Excretion of NaCl and KCl loads in mosquitoes. 2. Effects of the small molecule Kir channel modulator VU573 and its inactive analog VU342

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Rouhier MF, Hine RM, Park ST, Raphemot R, Denton J, Piermarini PM, Beyenbach KW. Excretion of NaCl and KCl loads in mosquitoes. 2. Effects of the small molecule Kir channel modulator VU573 and its inactive analog VU342. Am J Physiol Regul Integr Comp Physiol 307: R850–R861, 2014. First published July 23, 2014; doi:10.1152/ajpregu.00106.2014.—The effect of two small molecules VU342 and VU573 on renal functions in the yellow fever mosquito Aedes aegypti was investigated in vitro and in vivo. In isolated Malpighian tubules, VU342 (10 μM) had no effect on the transepithelial secretion of Na+, K+, Cl−, and water. In contrast, 10 μM VU573 first stimulated and then inhibited the transepithelial secretion of fluid when the tubules were bathed in Na+-rich or K+-rich Ringer solution. The early stimulation was blocked by bumetanide, suggesting the transient stimulation of Na-K-2Cl cotransport, and the late inhibition of fluid secretion was consistent with the known block of AeKir1, an Aedes inward rectifier K+ channel, by VU573. VU342 and VU573 at a hemolymph concentration of about 11 μM had no effect on the diuresis triggered by hemolymph Na+ or K+ loads. VU342 at a hemolymph concentration of 420 μM had no effect on the diuresis elicited by hemolymph Na+ or K+ loads. In contrast, the same concentration of VU573 significantly diminished the Na+ diuresis by inhibiting the urinary excretion of Na+, Cl−, and water. In K+-loaded mosquitoes, 420 μM VU573 significantly diminished the K+ diuresis by inhibiting the urinary excretion of K+, Na+, Cl−, and water. We conclude that 1) the effects of VU573 observed in isolated Malpighian tubules are overwhelmed in vivo by the diuresis triggered with the coinjection of Na+ and K+ loads, and 2) at a hemolymph concentration of 420 μM VU573 affects Kir channels systemically, including those that might be involved in the release of diuretic hormones.

K+ is secreted into the tubule lumen passing largely through principal cells [see Fig. 8 in companion paper (13)]. The entry step of K+ into principal cells is passive and mediated by barium-sensitive K+ channels that account for more than 60% of the basolateral membrane conductance (2). In addition, K+ can enter epithelial cells by electroneutral, bumetanide-sensitive transport, presumably mediated by a SLC12-like Na-K-Cl cotransporter (11, 14). Recent studies in our laboratory suggest that the large K+ conductance of the basolateral membrane of principal cells may stem from the abundant expression of inward-rectifying K+ (Kir) channels, two of which (AeKir1 and AeKir2B) we have functionally characterized (20).

When AeKir1 and AeKir2B are expressed heterologously in Xenopus oocytes, the macroscopic currents of AeKir1 are larger in magnitude than those of AeKir2B, but both Kir currents are blocked by barium (20). Moreover, the expression of AeKir1 in HEK293 cells confirmed K+ transport in patch-clamp studies and in Tl+ flux assays (22). The small molecule VU573 (1) potently inhibits AeKir1 in vitro (IC50 = 5.14 μM) but activates AeKir2B in vitro; 2) inhibits the production and excretion of urine in isolated mosquito Malpighian tubules and in intact mosquitoes; and 3) disrupts hemolymph K+ homeostasis in mosquitoes (22, 24). It was therefore of interest to investigate in more detail the mechanism of action of VU573 and its inactive analog VU342 on 1) transepithelial electrolyte and fluid secretion in isolated Malpighian tubules, and 2) the urinary excretion of K+ and Na+ loads in intact mosquitoes. The companion paper (13) documents how the tubules and the mosquito deal with Na+ and K+ challenges.

In the present paper we show that VU342 has no effect on fluid and electrolyte secretion in isolated Malpighian tubules and no effect on the excretion of Na+ and K+ loads in the mosquito. In contrast, VU573 (10 μM) first stimulates and then inhibits fluid secretion in isolated Malpighian tubules. The stimulation of fluid secretion is due in part to the activation of a Na-K-2Cl transporter (NKCC) either directly by VU573 or indirectly via the interaction of AeKir1 and/or AeKir2B with NKCC. The inhibition of fluid secretion is consistent with the known block of AeKir1 by VU573. However, in vivo, where VU573 is injected with a Na+- or K+-load for a hemolymph concentration of about 10 μM, VU573 does not interfere with the Na+ or K+ diuresis. In contrast, at a hemolymph concentration of 420 μM, VU573 significantly diminishes the Na+ or K+ diureses triggered respectively by the Na+ or K+ loads. We conclude that high concentrations of VU573 in the hemolymph affect Kir channels not only in Malpighian tubules but...
also in other tissues that may be involved in the release of diuretic hormones.

MATERIALS AND METHODS

Mosquitoes

Mosquitoes (Aedes aegypti) were reared and maintained in the laboratories of Piermarini and Beyenbach as described in the companion paper (13). Mosquitoes from the same brood and of the same approximate age (3–5 old adults) were used for in vitro studies in the laboratory of Beyenbach and for in vivo studies in the laboratory of Piermarini.

VU Compounds

The small molecules VU342 and VU573 were synthesized at Vanderbilt University (VU) and stored at a concentration of 100 mM in anhydrous dimethyl sulfoxide (DMSO) (22). The use of VU compounds in studies of isolated Malpighian tubules was limited by the tolerance of DMSO by the tubules. Since the tubules tolerate DMSO concentration of 0.05% (vol/vol) without detrimental effects (34), the maximum concentration of VU concentrations that could be tested was 50 μM. For routine studies in isolated Malpighian tubule we chose a VU concentration of 10 μM.

To test the effects of VU compounds in intact mosquitoes, we injected VU compounds at two different concentrations, 20 μM or 0.77 mM. After dilution in the hemolymph, the VU compounds are expected to have a concentration of 11 and 420 μM, respectively. The latter high concentration of VU573 (but not VU342) was found to induce renal failure in the mosquito in a previous study (22).

Study of Isolated Malpighian Tubules

We used “sister tubules” from the same female mosquito to evaluate the effects of VU compounds on epithelial transport in isolated Malpighian tubules. The sister tubules were studied in parallel; one served as the sham control and the other as the experimental tubule (3, 13). The parallel study of sister tubules eliminates the variation among tubules isolated from different mosquitoes. Isolated Malpighian tubules were prepared for the study of fluid secretion as described in the companion paper (13). Secreted fluid was collected for the measurement of its ionic composition by the method of electron probe [see companion paper (13)].

Study of Intact Mosquitoes

To evaluate the effects of VU342 and VU573 on urine excretion in intact mosquitoes, we used the methods and solutions described in the companion paper (13). In brief, after female mosquitoes were cold anesthetized, each received a hemolymph injection of 900 nl Na+-HEPES-buffered solution (Na+-HBS) or K+-HEPES-buffered solution (K+-HBS) to trigger a Na+ and K+ diuresis, respectively. Na+-HBS consisted of 146 mM NaCl, 25 mM HEPES, 4.2 mM NMDG, and 1.8% DMSO, pH 7.5, and K+-HBS consisted of 10 mM NaCl, 75 mM KCl, 25 mM HEPES, 65.2 mM NMDG, and 1.8% DMSO, pH 7.5. DMSO served as the vehicle of VU compounds.

After hemolymph injections, the mosquitoes were placed immediately in a graduated, packed cell volume tube for 2 h (MidSci, St. Louis, MO). Previous studies have shown that the yellow fever mosquito excretes the excess salt and water of a blood meal within 2 h (32). After 2 h, the mosquitoes were removed from the tube, and the tube was centrifuged to produce one collective volume of urine droplets sticking to the wall of the vial. The volume of urine was measured, and an aliquot was prepared for electron probe analysis of urine Na+, K+, and Cl− concentrations.

The above protocol was also used to examine the effect of 20 μM VU injections on the time course of Na+ and K+ diuresis triggered by the hemolymph injections of Na+-HBS and K+-HBS, respectively. We used five mosquitoes per graduated, packed cell volume tube in these studies.

Statistics

In studies of whole mosquitoes we used the Student’s t-test for sample means. Time-dependent data were examined for statistical significance using analysis of variance (ANOVA). In other studies we set the 99% confidence interval to sample means.

Data obtained from “sister” tubules studied in vitro were analyzed using the Student’s t-test for paired samples. The paired comparison in these studies is appropriate because 1) there is an underlying relationship between the subjects in each experiment, both tubules come from the same mosquito; 2) the subjects within each group, however, are independent, the two tubules are selected randomly from the five tubules in the mosquito; and 3) the total number of experiments is less than 30 (Cornell Statistical Consulting Service). The paired t-test evaluates the significance of the difference within the pair of tubules isolated from the same mosquito.

RESULTS

Experiments in Isolated Malpighian Tubules

Lack of effect of VU342 on transepithelial fluid and electrolyte secretion. To evaluate the effects of VU342 (10 μM) on the rate of transepithelial fluid secretion, we studied seven “sister tubules.” The tubules were isolated and studied in K+-rich Ringer as described in the companion paper. The K+-rich Ringer solution was chosen over a Na+-rich Ringer solution to amplify the effects of VU compounds on transepithelial K+ secretion. Each tubule was first studied for a 30-min control period (P1). Thereafter, the sham tube was treated with 0.05% DMSO, and the sister tube was treated with 10 μM VU342 in 0.05% DMSO (Fig. 1). We measured the rate of transepithelial fluid secretion for four more consecutive 30-min periods, P2 through P5 (Fig. 1). The mean rate of transepithelial fluid secretion ranged between 0.62 and 0.72 nl/min. Analysis of variance did not detect statistical significance in the rate of fluid secretion with time, neither in the sham nor the experimental tubule. Thus VU342 had no effect of the rate of fluid secretion in isolated Malpighian tubules.

A lack of effect of VU342 on the rate of transepithelial fluid secretion may result from reciprocal effects on the rates of transepithelial Na+ and K+ secretion (11). For example, as the transepithelial secretion of Na+ increases, the transepithelial secretion of K+ may decrease with no net effect on the secretion of Cl− and water. For this reason we repeated the experiment shown in Fig. 1 in five pairs of sister tubules and collected fluid secreted by both tubules for the measurement of secreted Na+, K+, and Cl− concentrations by the methods of electron probe [see companion paper (13)].

Table 1 applies the Student’s t-test to compare sham and experimental sister tubules in periods P1 and P2. Again, VU342 (10 μM) had no significant effect on the rate of
transepithelial fluid secretion. Moreover, VU342 had no effect on 1) the concentrations of Na⁺, K⁺, and Cl⁻ in secreted fluid; and 2) the rate of transepithelial secretion of Na⁺, K⁺, and Cl⁻.

Effect of the VU573 on transepithelial fluid secretion. The effect of VU573 (10 μM) on the rate of transepithelial fluid secretion in isolated Malpighian tubules was studied next (Fig. 2). Sham tubules exhibited constant fluid secretion rates with time and with no significant effects of DMSO, as determined by an ANOVA (Fig. 2). In contrast, VU573 elicited significant effects on fluid secretion in the experimental tubules (ANOVA, \( P < 0.002 \)). Moreover, the paired Student’s t-test of sham and experimental sister tubules revealed an unexpected stimulation of fluid secretion in periods P2 and P3, followed by a significant inhibition of fluid secretion in period P5 (Fig. 2). Not all 21 tubule pairs could be studied for five consecutive 30-min periods because some tubules came off the glass hook in the Ramsay assay (see Fig. 1, companion paper (13)).

Early stimulation of transepithelial electrolyte secretion by VU573. The early stimulation of fluid secretion by VU573 was studied further by analyzing the composition of secreted fluid in 9 of 21 tubules shown in Fig. 2. The addition of 10 μM VU573 to the peritubular K⁺-rich Ringer solution significantly (\( P < 0.004 \)) increased the rate of transepithelial fluid secretion from 0.81 ± 0.11 to 1.00 ± 0.09 nl/min in period P2 (Fig. 3). However, the concentrations of Na⁺, K⁺, and Cl⁻ in secreted fluid were not affected by VU573 (Fig. 3).

The product of the fluid secretion rate and the concentrations of Na⁺, K⁺, and Cl⁻ yield the transepithelial ion secretion rate. VU573 significantly stimulated transepithelial K⁺ and Cl⁻ secretion but not Na⁺ secretion (Fig. 3). In particular, 10 μM VU573 significantly (\( P < 0.03 \)) increased the transepithelial secretion of K⁺ from 127.8 ± 17.6 to 154.3 ± 13.0 pmol/min and significantly (\( P < 0.02 \)) increased the transepithelial secretion of Cl⁻ from 132.6 ± 21.5 to 159.5 ± 17.5 pmol/min (Fig. 3).

To examine whether the initial stimulation of fluid secretion by VU573 was influenced by the high peritubular K⁺ concentration (34 mM) in K⁺-rich Ringer solution, we repeated the experiment of Fig. 3 in the presence of Na⁺-rich Ringer solution containing 3.4 mM K⁺. As shown in Fig. 4, the addition of 10 μM VU573 to the peritubular bath containing Na⁺-rich Ringer solution significantly (\( P < 0.02 \)) increased the transepithelial secretion of fluid from 0.38 ± 0.05 to 0.63 ± 0.09 nl/min in seven tubule pairs without affecting the concent-

Table 1. Lack of effect of VU342 (10 μM) on transepithelial electrolyte secretion in isolated Malpighian tubules of Aedes aegypti bathed in K⁺-rich Ringer solution

<table>
<thead>
<tr>
<th></th>
<th>Sham Tubule</th>
<th>Sister Tubule</th>
<th>( P &lt; )</th>
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<tbody>
<tr>
<td></td>
<td>Control (P1)</td>
<td>DMSO (P2)</td>
<td>Control (P1)</td>
</tr>
<tr>
<td>Fluid secretion, nl/min</td>
<td>0.68 ± 0.2</td>
<td>0.66 ± 0.2</td>
<td>0.75 ± 0.2</td>
</tr>
<tr>
<td>([\text{Na}^+]_o), mM</td>
<td>62.6 ± 13.1</td>
<td>52.1 ± 10.6</td>
<td>77.1 ± 17.0</td>
</tr>
<tr>
<td>([\text{K}^+]_o), mM</td>
<td>105.8 ± 12.1</td>
<td>104.0 ± 15.2</td>
<td>111.4 ± 20.0</td>
</tr>
<tr>
<td>([\text{Cl}^-]_o), mM</td>
<td>145.3 ± 9.8</td>
<td>140.0 ± 8.4</td>
<td>155.4 ± 7.0</td>
</tr>
<tr>
<td>Na⁺ secretion, pmol/min</td>
<td>50.4 ± 22.2</td>
<td>41.3 ± 18.1</td>
<td>65.2 ± 24.4</td>
</tr>
<tr>
<td>K⁺ secretion, pmol/min</td>
<td>64.8 ± 11.3</td>
<td>61.3 ± 11.4</td>
<td>73.0 ± 13.3</td>
</tr>
<tr>
<td>Cl⁻ secretion, pmol/min</td>
<td>98.8 ± 26.3</td>
<td>90.4 ± 22.5</td>
<td>113.3 ± 28.4</td>
</tr>
</tbody>
</table>

Data are means ± SE; \( n = 5 \) tubule pairs. sf, secreted fluid. VU, Vanderbilt University. The concentrations of Na⁺, K⁺, and Cl⁻ were measured after the control period (P1) and the experimental period (P2) lasting 30 min each in 5 tubule pairs; each pair from the same mosquito. Sham tubules were treated with 0.05% DMSO in P2, and sister tubules were treated with 10 μM VU342 in 0.05% DMSO. \( P \), paired Student’s t-test.
VU573 impairs renal functions in mosquitoes

In previous studies we have observed that a significant increase in transepithelial fluid secretion with insignificant changes in the concentrations of secreted Na⁺, K⁺, and Cl⁻, as observed in Figs. 3–5, can derive from the stimulation of bumetanide-sensitive Na-K-Cl cotransport (5, 11). Accordingly, it was of interest whether the early stimulatory effect of VU573 could be blocked by bumetanide. As shown in Table 2, the coadministration of 10 μM VU573 and 100 μM bumetanide prevented the stimulation of the transepithelial secretion of Na⁺, K⁺, Cl⁻ and fluid by VU573.

Late inhibition of transepithelial electrolyte secretion by VU573 in period P5. The inhibition of fluid secretion by VU573 in period P5 (Fig. 2) was studied in six tubule pairs. After an initial 30-min control P1 period, the sham tubule was treated with 0.05% DMSO (control) and the sister tubule was treated with VU573 (10 μM) in 0.05% DMSO. Two hours later, corresponding to period P5 in Fig. 2, we measured the fluid secretion rate again together with the concentrations of Na⁺, K⁺, and Cl⁻ in secreted fluid. Figure 6 summarizes the effects of VU573 in period P5. The rate of transepithelial fluid secretion was significantly (P < 0.05) lower in the experimental tubules (0.56 ± 0.17 nl/min) treated with VU573 compared with the sham tubules (0.74 ± 0.11 nl/min) treated with DMSO. Moreover, the Na⁺ concentration in secreted fluid was significantly higher in the VU573-treated tubules (30.2 ± 13.8 mM) than in the DMSO-treated sham tubules (7.7 ± 7.3 mM). The K⁺ concentration was significantly lower in the VU573-treated tubules (158.6 ± 19.5 mM) compared with the sham tubules (180.4 ± 19.5 mM). The effect of VU573 on the

Tubule pairs (n), each from the same female mosquito, were studied for an initial 30-min control P1 period followed by the experimental P2 period. The sham tubule was treated with 0.05% DMSO, and the sister tubule was treated with VU573 in 0.05% DMSO at the beginning of P2. Data are means ± SE; sf, secreted fluid; t, paired Student’s t-test, two tail distribution of sham and sister tubule.

Concentrations of Na⁺, K⁺, and Cl⁻ in secreted fluid. As a result, VU573 significantly increased the rate of 1) transepithelial Na⁺ secretion from 70.6 ± 10.3 to 101.5 ± 18.0 pmol/min, 2) transepithelial K⁺ secretion from 4.5 ± 1.3 to 6.8 ± 2.1 pmol/min, and 3) transepithelial Cl⁻ secretion from 72.3 ± 10.0 to 108.3 ± 19.3 pmol/min (Fig. 4).

Similar observations were made when the concentration of VU573 was increased fivefold (Fig. 5). At a concentration of 50 μM, VU573 significantly (P < 0.01) increased the rate of transepithelial fluid secretion from 0.46 ± 0.06 to 0.64 ± 0.04 nl/min without an effect on the concentrations of secreted Na⁺, K⁺, and Cl⁻ (Fig. 5). As a result, the transepithelial secretion of Na⁺ significantly (P < 0.04) increased from 53.2 ± 10.0 to 77.4 ± 12.6 pmol/min, the transepithelial secretion of K⁺ significantly (P < 0.02) increased from 26.9 ± 5.8 to 37.4 ± 7.7 pmol/min, and the transepithelial secretion of Cl⁻ significantly (P < 0.002) increased from 79.7 ± 9.0 to 117.6 ± 12.4 pmol/min (Fig. 5).

Early stimulation of transepithelial electrolyte secretion by VU573 is inhibited by bumetanide. In previous studies we have observed that a significant increase in transepithelial fluid secretion with insignificant changes in the concentrations of secreted Na⁺, K⁺, and Cl⁻, as observed in Figs. 3–5, can derive from the stimulation of bumetanide-sensitive Na-K-Cl cotransport (5, 11). Accordingly, it was of interest whether the early stimulatory effect of VU573 could be blocked by bumetanide. As shown in Table 2, the coadministration of 10 μM VU573 and 100 μM bumetanide prevented the stimulation of the transepithelial secretion of Na⁺, K⁺, Cl⁻ and fluid by VU573.

Late inhibition of transepithelial electrolyte secretion by VU573 in period P5. The inhibition of fluid secretion by VU573 in period P5 (Fig. 2) was studied in six tubule pairs. After an initial 30-min control P1 period, the sham tubule was treated with 0.05% DMSO (control) and the sister tubule was treated with VU573 (10 μM) in 0.05% DMSO. Two hours later, corresponding to period P5 in Fig. 2, we measured the fluid secretion rate again together with the concentrations of Na⁺, K⁺, and Cl⁻ in secreted fluid. Figure 6 summarizes the effects of VU573 in period P5. The rate of transepithelial fluid secretion was significantly (P < 0.05) lower in the experimental tubules (0.56 ± 0.17 nl/min) treated with VU573 compared with the sham tubules (0.74 ± 0.11 nl/min) treated with DMSO. Moreover, the Na⁺ concentration in secreted fluid was significantly higher in the VU573-treated tubules (30.2 ± 13.8 mM) than in the DMSO-treated sham tubules (7.7 ± 7.3 mM). The K⁺ concentration was significantly lower in the VU573-treated tubules (158.6 ± 19.5 mM) compared with the sham tubules (180.4 ± 19.5 mM). The effect of VU573 on the
concentration of Cl\textsuperscript{-} in secreted fluid was not statistically significant (Fig. 6).

As expected from tubules bathed in K\textsuperscript{+}-rich Ringer solution, the rate of transepithelial Na\textsuperscript{+} secretion was low in both sham and VU573-treated sister tubules, 2.5 ± 2.4 and 7.61 ± 1.96 pmol/min, respectively, and not significantly different (Fig. 6). In contrast, the rate of transepithelial K\textsuperscript{+} secretion was significantly lower in the VU573-treated tubules (73.1 ± 24.8 pmol/min) compared with the sham tubule (118.8 ± 22.1 pmol/min). Likewise, the rate of transepithelial Cl\textsuperscript{-} secretion, 52.8 ± 20.1 pmol/min, was significantly lower in the VU573-treated tubules compared with 96.1 ± 17.0 pmol/min in the sham tubules. Thus VU573 exerted inhibitory effects on fluid secretion in period P5 by reducing the rate of transepithelial K\textsuperscript{+} and Cl\textsuperscript{-} secretion (Fig. 6).

Experiments in Mosquitoes

Effect of low VU doses in vivo. To evaluate whether the effects of VU concentrations in vivo are similar to those observed in vitro, we injected female mosquitoes with 900 nl Na\textsuperscript{+}-HBS or K\textsuperscript{+}-HBS containing 20 μM VU342 or VU573 and 1.8% DMSO. Dilution in the hemolymph is expected to yield a hemolymph VU concentration of about 11 μM, similar to 10 μM used in the study of isolated Malpighian tubules. As

![Fig. 5. Significant early stimulation by 50 μM VU573 of the transepithelial secretion of Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{-}, and fluid in Malpighian tubules bathed in Na\textsuperscript{+}-rich Ringer. Tubule pairs (n), each from the same female mosquito were studied, first in a 30-min control period (P1). Thereafter, the sham tubule was treated with 0.05% DMSO, and the experimental sister tubule was treated with 50 μM VU573 in 0.05% DMSO and studied for the next 30-min period (P2). Secreted fluid from a subset of 8 tubule pairs was investigated for ionic composition. Data are means ± SE; P, paired Student’s t-test, two-tail distribution.](image)

![Fig. 6. Late inhibitory effects of VU573 (10 μM) on fluid and electrolyte secretion in isolated Malpighian tubules of Aedes aegypti. The tubules were studied in the presence of K\textsuperscript{+}-rich Ringer solution in the absence (control) and presence of VU573. Data are means ± SE of measurements during period 5 (see Fig. 2) for both control and experimental tubules (n, number sister tubule pairs). Statistical significance was evaluated by the paired Student’s t-test comparing control and sister tubules isolated from the same mosquito.](image)

Table 2. Bumetanide blocks the early VU573 stimulation of the transepithelial ion and fluid secretion

<table>
<thead>
<tr>
<th></th>
<th>Sham Tubule</th>
<th>Sister Tubule</th>
<th>P &lt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (P1)</td>
<td>DMSO (P2)</td>
<td>VU573 plus bumetanide in DMSO (P2)</td>
</tr>
<tr>
<td>Fluid secretion, nl/min</td>
<td>0.60 ± 0.1</td>
<td>0.62 ± 0.1</td>
<td>0.60 ± 0.1</td>
</tr>
<tr>
<td>[Na\textsuperscript{+}]&lt;sub&gt;i&lt;/sub&gt;, mM</td>
<td>31.5 ± 9.6</td>
<td>30.4 ± 10.5</td>
<td>19.7 ± 5.2</td>
</tr>
<tr>
<td>[K\textsuperscript{+}]&lt;sub&gt;i&lt;/sub&gt;, mM</td>
<td>124.5 ± 15.1</td>
<td>115.6 ± 9.5</td>
<td>117.4 ± 14.9</td>
</tr>
<tr>
<td>[Cl\textsuperscript{-}]&lt;sub&gt;i&lt;/sub&gt;, mM</td>
<td>171.6 ± 14.7</td>
<td>151.0 ± 12.3</td>
<td>170.9 ± 12.1</td>
</tr>
<tr>
<td>Na\textsuperscript{+} secretion, pmol/min</td>
<td>19.4 ± 6.2</td>
<td>12.9 ± 3.6</td>
<td>17.4 ± 6.2</td>
</tr>
<tr>
<td>K\textsuperscript{+} secretion, pmol/min</td>
<td>71.4 ± 8.0</td>
<td>72.1 ± 9.3</td>
<td>70.1 ± 10.8</td>
</tr>
<tr>
<td>Cl\textsuperscript{-} secretion, pmol/min</td>
<td>101.7 ± 12.6</td>
<td>97.0 ± 15.1</td>
<td>102.6 ± 12.0</td>
</tr>
</tbody>
</table>

Data are means ± SE; 6 tubule pairs, each from the same female mosquito, were studied for an initial 30-min P1 control period in K\textsuperscript{+}-rich Ringer. Thereafter, the sham tubule was treated with 0.05% DMSO, and the experimental sister tubule was treated with VU573 (10 μM) plus 100 μM bumetanide and 0.05% DMSO. P, paired Student’s t-test, two-tail distribution.
When the hemolymph of female mosquitoes was loaded with mosquito excreted on average 725.0 nmol Na\(^+\) and 131.4 nmol K\(^+\) was excreted over the 2-h postinjection period are shown in Fig. 7, low hemolymph concentrations of VU342 and VU573 had no effect on the diuresis triggered by volume-loading mosquitoes with either Na\(^+\)-HBS or K\(^+\)-HBS (vehicle, DMSO).

**Effects of VU342 and VU573 on the excretion of a Na\(^+\) load.** When the hemolymph of female mosquitoes was loaded with 900 nl of Na\(^+\)-HBS containing 1.8% DMSO (control), the mosquito excreted on average 725.0 ± 32.0 nl of a NaCl-rich urine in the next 2 h (Fig. 8). Since the 99% confidence interval (CI) of this mean does not include 900 nl, the retention of 175 nl of the injected volume is significant. Injected with 900 nl Na\(^+\)-HBS containing 0.77 mM VU342, the mosquito excreted on average 654.2 ± 46.7 nl, which is not significantly different from the control injection (Fig. 8). Again, the 99% CI does not include the injected volume. Accordingly, the mosquito retained 245.8 nl or 27% of the injected volume. The hemolymph injection of Na\(^+\)-HBS with 0.77 mM VU573 in DMSO significantly (\(P < 10^{-5}\)) reduced the urine volume to 245.8 ± 50.8 nl (Fig. 8). Since the 99% CI of this mean does not include 900 nl, the retention of 654.2 nl of the injected volume is significant; i.e., the mosquito is unable to excrete 73% of the injected volume.

The concentration of Na\(^+\) in the urine was 226.9 ± 12.5 mM under control conditions, and 203.6 ± 16.6 mM and 308.4 ± 41.8 mM when the Na\(^+\)-HBS injection included VU342 and VU573, respectively (Fig. 8). The concentration of K\(^+\) in the urine was low (13.9 ± 1.7 mM) under control conditions and did not change significantly (15.8 ± 1.5 mM) when Na\(^+\)-HBS was injected with VU342 (Fig. 8). The concentration of K\(^+\) in excreted urine rose significantly to 68.1 ± 12.3 mM when the injected Na\(^+\)-HBS contained VU573. The urinary Cl\(^-\) concentration was 190.4 ± 11.4 mM after the control injection of Na\(^+\)-HBS and did not change significantly (176.9 ± 13.4 mM) in the presence of VU342. The Cl\(^-\) concentration significantly increased to 298.6 ± 42.6 mM when the injected Na\(^+\)-HBS included VU573. The 99% CI does not include the injected concentrations for Na\(^+\), K\(^+\), and Cl\(^-\) (Fig. 8). Accordingly, the urinary concentrations of Na\(^+\), K\(^+\), and Cl\(^-\) were significantly above the concentrations injected into the hemolymph with the exception of the Cl\(^-\) concentration coinjected with VU342 (Fig. 8).

The control injection of Na\(^+\)-HBS containing 131.4 nmol Na\(^+\) caused the mosquito to excrete 165.8 ± 13.9 nmol Na\(^+\).
During the next 2 h (Fig. 8). Likewise, when the injected Na\textsuperscript{+}-HBS included VU342, the mosquito excreted the injected Na\textsuperscript{+} load, on average 132.2 ± 13.6 nmol Na\textsuperscript{+}. In contrast, the coinjection of Na\textsuperscript{+}-HBS and VU573 significantly (P < 10\textsuperscript{-4}) dropped the excreted Na\textsuperscript{+} load to 67.0 ± 12.0 nmol (Fig. 8). The 99% CI of this mean does not include the injected Na\textsuperscript{+} load. Accordingly, the mosquito was unable to excrete 49% of the injected Na\textsuperscript{+} load.

As observed in the companion paper (13), the mosquito excreted very little K\textsuperscript{+} when injected with Na\textsuperscript{+}-HBS. Under control conditions the mosquito excreted 10.1 ± 1.3 nmol K\textsuperscript{+} (Fig. 8). The mosquito excreted 10.5 ± 1.4 nmol K\textsuperscript{+} when Na\textsuperscript{+}-HBS included VU342 and 12.9 ± 2.2 nmol K\textsuperscript{+} when Na\textsuperscript{+}-HBS included VU573 (Fig. 8). The 99% CI of the above 3 means does not include 0 nmol (amount of K\textsuperscript{+} injected), indicating that the urinary K\textsuperscript{+} excretion constitutes a significant loss of K\textsuperscript{+} under control and experimental conditions. The urinary excretion of Cl\textsuperscript{−} mirrored the urinary excretion of Na\textsuperscript{+} (Fig. 8). The control injection of Na\textsuperscript{+}-HBS triggered the mosquito to excrete 138.1 ± 10.5 nmol Cl\textsuperscript{−} during the next 2 h. The 99% CI of this mean includes the injected Cl\textsuperscript{−} load of 135.2 nmol. Likewise, the excreted Cl\textsuperscript{−} was 114.5 ± 10.1 nmol when Na\textsuperscript{+}-HBS included VU342 and not significantly different from the injected Cl\textsuperscript{−} load (99% CI). In contrast, the urinary Cl\textsuperscript{−} excretion decreased significantly (P < 0.0005) to 65.4 ± 12.2 nmol when Na\textsuperscript{+}-HBS was coinjected with VU573 (Fig. 8). The 99% CI does not include the injected Cl\textsuperscript{−} load. Accordingly, the mosquito was unable to excrete 52% of the injected Cl\textsuperscript{−}.

In summary, the coinjection of Na\textsuperscript{+}-HBS and VU342 had no significant effect on any measured variable (Fig. 8). In contrast, the coinjection of Na\textsuperscript{+}-HBS and VU573 significantly reduced the urine volume and the urinary excretion Na\textsuperscript{+} and Cl\textsuperscript{−}. In parallel, the concentrations of K\textsuperscript{+} and Cl\textsuperscript{−} in the urine increased significantly (Fig. 8).

**Effects of VU342 and VU573 on the excretion of a K\textsuperscript{+} load.** When the hemolymph of female mosquitoes was injected with 900 nl K\textsuperscript{+}-HBS, the mosquito excreted a urine volume of 466.7 ± 45.0 nl (control) in the next 2 h (Fig. 9). The 99% CI of this mean does not include the injected volume. Thus the mosquito held on to 48% of the injected volume. When the K\textsuperscript{+}-HBS injection included 0.77 mM VU342, the mosquito excreted a volume of 345.8 ± 50.4 nl, which is not significantly different from the control volume (Fig. 9). Again, the 99% CI of this mean does not include the injected volume. Thus the mosquito did not excrete 62% of the injected volume. The coinjection of K\textsuperscript{+}-HBS and 0.77 mM VU573 significantly (P < 0.0002) reduced the urine volume to 157.1 ± 36.2 nl (Fig. 8). Since the 99% CI of this mean does not include the injected volume, the mosquito retained as much as 83% of the injected volume.

Injected with a K\textsuperscript{+} load (K\textsuperscript{+}-HBS), the mosquito responded by excreting KCl-rich urine (Fig. 9) as observed in the companion paper (13). The concentration of Na\textsuperscript{+} in the excreted urine was 39.0 ± 6.5 mM under control conditions and not significantly different from 1) 56.8 ± 15.6 mM after the injection of K\textsuperscript{+}-HBS with VU342, and 2) 23.3 ± 9.3 mM after the injection of K\textsuperscript{+}-HBS with VU573 (Fig. 9). The 99% CI of these three means does not include the Na\textsuperscript{+} concentration in K\textsuperscript{+}-HBS (10 mM), indicating a Na\textsuperscript{+} concentration in the urine that is significantly greater than that in the injected K\textsuperscript{+}-HBS (Fig. 9). The concentration of K\textsuperscript{+} in excreted urine was high (121.2 ± 12.0 mM) and not significantly different from 162.6 ± 21.3 mM after injecting K\textsuperscript{+}-HBS with VU342 (Fig. 9). However, the concentration of K\textsuperscript{+} in the urine rose significantly (P < 0.008) to 277.3 ± 51.8 mM when the injected K\textsuperscript{+}-HBS included VU573 (Fig. 9). The 99% CI of these three means does not include the K\textsuperscript{+} concentration in K\textsuperscript{+}-HBS (75 mM), indicating a K\textsuperscript{+} concentration in the urine significantly greater than that in the injected K\textsuperscript{+}-HBS (Fig. 9). The concent-

![Fig. 9. Effects of VU342 and VU573 on the renal handling of a K\textsuperscript{+} load injected into the hemolymph.](image-url)
tation of Cl⁻ in excreted urine was 164.0 ± 17.4 mM after the mosquitoes received the K⁺-HBS injection (control), which is not significantly different from 221.3 ± 35.9 mM, the urinary Cl⁻ concentration after injecting K⁺-HBS with VU342. The coinjection of K⁺-HBS and 0.77 mM VU573 significantly (P < 0.03) increased the urinary Cl⁻ concentration to 312.8 ± 62.2 mM (Fig. 9). The 99% CI of this mean does not include the Cl⁻ concentration in K⁺-HBS (150.2 mM), indicating a Cl⁻ concentration in the urine significantly greater than that in K⁺-HBS (Fig. 9).

The hemolymph injection of K⁺-HBS that added 9.0 nmol Na⁺ to the hemolymph caused the mosquito to excrete 19.0 ± 3.7 nmol Na⁺ in the next 2 h (Fig. 9). Since the 99% CI of this mean does not include the quantity of Na⁺ (9 nmol) injected into the hemolymph, the mosquito excreted significantly more Na⁺ than received with the K⁺-HBS injection. When K⁺-HBS included VU342, the mosquito excreted on average 18.6 ± 4.4 nmol Na⁺, which is not significantly different from control (Fig. 9). Moreover, the 99% CI of the excreted Na⁺ includes the injected Na⁺. The urinary Na⁺ excretion dropped significantly (P < 0.002) to 2.5 ± 1.0 nmol when K⁺-HBS contained VU573. Since the 99% CI of this mean does not include the injected Na⁺ load, the mosquito excreted significantly less than the injected Na⁺ load.

The mosquito excreted 56.4 ± 7.6 nmol K⁺ under control conditions, which is not significantly different from 52.3 ± 7.2 nmol when K⁺-HBS was coinjected with VU342 (Fig. 9). The 99% CI of the K⁺ excreted under control conditions and in the presence of VU342 includes the injected K⁺ load of 67.5 nmol. Thus the mosquito was able to excrete the K⁺ load. In contrast, the urinary K⁺ excretion dropped significantly (P < 0.04) to 34.1 ± 4.6 nmol when K⁺-HBS was coinjected with VU573. The 99% CI of this mean does not include 67.5 nmol. Thus VU573 significantly diminished the urinary excretion of K⁺. The urinary excretion of Cl⁻ mirrored the excretion of water (Fig. 9). Under control conditions, the mosquito excreted 76.7 ± 10.9 nmol Cl⁻. Since the 99% CI of this mean does not include the injected Cl⁻ load (135.2 nmol), the mosquito held on to 43% of the injected Cl⁻, similar to the percent water retention (48%). Likewise, the coinjection of K⁺-HBS and VU342 led to a Cl⁻ excretion of 70.4 ± 10.2 nmol, which is not significantly different from control. The 99% CI of this mean does not include the injected Cl⁻ load, indicating that the mosquito held on to 48% of the injected Cl⁻. The coinjection of K⁺-HBS and VU573 significantly (P < 0.008) reduced the urinary Cl⁻ excretion to 37.5 ± 4.8 nmol (Fig. 9). Since this mean does not include the 99% CI of the injected Cl⁻ load, the mosquito retained as much as 72% of the injected Cl⁻ (Fig. 9).

In summary, the hemolymph injection of a K⁺ load together with VU342 had no significant effect on any measured variable compared with the control injection (Fig. 9). However, compared with the hemolymph input, the mosquito excreted significantly less volume than injected under 1) the control injection of K⁺-HBS, and 2) the coinjection of K⁺-HBS and VU342. The coinjection of K⁺-HBS with VU573 reduced the volume of urine even more, down to 17% of the injected volume, and down to 34% of the urine volume excreted under control conditions. The coinjection of K⁺-HBS with VU573 elicited large and significant reductions in the urinary excretion of Na⁺, K⁺, and Cl⁻. In parallel, the concentrations of K⁺ and Cl⁻ in the urine increased significantly.

DISCUSSION

Small Molecules Developed at Vanderbilt University

The two small molecules VU342 and VU573 are guanidium compounds (Fig. 10). VU573 was developed in the effort of finding new agents for controlling G protein-coupled inward rectifier K (GIRK) channels in the potential treatment of atrial fibrillation in humans. With the use of a high-throughput screen of a Kir1.1 inhibitory library for modulators of GIRK in mammals, VU573 was found to be 10 times more selective for GIRK over Kir1.1 channels (21). VU573 also inhibits the epithelial K⁺ channel AeKir1 in Malpighian tubules of the yellow fever mosquito (22), but it activates AeKir2B, which is another Kir channel we have cloned from Aedes Malpighian tubules (24). Structure-activity studies have identified VU342, an analog of VU573, in which the aryl ether is replaced with a morpholine moiety (Fig. 10). VU342 is 22 times less potent than VU573 at inhibiting AeKir1 (22). We therefore consider VU342 as the best “inactive analog” of VU573 we presently have. In the present study, VU342 has no effect on transepithelial electrolyte and fluid secretion in isolated Malpighian tubules (Fig. 1, Table 1) and no effect on the diuresis triggered in intact mosquitoes by the hemolymph injections of Na⁺ and K⁺ loads (Figs. 7–9).

VU573 and VU342 are expected to be protonated at pH 7. Since protonation enhances solubility in water, VU342 should be more water soluble than VU573, which we have observed when handling the compounds in the laboratory. Nevertheless, the limited solubility of VU573 in water requires the use of DMSO. Isolated Malpighian tubules tolerate DMSO up to 0.1% without ill effect (34). Accordingly, we used VU compounds in 0.05% DMSO, which limited the maximal VU573 concentration to 50 µM in studies of isolated Malpighian tubules.

Much higher concentrations of DMSO were tolerated by mosquitoes. The hemolymph injection of as much as 1.9% in 900 nl of Na⁺- or K⁺-HBS had no significant effect on the urinary excretion of the Na⁺ or K⁺ loads (see Fig. 4 in Ref. 13), which consequently allowed us to use greater concentrations of VU573 and VU342 in vivo than in isolated Malpighian tubules. Upon dilution in the hemolymph, 1.9% DMSO is expected to decrease to about 0.8% DMSO and to decrease further as it distributes in the intracellular fluid compartment. The uptake of DMSO by fat cells, the reduction of DMSO to dimethyl sulfide or dimethyl sulfone, and metabolic detoxification and excretion may further lower hemolymph DMSO concentrations and account for the greater tolerance of DMSO in vivo than in Malpighian tubules in vitro.
In isolated Malpighian tubules, 10 μM VU342 had no effect on the rate of transepithelial fluid secretion (Fig. 1), confirming previous results (22). Moreover, VU342 had no effect on the concentrations of Na⁺, K⁺, and Cl⁻ in fluid secreted by the tubules and no effect on transepithelial Na⁺, K⁺, and Cl⁻ secretion in vitro (Table 1). Likewise, a low concentration of VU342 in the hemolymph of mosquitoes (∼11 μM) had no effect on the diuresis triggered by Na⁺ or K⁺ loading the hemolymph (Fig. 7). Moreover, the presence of VU342 in the hemolymph at a concentration of 420 μM had no significant effect on the volume of urine or the quantities of Na⁺, K⁺, and Cl⁻ excreted in 2 h after injecting Na⁺ or K⁺ loads into the hemolymph (Figs. 8 and 9). From the structural similarity of VU342 and VU573, the former is thus the “inactive” analog of the latter in vivo as well as in vitro.

Early, Stimulatory Effects of VU573 in Isolated Malpighian Tubules

VU573 (10 μM) first stimulated and then inhibited transepithelial electrolyte and fluid secretion in isolated Malpighian tubules (Fig. 2). The stimulation occurred within the first 60 min of adding VU573 to the peritubular bath containing K⁺-rich or Na⁺-rich Ringer solution (Figs. 3 and 4). In both media, the rate of fluid secretion increased significantly, but the concentration of the three major osmolytes in the secreted fluid (Na⁺, K⁺, and Cl⁻) did not change (Figs. 3 and 4). Accordingly, VU573 significantly stimulated the isosmotic secretion of Na⁺, K⁺, and Cl⁻ (Figs. 3 and 4), suggesting the involvement of a Na-K-2Cl cotransporter. Indeed, bumetanide, a known blocker of Na-K-2Cl cotransport, prevented the stimulation of isosmotic fluid secretion (Table 2). Thus VU573 may stimulate NKCC directly or indirectly via the block of AeKir1. K⁺ channels are known to interact with NKCCs in other epithelia (7, 10, 12, 33).

The activation of AeKir2B by VU573 (24) could also contribute to the stimulation of isosmotic fluid secretion, especially in tubules bathed in K⁺-rich Ringer solution (Fig. 3). Additional experiments are necessary to elucidate the mechanism(s) by which VU573 brings about the transient stimulation of electrolyte and fluid secretion.

Studies in intact mosquitoes do not reveal the transient stimulation of fluid secretion by VU573 we observe in isolated Malpighian tubules (Figs. 2–5, and 8). As a first hypothesis, much higher doses of VU573 used in vivo (420 μM) than in vitro (10–50 μM) may have elicited net inhibitory effects on urine production in the mosquito, which would mask any stimulatory effect in distal Malpighian tubules.

Late Inhibitory Effect of VU573 on K⁺ Transport in Isolated Malpighian Tubules

The inhibition of transepithelial fluid secretion in isolated Malpighian tubules set in 1.5 h after the addition of VU573 to the peritubular medium concomitant with the inhibition of transepithelial secretion of K⁺ and Cl⁻ (Figs. 2 and 6), similar to the inhibitory effects of Ba²⁺ (2, 15, 25) and consistent with the inhibition of AeKir1 K⁺ channels (22). However, an inhibition that takes 2 h to develop indicates a channel block different from the rapid and reversible block of Ba²⁺ (2, 15). It suggests that the binding sites of VU573 are located in membrane and/or cytosolic domains of K⁺ channels. Nevertheless, the VU573 inhibition of transepithelial K⁺ secretion, though significant, was partial. At a concentration of 10 μM, VU573 inhibited only 38% of the transepithelial K⁺ secretion in isolated Malpighian tubules (Fig. 6) but more than 60% of the whole cell current in T-REx-HEK293 cells expressing AeKir1 channels (22). Moreover, whole cell currents were completely inhibited by a VU573 concentration of 100 μM. Although VU573 fully blocks AeKir1 channels in T-REx-HEK293 cells, it cannot be expected to fully block transepithelial K⁺ secretion in Malpighian tubules. For one reason, Aedes Malpighian tubules express AeKir2B and AeKir3 next to AeKir1 (20). For another reason, NKCC makes important contributions to transepithelial K⁺ secretion [see Fig. 8 in companion paper (13)], and Aedes Malpighian tubules may also express voltage-gated K⁺ (Kv) channels, the transcripts of which have been identified in Drosophila Malpighian tubules (28).

The expression of several K⁺ transporters in Malpighian tubules offers a functional reserve for removing K⁺ from the hemolymph of the mosquito. It increases the reliability on Malpighian tubules for handling K⁺ challenges to the extracellular fluid compartment. For example, the injection of 67.5 nmol K⁺ into the hemolymph (750 nl) of the mosquito (Figs. 7 and 9) is expected to increase the hemolymph K⁺ concentration instantaneously from 6.5 (control) to 43.8 mM (26, 32). A vertebrate system would be unlikely to survive a sudden sixfold increase in extracellular K⁺ concentration. The redundancy of epithelial K⁺ transport mechanisms contributes to the prompt and powerful response of Malpighian tubules faced with extracellular K⁺ concentrations as high as 34 mM in vitro [see Fig. 3 in companion paper (13)] and with about 44 mM in vivo (Fig. 9). Even in the presence of high concentrations of VU573 in the hemolymph of mosquitoes (∼420 μM), which should block all AeKir1 channels, the mosquito is able to excrete 50% of the injected K⁺ load in the first 2 h after the injection (Fig. 9).

Effect of VU573 on the Excretion of a Na⁺ Load in the Mosquito

We show in Fig. 7 of the companion paper (13) that the mosquito completely clears the injected Na⁺ load from the hemolymph in the time of 2 h. Most of the Na⁺ load is excreted in the first 30 min of the diuresis (Fig. 7). To do so, the mosquito must release the calcitonin-like diuretic peptide [formerly the mosquito natriuretic peptide MNP (6)] because transepithelial secretion rates of Na⁺ and Cl⁻ measured in isolated Malpighian tubules cannot account for urinary Na⁺ and Cl⁻ excretion rates measured in the mosquito [see Fig. 5 in companion paper (13)].

The hemolymph injection of a Na⁺ load mimics the Na⁺ load of a blood meal, which is known to trigger the release of MNP (17–19, 29). The present study shows that the coinjection of the Na⁺ load with VU573 prevented the mosquito from excreting the injected Na⁺ load within 2 h. Compared with the 100% clearance of the injected Na⁺ and Cl⁻ loads in the absence of VU573 (Fig. 8), the mosquito cleared from the hemolymph only 44% of the injected Na⁺ and only 47% of the injected Cl⁻ in the presence of VU573 (Fig. 8). As a
result, the mosquito excreted only 33% of the volume injected with the Na+ load and VU573 (Fig. 8). Since VU573 seriously diminished the excretion of Na+, Cl-, and water but not K+, we suggest that VU573 interferes in the normal natriuretic response of the mosquito, possibly by diminishing or blocking the release of calcitonin-like diuretic hormone (CT-like diuretic peptide) into the hemolymph. Without stimulation by this hormone, the secretory activity of Malpighian tubules falls short of excreting the injected loads of Na+, Cl-, and water (Fig. 8). Significantly, we have detected the mRNA expression of two or more Kir channel subunits in the head, thoracic/abdominal musculature, midgut, and hindgut of adult female Aedes aegypti (23), and the transcripts of Kir channels have been detected in the brain and cardiac tissue of Drosophila (8, 9). The block or activation of these Kir channels may thus inhibit the release of the CT-like diuretic peptide thereby leading to the renal failure we have observed previously upon the hemolymph injection of VU573 together with a Na+ load (22).

**Effect of VU573 on the Excretion of a K+ Load in the Mosquito**

We show in the companion paper that mosquitoes injected with a K+ load (67.5 nmol) completely clear the injected K+ from the hemolymph together with a minimum of Cl- and water during the next 2 h [see Fig. 6 in companion paper (13)]. Since K+ secretion rates measured in isolated Malpighian tubules can account for the urinary K+ excretion in vivo, the stimulation of K+ secretion by diuretic hormone(s) is apparently not required. Malpighian tubules seem to autoregulate the transepithelial secretion of K+ by responding directly to changes in tubular K+ concentrations (see Fig. 6 in companion paper (13)]. However, the tubular autoregulation of K+ secretion was disrupted by VU573 in vitro as well as in vivo (Figs. 6 and 9). Of the 67.5 nmol K+ coinjected into the hemolymph with VU573, the mosquito excreted only 34.4 nmol K+ in the time of 2 h, i.e., only 51% of the injected K+ load compared with 100% in the absence of VU573 (Fig. 9). As a first hypothesis, the significant reduction in the urinary excretion of K+, and consequently Cl- and water, can be attributed to VU573 blocking AeKir1 channels in the tubules (Figs. 6 and 9). The block would give Na+ a competitive advantage as illustrated by the rise of the Na+ concentration together with the fall of the K+ concentration in the fluid secreted by isolated Malpighian tubules (Fig. 6). However, these changes reverse in intact mosquitoes where VU573 decreases the Na+ concentration and increases the K+ concentration in the urine (Fig. 9). The reversal may be explained by the role of the hindgut, which reabsorbs Na+, Cl-, and water in the attempt to minimize volume loss with the excretion of the K+ load (Fig. 9). The significant reduction in the urinary excretion of Na+, Cl-, and water (Fig. 9) suggests that the hemolymph injection of VU573 augments the antidiuretic function of the hindgut. The antidiuretic agent stimulating the reabsorption of Na+, Cl-, and water in the hindgut is unknown. Although the antidiuretic effect of the hindgut increases the concentration of K+ in the urine, VU573 caused the urinary excretion of K+ to be significantly diminished (Fig. 9).

The continued disruption of urinary output beyond 2 h is what likely leads to the more serious consequences of VU573 observed a day later, when close to 60% of the mosquitoes injected with VU573 and a K+ load are either dead or unable to fly (22). At that time, some mosquitoes exhibit bloated abdomens as the obvious sign of renal failure (22). In contrast, mosquitoes tend to survive the injection of VU573 with a Na+ load (22). The comparison underscores the principal mechanism of action of high concentrations of VU573 in vivo, namely the block of K+ channels not only in Malpighian tubules but in other tissues where VU573-sensitive channels may be involved in the release of diuretic factors. Low concentrations of VU573 (~10 μM) in vivo have no effect on clearing Na+ and K+ loads from the hemolymph (Fig. 7) even though the transepithelial secretion of K+, Cl-, and water is significantly inhibited by 10 μM VU573 in isolated Malpighian tubules (Fig. 6). Apparently, low concentrations of VU573 in the hemolymph do not interfere in the release of diuretic factors. Thus the stimulation of transepithelial electrolyte and fluid secretion by diuretic peptides in vivo appear to overpower the (partial) block of AeKir1 channels in Malpighian tubules.

**Concluding Summary**

We have concluded in the companion paper (13) that the mosquito gets rid of the Na+ load by releasing the CT-like diuretic hormone that substantially increases rates of Na+, K+, and water secretion in distal Malpighian tubules. Subsequently, the reabsorption of Na+ is completely inhibited in the “hindgut,” the reabsorption of Cl- and water is reduced, and the reabsorption of K+ is stimulated. In the present study we find that VU573 appears to inhibit the release of CT-like diuretic hormone thereby significantly reducing the secretory diuresis in distal Malpighian tubules. In addition, in the presence of VU573, the reabsorption of Na+ in the “hindgut” is not inhibited, and the reabsorption of K+, Cl-, and water is markedly reduced. Thus VU573 exacerbates the problem of the Na+ load by diminishing the Na+ diuresis upstream and increasing the reabsorption of Na+ downstream. As a result, the urinary excretion of Na+, Cl-, and water is markedly reduced.

The companion paper also elucidates the excretory handling of a K+ load injected into the hemolymph. Rates of the transepithelial secretion of KCl and water in distal Malpighian tubules increase spontaneously with elevation in the peritubular K+ concentration. A kaliuretic hormone does not appear necessary. Thus the K+ diuresis in the mosquito stems largely from modified transport functions in the “hindgut.” Here, the reabsorption of Na+ seems completely inhibited again while the reabsorption of K+, Cl-, and water is strongly inhibited. As a result, the mosquito excretes urine containing largely KCl and some NaCl, isosmotic to the hemolymph. In the present study we find that VU573 reduces the transepithelial secretion of K+, Cl-, and water at the level of Malpighian tubules as AeKir1 channels are blocked by VU573. In addition, in the presence of VU573, the “hindgut” increases the reabsorption of K+ and water, thereby exacerbating the problem of the hemolymph K+ load.

Taken together, our two papers demonstrate that the excretory system of the yellow fever mosquito has the mechanisms for dealing effectively with Na+ and K+ assaults in the
hemolymph. VU573, the small molecule inhibitor of AeKir1 and activator of AeKir2B, disrupts these mechanisms of homeostasis in both Na\textsuperscript{+} - and K\textsuperscript{+}-loaded mosquitoes by modulating K\textsuperscript{+} channels not only in distal Malpighian tubules but also elsewhere in the body where these channels may have a role in the release of diuretic and antidiuretic peptides or hormones.

**Perspective and Significance**

In electrophysiological studies, the small molecule VU573 blocks AeKir1 channels but activates AeKir2B channels cloned from mosquito Malpighian tubules. The response of isolated Malpighian tubules to VU573 is biphasic. VU573 first stimulates transepithelial fluid secretion in the first hour of exposure to VU573 and then inhibits fluid secretion after 1.5 h. The early stimulation of fluid secretion involves the activation of NKCC; to VU573 and then inhibits fluid secretion after 1.5 h. The early inhibition of an unknown diuretic peptide diuretic hormone. In the case of the K\textsuperscript{+}-loaded mosquitoes by modulating K\textsuperscript{+} channels not only in distal Malpighian tubules but also elsewhere in the body where these channels may have a role in the release of diuretic and antidiuretic peptides or hormones.


