immunoreactivity localizes to principal cells, whereas stellate cells (asterisks) contain no detectable labeling (Figure 4). An optical section through a tubule—in the plane indicated by the dashed line in Figure 4B—shows discontinuous labeling along the basolateral surface of the tubule (Figure 4C). The areas without staining (asterisks) correspond to the ‘arms’ of a stellate cell. Figure 4D demonstrates the accumulation of Kir2B immunoreactivity along the basolateral aspect of a principal cell. When the Kir1 and Kir2B antibodies are pre-adsorbed with their respective

immunogenic peptides, the immunolabeling of stellate cells and principal cells is respectively blocked (data not shown).

To verify the inferred cell- and membrane-specific expression of *AeKir1* and *AeKir2B*, we conducted double-immunolabeling experiments. As shown in Figure 5A, the labeling of Kir1 (magenta) is restricted to a stellate cell, whereas that of Kir2B is restricted to surrounding principal cells. An optical section through the tubule—in the plane indicated by the dashed line in Figure 5A—clearly shows the basolateral distribution of the labeling in the respective cell types, confirming the cell and membrane specific localizations of these two subunits (Figure 5B).

In contrast to Kir1 and Kir2B, Kir3 immunoreactivity (yellow) occurs in both principal and stellate cells (Figure 6). In principal cells, the labeling is punctate throughout the cytoplasm and not associated with plasma membranes (Figure 6A, B). In stellate cells, the intracellular localization is difficult to resolve due to their limited cytoplasmic volume, but labeling is apparent near the nucleus (Figure 6C). When the Kir3 antibody is pre-adsorbed with its immunogenic peptide, the immunolabeling of principal and stellate cells is diminished (data not shown).

The effect of VU small molecules on transepithelial fluid secretion

The increase of the peritubular $[K^+]$ from 3.4 mM to 34 mM in the peritubular medium of isolated Malpighian tubules triggers a kaliuresis (Hine et al., 2014). Rates of transepithelial K^+ secretion increase nearly 6-fold at the expense of Na^+ secretion, which drops nearly 4-fold (Hine et al., 2014). As a result, rates of transepithelial cation secretion (K^+ plus Na^+) increase 1.13 fold and rates of transepithelial Cl^- and fluid secretion increase 1.21 fold and 1.33 fold respectively (Hine et al., 2014). The K^+ specificity of this diuresis can be attributed to the large K^+ conductance of the basolateral membranes of the tubule (Beyenbach and Masia, 2002), which is presumably mediated by *AeKir2B* in principal cells and *AeKir1* in stellate cells (Figure 3-Figure 5). Since the effect of VU small molecules on K^+ transport mediated by these two Kir channels was our primary interest, we studied tubules bathed in a peritubular medium containing a $[K^+]$ of 34 mM.

We examined the effect of four VU small molecules on transepithelial fluid secretion: VU590, VU608, VU625, and VU688. VU590 is a selective inhibitor of *AeKir1* ($IC_{50} \sim 5 \mu M$) vs. *AeKir2B*, and VU608 is an analog of VU590 that weakly inhibits *AeKir1* ($IC_{50} > 100 \mu M$) (Rouhier et al., 2014b). In contrast, VU625 is a highly potent inhibitor of *AeKir1* ($IC_{50} \sim 100 nM$) that also inhibits *AeKir2B* (estimated $IC_{50} \sim 4 \mu M$), and VU688 (a.k.a. VU0477688) is an analog of VU625 that weakly inhibits *AeKir1* ($IC_{50} > 30 \mu M$) (Raphemot et al., 2014b). To evaluate the effects of a small molecule on fluid secretion, two tubules were isolated from the same mosquito and studied in parallel to compare the sham treatment with the experimental treatment in 5 consecutive 30-min periods, P1 through P5. P1 served as the control period in the absence of the VU small molecule. The VU small molecule ($10 \mu M$) was added to the peritubular bath of the experimental tubule beginning at P2, whereas the vehicle (0.05% DMSO) was added to the peritubular bath of the sham tubule beginning at P2.

In general, the small molecules affected fluid secretion as expected based on their pharmacological properties. In particular, peritubular VU590 first caused a significant increase in the rate of fluid secretion in P2, but then led to a significant decrease of fluid secretion in P5 (Table 1). On the other hand, peritubular VU608 elicited a significant increase in the rate of fluid secretion compared to sham tubules, but only in P3, and did not lead to an inhibition (Table 1). Peritubular VU625 significantly reduced the rates of transepithelial fluid secretion in P3, P4 and P5, whereas peritubular VU688 had no significant effect on transepithelial fluid secretion in any 30-min period (Table 1). Thus, the inhibition of *AeKir1* by VU590 and the inhibition of *AeKir1* and *AeKir2B* by VU625 lead to reduced rates of transepithelial fluid secretion within 2 h. Given that we were primarily interested in the mechanisms of inhibition by the VU compounds, we next examined the effects of VU590 and VU625 on the transepithelial secretion of the three major osmolytes in secreted fluid (Na^+ , K^+ and Cl^-), with a focus in P5 only.

VU590 inhibits the transepithelial secretion of KCl

As shown in Figure 7, the rate of transepithelial fluid secretion was 1.18 ± 0.16 nl/min in the sham tubules during P5 and significantly ($p < 0.012$) lower (0.64 ± 0.10 nl/min) in six pairs of sister tubules treated with VU590. In the same tubule pairs, the sham tubules secreted fluid with a $[\text{Na}^+]$ of 12.8 ± 5.4 mM, a $[\text{K}^+]$ of 131.1 ± 6.2 mM, and a $[\text{Cl}^-]$ of 158.9 ± 6.7 mM (Figure 7). These concentrations were not significantly different in tubules treated with VU590 during P5 (Figure 7).

The product of fluid secretion rate and the concentration of secreted ions yields the rate of transepithelial ion secretion. In P5, VU590 had no significant effect on the rate of transepithelial Na^+ secretion; rates were 14.0 ± 5.1 pmol/min in the sham tubules and 9.45 ± 4.2 pmol/min in the experimental tubules (Figure 7). However, as shown in Figure 7, the rate of K^+ secretion was significantly ($p < 0.0111$) greater in the sham tubules (155.5 ± 22.4 pmol/min) compared to the sister experimental tubules treated with VU590 (83.8 ± 14.6 pmol/min), and the rate of Cl^- secretion was significantly ($p < 0.009$) greater in the sham tubules (189.1 ± 29.6 pmol/min) relative to the VU590-treated tubules (103.1 ± 20.4 pmol/min). In summary, VU590 significantly reduces the transepithelial secretion rates of K^+ , Cl^- and water, respectively, to levels that are 53.8 %, 54.5 %, and to 54.2% of those in the corresponding sham tubules. In general, nearly identical reductions of these three rates indicate an inhibition of water secretion secondary to KCl secretion, and an inhibition of K^+ secretion in particular.

VU625 inhibits the transepithelial secretion of KCl

As shown in Figure 8, the rate of transepithelial fluid secretion was 0.83 ± 0.15 nl/min in seven sham tubules during P5 and significantly ($p < 0.002$) lower (0.32 ± 0.08 nl/min) in seven sister tubules treated with VU625. In the sham tubules, secreted fluid had a $[\text{Na}^+]$ of 12.6 ± 7.2 mM, a $[\text{K}^+]$ of 135.9 ± 21.4 mM K and a $[\text{Cl}^-]$ of 139.1 ± 6.5 mM in P5 (Figure 8). In contrast, in the experimental tubules treated with VU625, the $[\text{Na}^+]$ in secreted fluid was significantly ($p < 0.019$) higher (20.3 ± 8.7 mM) and the $[\text{K}^+]$ was significantly ($p < 0.019$) lower (111.4 ± 20.6 mM) compared to the sister sham tubules in P5 (Figure

8). The concentration of Cl^- in the secreted fluid was not significantly different between the sham and experimental tubules (Figure 8).

The product of fluid secretion and ion concentration shows that VU625 had no significant effect on the rate of transepithelial Na^+ secretion in P5 (Figure 8), which was 10.0 ± 8.1 pmol/min in the seven sham tubules and 6.9 ± 5.2 pmol/min in the seven experimental tubules. However, as shown in Figure 8, the rate of K^+ secretion in the sham tubules (115.7 ± 23.8 pmol/min) was significantly ($p < 0.006$) lower in the experimental sister tubules treated with VU625 (39.2 ± 10.1 pmol/min) in P5 (Figure 8). Likewise, rates of Cl^- secretion in the experimental tubules was significantly ($p < 0.002$) lower (117.0 ± 22.4 pmol/min) than that in the sham tubules (41.7 ± 12.5 pmol/min) (Figure 8). In summary, VU625 significantly reduces the transepithelial secretion rates of K^+ , Cl^- and water, respectively, to levels that are 33.8 %, 35.6 %, 38.6 % of those in the corresponding sham tubules. Again, the similar percent reductions of these three rates indicate the inhibition of water secretion secondary to KCl secretion, and the inhibition of K^+ secretion in particular.

Electrophysiological studies

Since the inhibitory effects of VU small molecules are best observed after about one hour, it was necessary to record the basolateral membrane voltage (V_{bl}) and the cell input conductance (g_{in}) of a single principal cell for an extended time period. Fortunately, the large size of principal cells (Figure 4-Figure 6) permits the stable impalement of a single principal cell with voltage and current microelectrodes for over 3 hours (Figure 9). The initial impalement of a principal cell of the tubule with voltage and current electrodes revealed a V_{bl} of -84 mV and a g_{in} of 2.15 μS at time 5 min, when the peritubular Ringer solution had a K^+ concentration of 3.4 mM (Figure 9). The isosmotic increase of the peritubular K^+ concentration to 34 mM promptly depolarized V_{bl} to -43 mV and increased g_{in} to 2.60 μS (Figure 9). Thereafter, V_{bl} remained within a few millivolts of -40 mV and g_{in} remained elevated for more than 3 hours. The withdrawal of the voltage electrode from the cell returned V_{bl} to 0 mV, the value before cell impalement (Figure 9).

In a total of 31 experiments, V_{bl} was -65.6 ± 3.8 mV and g_{in} was 2.82 ± 0.22 μ S in the presence of 3.4 mM K^+ Ringer solution. In the same tubules, V_{bl} significantly ($p < 8.8 \times 10^{-12}$) depolarized to -34.5 ± 1.9 mV and g_{in} significantly ($p < 2.2 \times 10^{-7}$) increased to 3.44 ± 0.29 μ S when the tubules were bathed in 34 mM K^+ Ringer solution. The relatively constant values of V_{bl} and g_{in} for long periods of time serve as references for the long-term effects of VU590 and VU625 shown below.

Effects of VU590 on tubule electrophysiology

The addition of VU590 to the peritubular medium of isolated Malpighian tubules had little effect on V_{bl} measured in principal cells, but it caused g_{in} to decrease with time. In the representative experiment shown in Figure 10, V_{bl} was -77 mV in the presence of 3.4 mM K^+ and -44 mV in the presence of 34 mM K^+ . The increase in the peritubular $[K^+]$ increased the cell input conductance from 4.69 μ S to 5.90 μ S. Subsequently, the addition of 10 μ M VU590 to the peritubular medium had no immediate effect on V_{bl} and g_{in} (Figure 10). However, in the next 200 min, the average value of g_{in} was 4.84 μ S compared to 5.90 μ S in the absence of VU590; values of V_{bl} did not change appreciably with time in the presence of VU590. The return of the bath $[K^+]$ to 3.4 mM (which also washed out VU590) at time 210 min yielded a K^+ diffusion potential of 37 mV, which is similar the K^+ diffusion potential measured before VU590 was added to the peritubular bath (i.e., 33 mV; Figure 10). Moreover, adding peritubular Ba^{2+} (5 mM) in the presence of VU590 significantly hyperpolarized V_{bl} and significantly reduced g_{in} (data not shown). Thus, principal cells of the tubule retain substantial K^+ conductances in the presence of VU590 (a selective inhibitor of *AeKir1*), which is consistent with the localization of *AeKir1* to stellate cells and not principal cells (Figure 3, Figure 5).

Summarizing observations in eleven tubule experiments, V_{bl} was -36.7 ± 2.0 mV immediately after the addition of VU590 to the peritubular bath and was not significantly different (-37.0 ± 2.9 mV) on average 93 min later. In contrast, in a subset of 7 tubules, g_{in} was 5.34 ± 0.71 μ S immediately after adding VU590 and significantly ($p < 0.03$) lower (3.38 ± 0.63 μ S) on average 118 min later. Thus, VU590 has no significant effect on V_{bl} , but it slowly and significantly reduces g_{in} by 37% within ~2 h.

Effects of VU625 on tubule electrophysiology

On a molar basis VU625 was considerably more potent than VU590 on tubule electrophysiology. The time course of the effect of VU625 on V_{bl} and g_{in} in a typical experiment is shown in Figure 11. When the tubule was bathed in 3.4 mM K^+ Ringer solution, V_{bl} was close to -80 mV and g_{in} was between 2.2 μ S and 2.5 μ S. Replacing the peritubular bath with 34 mM K^+ Ringer solution (at 10 min, Figure 11) depolarized V_{bl} to -41 mV and increased g_{in} to 3.2 μ S. The addition of 10 μ M VU625 (at 21 min, Figure 11) triggered a slow, progressive depolarization of V_{bl} and a slow loss of g_{in} until 50 min. Thereafter, V_{bl} depolarized at an accelerated rate concomitant with reductions of g_{in} . The progressive effects of VU625 on tubule electrophysiology mirror those observed in the fluid secretion assays (Table 1). After reaching a new steady state at ~60 min (Figure 11), V_{bl} and g_{in} no longer responded to 1) a change in peritubular K^+ concentration, or 2) the addition of 5 mM Ba^{2+} to the peritubular bath, suggesting a complete shutdown of principal cell K^+ -channel activity by VU625, presumably due to its inhibition of *AeKir2B*, which is expressed exclusively in principal cells (Figure 4, Figure 5).

To summarize the effects of VU625, we compared the values of V_{bl} and g_{in} immediately after adding VU625 to the peritubular bath with those at the conclusion of the experiment, which was on average 28 min later. In 14 experiments, V_{bl} was -36.1 ± 3.5 mV and g_{in} was 2.69 ± 0.16 μ S shortly after adding 10 μ M VU625 to the peritubular bath. At the end of the experiment (~28 min later), V_{bl} had significantly ($p < 0.035$) depolarized to -22.5 ± 3.9 mV and g_{in} had significantly ($p < 0.0008$) decreased to 1.50 ± 0.24 μ S. Thus, the effects of VU625 on V_{bl} and g_{in} are stronger and faster than those of VU590. Moreover, in the presence of VU625, the tubules lose their ability to respond to changes in peritubular $[K^+]$ and to the addition of Ba^{2+} to the peritubular bath.

Effect of barium on tubule electrophysiology

Next we examined the effects of peritubular Ba^{2+} on V_{bl} and g_{in} in tubules bathed in the Ringer solution containing 34 mM K^+ . Adding 10 μ M Ba^{2+} to the peritubular medium had no effect on V_{bl} and

g_{in} . In the representative experiment shown in Figure 12, V_{bl} was about -90 mV and g_{in} was 1.93 μ S when the tubule was bathed in low K^+ Ringer solution containing 3.4 mM K^+ . Replacing the peritubular bath with high K^+ Ringer solution containing 34 mM K^+ depolarized V_{bl} to -41 mV and increased g_{in} to 2.42 μ S. The addition of 10 μ M Ba^{2+} to the peritubular bath had no effect on V_{bl} and g_{in} . In contrast, the addition of 5 mM Ba^{2+} to the peritubular bath repolarized V_{bl} to -62 mV and decreased g_{in} to 1.96 μ S. The washout of 5 mM Ba^{2+} returned V_{bl} to -43 mV and increased g_{in} to 1.98 μ S.

Together with 6 other observations, V_{bl} and g_{in} were respectively -34.4 ± 3.2 mV and 3.06 ± 0.24 μ S in high K^+ Ringer solution in the absence of Ba^{2+} and not significantly different from -33.7 ± 5.4 mV and 3.13 ± 0.31 μ S in the presence 10 μ M Ba^{2+} ($n = 7$). In contrast, 5 mM Ba^{2+} significantly hyperpolarized V_{bl} and decreased g_{in} . In the absence of 5 mM Ba^{2+} (control), V_{bl} and g_{in} were -35.7 ± 6.1 mV and 2.99 ± 0.39 μ S, respectively, and in the presence of 5 mM Ba^{2+} , V_{bl} and g_{in} were -46.0 ± 8.5 mV and 2.41 ± 0.27 μ S ($n = 7$), consistent with the Ba^{2+} block of K^+ channels observed in previous studies (Beyenbach and Masia, 2002; Masia et al., 2000; Scott et al., 2004; Wu and Beyenbach, 2003).

In summary, Ba^{2+} , the well-known blocker of K^+ channels, had no effect on V_{bl} and g_{in} at a concentration of 10 μ M, but it significantly hyperpolarized V_{bl} and reduced g_{in} at a concentration of 5 mM. At this concentration, the effects of Ba^{2+} were immediate and reversible, in contrast to the slow channel inhibition in the presence of VU590 (Figure 10) or VU625 (Figure 11).

Discussion

Localization of Kir channel subunits in A. aegypti Malpighian tubules

The genome of the yellow fever mosquito *A. aegypti* has five genes encoding Kir channel subunits: *AeKir1*, *AeKir2A*, *AeKir2B*, *AeKir2B'*, and *AeKir3* (Piermarini et al., 2013). The Malpighian tubules primarily express mRNAs for *AeKir1*, *AeKir2B*, and *AeKir3* (Piermarini et al., 2013). The present study provides the first immunochemical localization of Kir channel subunits in any insect tissue. Notably, *AeKir1* and *AeKir2B* localize to the basolateral membranes of stellate and principal cells, respectively (Figure 3-Figure 5). In contrast, *AeKir3* localizes to intracellular compartments of both principal and stellate cells (Figure 6). Given that principal cells comprise over 80% of the total cell population and 90% of the tubule mass in mosquito Malpighian tubules (Beyenbach and Piermarini, 2011), it can be inferred that *AeKir2B* and *AeKir3* are the most abundant Kir subunits in the epithelium. This inference is consistent with RT-PCR data that indicate the most abundant Kir subunit mRNAs expressed in mosquito Malpighian tubules are *Kir2B* and *Kir3* (Piermarini et al., 2013; Raphemot et al., 2014a).

The cellular localization of Kir subunit proteins that we have elucidated in mosquito Malpighian tubules contrasts with that of Kir subunit mRNAs in fruit fly (*D. melanogaster*) Malpighian tubules. That is, the Dow laboratory has localized *Kir1* (*ir*), *Kir2* (*irk2*), and *Kir3* (*irk3*) mRNAs exclusively to principal cells in Malpighian tubules of *D. melanogaster* (Evans et al., 2005). Thus, the localization of *AeKir1* in the basolateral membranes of stellate cells in mosquito Malpighian tubules (Figure 3, Figure 5) suggests that these cells have evolved an additional role in epithelial cation transport not found in the stellate cells of fruit flies. Further supporting this notion is the presence of the alpha subunit of the Na^+, K^+ -ATPase (α -NKA) and a cation-proton antiporter (NHA2) in, respectively, the basolateral and apical membranes of stellate cells in the mosquito (Patrick et al., 2006; Xiang et al., 2012). In contrast, α -NKA and NHA2 respectively localize to the basolateral and apical membranes of principal cells in *D. melanogaster* (Day et al., 2008; Torrie et al., 2004). The absence of these cation transporters in stellate

cells of *D. melanogaster* may reflect the specialized role of these cells in anion transport, namely transcellular Cl⁻ secretion via CIC channels (Cabrero et al., 2014).

The localization of Kir subunits in the present study raises the question about heteromeric Kir channels in mosquito Malpighian tubules. The formation of heteromeric Kir channels in vivo requires the expression of two different Kir subunits in the same cell. Thus, the potential for heteromeric channels composed of *AeKir1* and *AeKir2B* subunits is very low in mosquito Malpighian tubules in view of the clear segregation of the *AeKir1* subunit to stellate cells and the *AeKir2B* subunit to principal cells (Figure 3-Figure 5). On the other hand, the *AeKir1* and *AeKir2B* subunits have the potential to form heteromeric channels with *AeKir3*. However, if present, such channels must localize to intracellular compartments, because we did not detect *AeKir3* immunoreactivity in the basolateral membranes of stellate or principal cells, where *AeKir1* and *AeKir2B* respectively reside.

The segregation of the *AeKir1* and *AeKir2B* subunits in different cell types of the tubule may reflect different roles in transepithelial K⁺ transport, because these subunits display remarkable functional and pharmacological differences when expressed heterologously in *Xenopus* oocytes. *AeKir1* subunits form homomeric channels that mediate robust, inward-rectifying K⁺ currents that are blocked by Ba²⁺, enhanced by extracellular Na⁺, and inhibited by the small molecules VU573, VU590, and VU625 (Piermarini et al., 2013; Raphemot et al., 2011; Rouhier et al., 2014b). In contrast, *AeKir2B* subunits form homomeric channels that mediate weak, inward-rectifying K⁺ currents that are blocked by Ba²⁺, unaffected by extracellular Na⁺, enhanced by VU573, unaffected by VU590, and inhibited by VU625 (Piermarini et al., 2013; Raphemot et al., 2011; Rouhier et al., 2014b). Moreover, the homomeric *AeKir2B* channels are unique in that they allow the passage of Cs⁺ (Piermarini et al., 2013). Thus, the immunolocalization of Kir channel subunits in the present study assigns the unique functional activities and pharmacological sensitivities of *AeKir1* to the basolateral membrane of stellate cells and *AeKir2B* to the basolateral membrane of principal cells.

529 *What is the function of AeKir3?*

530 To date, the functional role(s) of insect Kir3 subunits have remained enigmatic. In insects, the Kir3
531 subunits evolved independently from Kir1 and Kir2 subunits, and the Kir3 subunits of mosquitoes possess
532 a non-canonical selectivity filter (Piermarini et al., 2013; Raphemot et al., 2014a), suggesting potentially
533 novel functions for these subunits. In a previous study, we were unable to measure Kir channel activity in
534 the plasma membrane of *Xenopus* oocytes injected with *AeKir3* cRNA, suggesting that this subunit does
535 not form functional homomeric channels. Similar negative results have been found in attempts to
536 functionally characterize Kir3 subunits (*irk3*) of *D. melanogaster* expressed in *Xenopus* oocytes or *D.*
537 *melanogaster* S2 cell lines (Doring et al., 2002). In the present study, we confirm that *Xenopus* oocytes
538 translate the injected *AeKir3* cRNA into protein (Figure 1). Thus, the lack of function in oocytes is not
539 due to a lack of translated protein. This taken together with the localization of *AeKir3* immunoreactivity
540 to intracellular compartments in Malpighian tubules (Figure 6) suggests that in *Xenopus* oocytes the
541 encoded protein likely does not reach the plasma membrane, thereby explaining its lack of
542 electrophysiological activity.

543 The physiological role of an apparent intracellular Kir channel in Malpighian tubules remains unclear.
544 The distinct, punctate localization pattern of *AeKir3* throughout the cytoplasm of principal cells (Figure 6)
545 suggests it may be associated with the vesicular, mineralized concretions, which are temporary storage
546 sites for a variety of monovalent and divalent cations and anions, including K⁺ (Beyenbach and
547 Piermarini, 2009; Wessing et al., 1992). *AeKir3* could potentially be involved with modulating the [K⁺]
548 and/or voltage of these vesicular compartments. Alternatively, the intracellular localization could reflect
549 expression in vesicles that are trafficked to the plasma membrane upon receiving the appropriate stimulus.
550 Thus, *AeKir3* may be analogous to Kir3.1 of mammals, which is expressed in intracellular secretory
551 vesicles of pituitary gland thyrotroph cells (Morishige et al., 1999); these vesicles are trafficked to the
552 plasma membrane upon stimulation by thyrotropin-releasing hormone (Morishige et al., 1999).

553 The localization of *AeKir3* near the nucleus of stellate cells (Figure 6) suggests it may also be
554 associated with the endoplasmic reticulum (ER) and/or Golgi complex. In mammals, Kir2.6 localizes to

the ER where it interacts with Kir2.1 and Kir2.2 subunits and reduces their trafficking to the plasma membrane (Dassau et al., 2011). It will be interesting to determine if *AeKir3* convergently evolved a similar function for regulating the surface expression of *AeKir1* and/or *AeKir2B* in stellate and principal cells, respectively.

The effects of VU small molecules on Malpighian tubule physiology

VU625 (Figure 13) is the most potent inhibitor of *AeKir1* to date with an IC_{50} of 96.8 nM for *AeKir1* expressed in HEK-293 cells (Raphemot et al., 2014b). Experiments in *Xenopus* oocytes reveal that VU625 also inhibits *AeKir2B*, but with a 12-fold lesser potency than that for *AeKir1* (Raphemot et al., 2014b). In the present study, VU625 was the most effective inhibitor of Malpighian tubule physiology. At a concentration of 10 μ M, VU625 significantly inhibited transepithelial fluid secretion from 60-120 min after addition to the peritubular bath (Table 1 and Figure 8). The inhibition of fluid secretion stems from the inhibition of transepithelial K^+ and Cl^- secretion, which drop respectively to 34% and 36% of the control (Figure 8). Importantly, VU625 does not affect transepithelial Na^+ secretion. The specific inhibition of KCl secretion is consistent with the shutdown of channel-mediated K^+ secretion. Electrophysiological studies support this conclusion. Namely, the significant drop of g_{in} from 2.7 μ S (control) to 1.5 μ S within ~30 min after the addition of VU625. At this low g_{in} , the basolateral membrane of principal cells no longer displays K^+ diffusion potentials, and Ba^{2+} no longer has any effect on V_{bl} and g_{in} (Figure 11), which is consistent with the shutdown of both *AeKir1* and *AeKir2B* channels. The transepithelial secretion of K^+ that remains in the presence of VU625 (Figure 8) is most likely independent of channel-mediated transport and is presumably mediated by an electroneutral, bumetanide-sensitive Na,K,2Cl cotransporter (NKCC) and/or the NKA (Figure 14).

The replacement of the methyl group at the amide side of VU625 with toluene yields VU688 (Figure 13). VU688 is 100-fold less potent than VU625 in reducing the thallium flux in HEK-293 cells expressing *AeKir1* (Raphemot et al., 2014b). Furthermore, K^+ currents mediated by *AeKir1* or *AeKir2B* in *Xenopus* oocytes were not sensitive to VU688 (Piermarini, unpublished observations). In the present

study, VU688 did not affect the transepithelial secretion of fluid in isolated Malpighian tubules (Table 1), supporting the conclusion reached previously that aryl groups at the amide portion of VU625 substantially reduce its inhibitory effects on channel-mediated K^+ transport (Raphemot et al., 2014b).

VU590 distinguishes itself from VU625 in its structure (Figure 13) and pharmacology. VU590 was originally discovered in a high-throughput screen for modulators of mammalian Kir1.1 channels (Lewis et al., 2009), and has been shown to inhibit mammalian Kir1.1 and Kir 7.1 channels by blocking the intracellular pore near the selectivity filter of these channels (Bhave et al., 2010; Lewis et al., 2009). In spite of its marked structural differences from VU625, VU590 also inhibits *AeKir1* channels expressed in HEK-293 cells and *Xenopus* oocytes, but not *AeKir2B* channels expressed in *Xenopus* oocytes (Rouhier et al., 2014b). Our present study in isolated Malpighian tubules is consistent with *AeKir1* as the target of VU590. For example, the inhibition of *AeKir1* by VU590 is expected to be less effective on tubule physiology than the inhibition of both *AeKir1* and *AeKir2B* by VU625. To wit, VU590 reduced transepithelial K^+ secretion to 56% of control rates, while VU625 reduced the secretion of K^+ significantly further ($p < 0.04$) to 31% of control rates (Figure 7, Figure 8). Furthermore, VU590 had no effect on the V_{bl} of principal cells, whereas VU625 caused a significant depolarization (Figure 10, Figure 11). Although VU590 significantly decreased the g_{in} of principal cells like VU625, it took much longer to manifest (~118 min) compared to VU625 (~28 min). The relatively weak electrophysiological effects of VU590 on principal cell electrophysiology are also consistent with our immunolocalization data that indicate *AeKir1* is expressed exclusively in stellate cells (Figure 3, Figure 5).

VU608 is an analog of VU590 in which the nitrobenzyls are replaced with (methylsulfonyl)benzyl groups (Figure 13). The inhibition of *AeKir1*-mediated TI^+ -flux in HEK-293 cells by VU608 ($IC_{50} > 100 \mu M$) is much reduced compared to VU590 ($IC_{50} = 5.6 \mu M$) (Rouhier et al., 2014b). Furthermore, VU608 injected into the hemolymph of mosquitoes did not affect the diuretic capacity or survival of adult female mosquitoes (Rouhier et al., 2014b). Consistent with these findings, VU608 failed to inhibit the rate of fluid secretion in isolated Malpighian tubules (Table 1).

Roles of AeKir1 and AeKir2B in transepithelial K⁺ secretion.

The inhibition of AeKir1 in stellate cells by VU590 and the inhibition of both AeKir1 in stellate cells and AeKir2B in principal cells by VU625 allow us to estimate the relative contributions that principal and stellate cells make to transepithelial K⁺ secretion in the presence of elevated peritubular [K⁺] (34 mM).

With both AeKir1 and AeKir2B blocked by VU625, the tubules continue to secrete K⁺ at a rate that is 34% of the controls (Figure 8), which is presumably mediated via a NKCC and/or NKA (Figure 14).

Accordingly, AeKir1 and AeKir2B must collectively mediate 66% of the transepithelial secretory K⁺ flux.

During the selective block of AeKir1 by VU590, 54% of the transepithelial K⁺ secretion remains (Figure 7). Assuming that a NKCC and/or NKA contribute to 34% of the remaining K⁺ secretion (Figure 14), then 20% of the K⁺ secretion must be mediated by AeKir2B. Accordingly, AeKir1 mediates 46% of the transepithelial K⁺ secretion. These numbers surprise, because they suggest that AeKir1 located in stellate cells (the minority population of epithelial cells) mediates nearly half of the transepithelial secretion of K⁺, whereas AeKir2B located in principal cells (the majority population of epithelial cells) mediates but 1/5 of the transepithelial secretory K⁺ transport in the presence of elevated peritubular [K⁺] (Figure 14).

Our previous in vitro and in vivo studies are consistent with an important role of AeKir1 in renal K⁺ transport. First of all, when expressed heterologously in *Xenopus* oocytes, AeKir1 mediates a more pronounced macroscopic K⁺-conductance than AeKir2B (Piermarini et al., 2013); depending upon the concentrations of Na⁺ and K⁺ in the extracellular solutions the K⁺ conductance mediated by AeKir1 can exceed that mediated by AeKir2B by at least 3-fold and as much as 104-fold (Piermarini et al., 2013). Thus, the greater activity of AeKir1 channels may allow the minority stellate cells to carry the majority of the channel-mediated transepithelial K⁺ transport compared to principal cells, which possess the less active AeKir2B channels. Second, the injection of VU590, the selective inhibitor of AeKir1, into the hemolymph of adult female mosquitoes leads to abdominal bloating, death, and/or an inhibition of their diuretic capacity (Rouhier et al., 2014b). Third, when injected into the hemolymph of adult female mosquitoes, VU573—a small molecule inhibitor of AeKir1 and agonist of AeKir2B (Rouhier et al.,

2014b)—inhibits the urinary excretion of K^+ (Rouhier et al., 2014a) and exhibits an enhanced toxicity if delivered with a load of K^+ to the hemolymph (Raphemot et al., 2013). Fourth, in isolated Malpighian tubules bathed in a Ringer solution with elevated K^+ (34 mM K^+), the addition of peritubular VU573 inhibits transepithelial K^+ secretion (Rouhier et al., 2014a). Thus, our observations in vitro and in vivo are consistent with an important role of *AeKir1* in renal K^+ homeostasis, and confirm a direct role of stellate cells in transepithelial ion and fluid transport, which has only recently been recognized in Malpighian tubules of *A. aegypti* and *D. melanogaster* (Cabrero et al., 2014; Dow, 2012; Hine et al., 2014; Patrick et al., 2006; Piermarini et al., 2010; Xiang et al., 2012).

Barium and VU625: different mechanisms of action?

We know from a previous study that barium-sensitive K^+ channels account for as much as 64% of the conductance of the basolateral membrane of principal cells in Malpighian tubules of *A. aegypti* (Beyenbach and Masia, 2002). In the present study, the large K^+ conductance contributes to the large depolarization of V_{bl} from -66 mV to -34 mV as the peritubular $[K^+]$ increases from 3.4 mM to 34 mM. In parallel, the g_{in} increases from 2.8 μS to 3.4 μS as K^+ enters epithelial cells at increased rates (Figure 9- Figure 12). The extrusion of K^+ on the other side of epithelial cells brings about the nearly 6-fold increase in the transepithelial secretion of K^+ compared to secretion rates in the presence of the 3.4 mM K^+ Ringer solution (Hine et al., 2014).

The addition of 5 mM barium, the well-known blocker of K^+ channels, to the peritubular bath of isolated Malpighian tubules inhibits 80% of the transepithelial K^+ secretion (Scott et al., 2004), and hyperpolarizes the V_{bl} and decreases the g_{in} of principal cells (Beyenbach and Masia, 2002; Masia et al., 2000; Scott et al., 2004). In the present study, Ba^{2+} had no effect on V_{bl} and g_{in} when used at a peritubular concentration of 10 μM (the concentration of VU small molecules), but it significantly hyperpolarized V_{bl} and decreased g_{in} at a concentration of 5 mM (Figure 12). The hyperpolarization of V_{bl} comes about from the polarization of the basolateral membrane by the intraepithelial current I_{ie} . Equation 1 shows that V_{bl} hyperpolarizes as g_{in} decreases when K^+ channels are blocked by Ba^{2+} . I_{ie} originates at the apical

membrane where the V-type H⁺-ATPase transports protons from the cytosol to the tubule lumen (Figure 14) (Beyenbach, 1995, 2001, 2003; Beyenbach et al., 2000; Beyenbach and Piermarini, 2011; Beyenbach and Wieczorek, 2006; Scott et al., 2004). Thus, I_{ie} is carried by H⁺ across the apical membrane.

Returning to the cytoplasmic face of the V-type H⁺-ATPase, I_{ie} first passes through the paracellular pathway where it is carried by Cl⁻ ions and then by K⁺ passing through AeKir1 and AeKir2B channels embedded in basolateral membranes of stellate cells and principal cells, respectively (Figure 14). It follows that V_{bl} hyperpolarizes as g_{in} decreases consequent to channel block by Ba²⁺ (eq. 1).

$$V_{bl} = I_{ie}/g_{bl} \cong I_{ie}/g_{in} \quad (1)$$

The addition of VU625 to the peritubular medium of the tubule decreases g_{in} like Ba²⁺ and it inhibits 66% of the transepithelial K⁺ secretion (Figure 8, Figure 11). However, unlike Ba²⁺, VU625 depolarizes V_{bl} with time (Figure 11). The depolarization of V_{bl} indicates that VU625 has additional effects on the physiology of the tubule. According to eq. 1, VU625 must lower I_{ie} for V_{bl} to depolarize as g_{in} decreases. Clearly, Ba²⁺ and VU625 affect intact Malpighian tubules in different ways.

The effects of Ba²⁺ on V_{bl} and g_{in} are acute; i.e., they are immediate and reversible upon washout, consistent with the physical block of open K⁺ channels. Ba²⁺ is thought to block Kir channels from the extracellular side by obstructing the open channel pore. Ba²⁺ mimics a divalent potassium ion that competes with K⁺ for two binding sites in the selectivity filter (Chatelain et al., 2005; Gibor et al., 2004; Piasta et al., 2011). This mechanism of action probably accounts for the quick, potent, and reversible effects of Ba²⁺ in Malpighian tubules of *A. aegypti* observed in the present and previous studies (Beyenbach and Masia, 2002; Masia et al., 2000; Scott et al., 2004).

In contrast, the slow run-down of V_{bl} and g_{in} in the presence of VU625 stems from 1) the run-down of AeKir1 and AeKir2B channels, and 2) the inhibition of the I_{ie} (Figure 8, Figure 11). The run-down of AeKir1 and AeKir2B channels is clearly indicated by the failure of Ba²⁺ to affect V_{bl} and g_{in} after tubules have been exposed to VU625 for about an hour (Figure 11). The slow kinetics is not consistent with the

rapid, physical obstruction of open channels from the extracellular face of the channel. More likely, the slow kinetics suggests interactions of VU625 with intracellular parts of the channel protein, similar to the interaction of VU590 with mammalian Kir1.1, which first needs to cross the plasma membrane before it can block the intracellular pore of the channel (Bhave et al., 2010; Lewis et al., 2009). Alternatively, the slow kinetics may indicate that VU625 causes 1) C-type inactivation by deforming the selectivity filter of the channel and therefore impeding the passage of K^+ (Sepulveda et al., 2015), and/or 2) disruptions in Kir channel activity by interfering with the channel gating that is regulated by agents such as PIP_2 , G proteins, and intracellular pH (Sepulveda et al., 2015).

The mechanism of action by which VU625 inhibits I_{Kc} is not apparent, but we emphasize that the present study was performed in intact epithelial cells of Malpighian tubules engaged in transepithelial K^+ secretion. Here, Kir channels do not operate in isolation. The functional interactions of Kir channels with other transporters are observed in diverse tissues, including 1) Cl^- channels in the thick ascending limb (TAL) of the kidney (Lu et al., 2006), 2) NKCC and the NKA in the TAL (Hebert et al., 2005), 3) glutamate transporters in astroglial cells (Djukic et al., 2007), and 4) the H/K ATPase in H^+ -secreting parietal cells of the stomach (Fujita et al., 2002; Kaufhold et al., 2008). Interactions of Kir channels with the V-type H^+ -ATPase have not yet been reported, but could match the entry of K^+ into epithelial cells with the extrusion across the apical membrane, thereby affecting I_{Kc} (Figure 14). Such interactions between events at basolateral and apical membranes could be communicated via electrical coupling and/or intracellular messengers.

In summary, the segregation of *AeKir1* in stellate cells and *AeKir2B* in principal cells suggests different functional roles in Malpighian tubules consistent with the functional differences of these two Kir channels expressed in *Xenopus* oocytes. The selectivity of VU590 for *AeKir1* and the inhibition of both *AeKir1* and *AeKir2B* by VU625 allowed estimates of the relative roles of *AeKir1* and *AeKir2B* in transepithelial K^+ secretion. *AeKir1* contributes 46% and *AeKir2B* 20% to transepithelial K^+ secretion when the tubules are challenged to secrete K^+ at high rates in Ringer solution containing 34 mM K^+ . Thus, *AeKir1* localized exclusively to stellate cells, the minority population of epithelial cells, mediates

nearly half of the transepithelial secretory K^+ flux when the tubules are challenged to secrete K^+ at high rates. The physiological role of *AeKir3* in Malpighian tubules, which localizes to intracellular compartments of principal and stellate cells, remains to be elucidated.

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Tables

Table 1. The effect of VU small molecules (10 μM) on transepithelial fluid secretion (nl/min) in isolated Malpighian tubules of *A. aegypti* bathed in high K^+ (34 mM) Ringer solution. To prevent the transepithelial secretion of VU small molecules, they were studied in the presence of probenecid (100 μM). Pairs of sister tubules were studied for five consecutive 30 min periods: P1 through P5. At the beginning of P2, the sham tubule was treated to a sham volume change (5 μl) of the peritubular Ringer solution containing the vehicle (0.05% DMSO). In the experimental tubule, 5 μl of peritubular Ringer solution was replaced with an equal volume of Ringer solution containing the VU small molecule and probenecid such that experimental concentrations were 10 μM VU small molecule in 0.05% DMSO and 100 μM probenecid. Data are means $\pm$ SE, based on the number of pairs of sister tubules in parentheses. Sister tubules are tubules isolated from the same female mosquito. Statistical significance was evaluated with the paired Student's t-test comparing the sham tubule with the experimental tubule; NS, not significant.

	P1	P2	P3	P4	P5
Sister Tubules (11)					
Sham	0.74 $\pm$ 0.07	0.78 $\pm$ 0.09	0.91 $\pm$ 0.11	0.96 $\pm$ 0.13	0.94 $\pm$ 0.14
VU590	0.71 $\pm$ 0.05	0.93 $\pm$ 0.08	0.90 $\pm$ 0.08	0.71 $\pm$ 0.11	0.60 $\pm$ 0.10
p<	NS	0.049	NS	NS	0.013
Sister Tubules (8)					
Sham	0.73 $\pm$ 0.10	0.76 $\pm$ 0.12	0.87 $\pm$ 0.12	0.97 $\pm$ 0.11	0.96 $\pm$ 0.15
VU608	0.73 $\pm$ 0.08	0.94 $\pm$ 0.11	1.19 $\pm$ 0.16	1.32 $\pm$ 0.20	1.26 $\pm$ 0.25
p<	NS	NS	0.020	NS	NS
Sister Tubules (12)					
Sham	0.79 $\pm$ 0.06	0.76 $\pm$ 0.07	0.84 $\pm$ 0.10	0.83 $\pm$ 0.12	0.79 $\pm$ 0.11
VU625	0.81 $\pm$ 0.07	0.76 $\pm$ 0.09	0.60 $\pm$ 0.09	0.45 $\pm$ 0.08	0.31 $\pm$ 0.05
p<	NS	NS	0.0019	0.00095	0.00011
Sister Tubules (7)					
Sham	0.56 $\pm$ 0.10	0.45 $\pm$ 0.09	0.45 $\pm$ 0.07	0.54 $\pm$ 0.12	0.53 $\pm$ 0.12
VU688	0.70 $\pm$ 0.18	0.61 $\pm$ 0.15	0.62 $\pm$ 0.17	0.58 $\pm$ 0.18	0.57 $\pm$ 0.14
p<	NS	NS	NS	NS	NS

Figure Legends

Figure 1. Characterization of Kir1 (A), Kir2B (B), and Kir3 (C) antibodies in Western blots of total membrane fractions isolated from *Xenopus* oocytes injected with H₂O or cRNA encoding AeKir1, AeKir2B, or AeKir3 subunits.

Figure 2. Kir1 (A), Kir2B (B), and Kir3 (C) immunoreactivities in Western blots of crude lysates of Malpighian tubules isolated from adult female mosquitoes.

Figure 3. Immunofluorescent labeling of Kir1 (magenta) in whole mounts of isolated Malpighian tubules. Nuclei are counterstained with DAPI (cyan). (A, B) z-stacks of optical sections of tubules showing the labeling of stellate cells and not principal cells (diamonds). Overlays of the fluorescent signals with visible light are provided on the right to visualize the morphology of the unlabeled principal cells. C) zoom of the boxed area in (B). The dashed line represents the plane of the optical section shown in (D). D) optical section through the stellate cell shown in (C). 'IC' and 'EC' indicate direction of intracellular and extracellular regions, respectively. 'SC' and 'PC' indicate the nuclei of stellate and principal cells, respectively.

Figure 4. Immunofluorescent labeling of Kir2B (green) in whole mounts of isolated Malpighian tubules. Nuclei are counterstained with DAPI. (A, B) z-stack of optical sections of a tubule showing the labeling of principal cells and not a stellate cell (asterisks). In (B), the dashed line represents the plane of the optical section shown in (C). C) optical section through the tubule shown in (B). 'IC' and 'EC' indicate direction of intra- and extracellular regions, respectively. D) z-stack of optical sections through a principal cell showing labeling of the basolateral margins. An overlay of the fluorescent and light images reveals the morphology of unlabeled structures. 'SC' and 'PC' indicate the nuclei of stellate and principal cells, respectively.

Figure 5. Co-labeling of Kir1 (magenta) and Kir2B (green) in a whole mount of an isolated Malpighian tubule. Nuclei are counterstained with DAPI. (A) z-stack of optical sections of a tubule showing the labeling of principal cells compared to a stellate cell (asterisks). The dashed line represents the plane of the optical section shown in (B). (B) optical section through the tubule shown in (A). ‘IC’ and ‘EC’ indicate direction of intracellular and extracellular regions, respectively. ‘PC’ indicates the nucleus of a principal cell.

Figure 6. Immunofluorescent labeling of Kir3 (yellow) in whole mounts of isolated Malpighian tubules. Nuclei are counterstained with DAPI (cyan). (A) z-stack of optical sections through the distal end of a tubule showing the labeling of principal cells and a stellate cell. (B) z-stack of optical sections through a principal cell showing punctate labeling throughout the cytoplasm. (C) z-stack of optical section through a tubule showing labeling near the nucleus of a stellate cell and the cytoplasm of surrounding principal cells (diamonds). On the right, overlays of the fluorescent signals with visible light are provided to visualize the morphology of unlabeled structures. ‘SC’ and ‘PC’ indicate the nuclei of stellate and principal cells, respectively.

Figure 7. The effect of VU590 (10 μ M in the presence of 100 μ M probenecid) on transepithelial fluid and electrolyte secretion in 6 paired sister Malpighian tubules during period 5 (P5); i.e., 90 min after the addition of VU590 to the peritubular Ringer bath. Data are means $\pm$ SE. P-values indicate significant difference between sham and experimental tubules as determined by the paired Student’s t-test.

Figure 8. The effect of VU625 (10 μ M in the presence of 100 μ M probenecid) on transepithelial fluid and electrolyte secretion in 7 paired sister Malpighian tubules during period 5 (P5); i.e., 90 min after the addition of VU625 to the peritubular Ringer bath. Data are means $\pm$ SE. P-values indicate significant difference between sham and experimental tubules as determined by the paired Student’s t-test.

Figure 9. Effect of the 10-fold increase in the peritubular $[K^+]$ on the basolateral membrane voltage (V_{bl}) and the input conductance (g_{in}) of a principal cell in an isolated Malpighian tubule of the yellow fever mosquito. Downward and upward arrows indicate respectively the cell entry and withdrawal of the voltage electrode.

Figure 10. Effect of VU590 (10 μ M) on the basolateral membrane voltage (V_{bl}) and the input conductance (g_{in}) of a principal cell in an isolated Malpighian tubule of the yellow fever mosquito bathed in 34 mM K^+ -Ringer solution. The experiment began with the tubule bathed in Ringer solution containing 3.4 mM K^+ and the impalement of the cell with voltage and current microelectrodes (downward arrow). The experiment terminated with the withdrawal of the electrodes when the tubule was bathed in Ringer solution containing 34 mM K^+ (upward arrow).

Figure 11. The effect of 10 μ M VU625 on the basolateral membrane voltage (V_{bl}) and the cell input conductance (g_{in}) of a principal cell in an isolated Malpighian tubule of the yellow fever mosquito bathed in 34 mM K^+ -Ringer solution. The experiment began with the tubule bathed in Ringer solution containing 3.4 mM K^+ and the impalement of the cell with voltage and current microelectrodes (downward arrow). The experiment terminated with the withdrawal of the electrodes when the tubule was bathed in Ringer solution containing 34 mM K^+ (upward arrow).

Figure 12. The effect of low (10 μ M) and high Ba^{2+} (5 mM) concentrations on the basolateral membrane voltage (V_{bl}) and the input conductance (g_{in}) of a principal cell in an isolated Malpighian tubule of the yellow fever mosquito bathed in 34 mM K^+ -Ringer solution. The experiment began with the tubule bathed in Ringer solution containing 3.4 mM K^+ and the impalement of the cell with voltage and current microelectrodes (downward arrow). The experiment terminated with the withdrawal of the electrodes when the tubule was bathed in Ringer solution containing 34 mM K^+ (upward arrow).

Figure 13. VU small molecules used in the present study. VU625 and VU688 have no charge, and they share three regions: sulfonamide, central core, and amide (Raphemot et al., 2014b). The positively charged small molecules VU590 and VU608 share the same macrocycle core, but they differ in two nitrobenzyl groups of VU590 that are replaced with (methylsulfonyl)benzyl groups in VU608 (Rouhier et al., 2014b).

Figure 14. Hypothetical magnitudes of transepithelial K^+ secretion mediated by *AeKir1* and *AeKir2B* across the basolateral membranes of *A. aegypti* Malpighian tubules challenged to secrete K^+ at high rates (Hine et al., 2014). Relative magnitudes are estimated from the differential effects of VU590 and VU625 on *AeKir1* and *AeKir2B* (see text for details), and indicated by line thickness. VU625 inhibits 66% of transepithelial K^+ secretion, leaving 34% to secretion by presumably NKCC and/or NKA (see text for details). Of the VU625-sensitive K^+ secretion, 46% is mediated by *AeKir1* and 10% by *AeKir2B*. The relative contributions of the potential K^+ transporters illustrated at the apical membranes are unknown as indicated by the question marks. Note that gap junctions between stellate and principal cells may allow for principal cells to secrete K^+ that enters stellate cells via *AeKir1*. The model is reduced to focus on the transepithelial secretion of K^+ . ATPase, Na/K pump; AE, Cl/HCO₃ anion exchanger; NHA, Na/proton antiporters; NKCC, Na,K,Cl cotransporter; KCC, K,Cl cotransporter; V-ATPase, electrogenic proton pump; GJ, gap junction; SJ, septate junction.

A)

B)

C)

Figure 02

A)

B)

C)

Figure 02

A)

B)

C)

D)

Figure 04

A)

B)

C)

D)

Figure 05

A)

B)

Figure 06

A)

B)

C)

Figure 07

Figure 08

Figure 09

Figure 10

Figure 11

Figure 12

Figure 13

Figure 14

Localization of Kir1 and Kir2B channels in Malpighian tubules

Model of K⁺ transport in Malpighian tubules