Na\(^+\)/H\(^+\) exchange in mosquito Malpighian tubules

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Within minutes of taking a blood meal, a female yellow-fever mosquito excretes a large amount of sodium and water derived from the plasma portion of the ingested blood meal (30). The maintenance of fluid homeostasis after a blood meal in insects is dependent on ion transport by both the Malpighian tubules and the hindgut. In most insects, fluid is secreted from the hemolymph across the Malpighian tubules, whereas fluid reabsorption takes place across the hindgut. Factors released from the brain of the mosquito regulate the post-blood meal natriuresis by increasing rates of sodium and fluid secretion by the mosquito’s five Malpighian tubules (21). Fluid secretion is driven by a H\(^+\)-ATPase located in the apical membrane of the mosquito Malpighian tubule (20). To date, emphasis has been placed on the physiology of the H\(^+\)-ATPase in driving fluid secretion (29), whereas little is known concerning the secondary active transport of hydrogen ions driven by the H\(^+\)-ATPase. However, the role played by Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\) exchangers is critical in the net secretion of Na\(^+\) and K\(^+\) ions across the apical membranes of Malpighian tubules (18). Thus the regulation of a steady-state cellular pH may be primarily responsible for the regulation of Na\(^+\) and K\(^+\) ion secretion, which in turn drives fluid secretion.

Our previous measurements of the intracellular pH (pH\(_i\)) with microelectrodes and pH-sensitive fluorescent probes in yellow-fever mosquito Malpighian tubules revealed that the steady-state pH\(_i\) is 7.03 ± 0.05 and that pH\(_i\) is decreased by 0.2 pH units in the presence of 1 mM cAMP (22). The cellular mechanisms resulting in this steady-state pH\(_i\) in mosquito Malpighian tubules are unknown. In most cells, the “fundamental law of pHi regulation” governs the steady-state pH\(_i\), i.e., the steady-state pH\(_i\) is the difference between acid-extruding mechanisms, including the sodium/hydrogen (Na\(^+\)/H\(^+\)) exchanger and the H\(^+\)-ATPase pump, and acid-loading mechanisms, including the chloride/bicarbonate exchanger, divided by the intracellular buffering capacity. As a first step in identifying such acid-base regulatory mechanisms in the mosquito Malpighian tubule, we present evidence for the role of the Na\(^+\)/H\(^+\) exchanger in determining the steady-state pH\(_i\). Previously, fluid secretion studies in mosquito Malpighian tubules have indicated that fluid secretion is inhibited by amiloride (11) and 4-acetamido-4’-isothiocyanostilbene-2,2’-disulfonic acid, an inhibitor of chloride/bicarbonate exchange (12); yet, the effects of these inhibitors on pH\(_i\) were not investigated.

In the present study, the chloride/bicarbonate exchanger was not examined because extracellular bicarbonate was eliminated and the focus was on one of the acid-extrusion mechanisms, namely, Na\(^+\)/H\(^+\) exchange.

The results presented indicate that fluid secretion is inhibited by Na\(^+\)/H\(^+\) exchange inhibitors, including 5-(N-ethyl-n-isopropyl)-amiloride (EIPA). The steady-state pH\(_i\) and the rate of pH\(_i\) recovery from an acute acid load induced by NH\(_4\)Cl are dependent on the basolateral sodium concentration and are inhibited by EIPA.

METHODS

Mosquitoes

Aedes aegypti were reared according to the methods of Mustermann and Wasmuth (16). The larvae were fed liver...
powder, and adults were fed a 3% solution of sucrose in tap water. The mosquitoes were maintained at 30°C, 80% relative humidity, and a 16:8-h light-dark photoperiod schedule. Assays were performed on adult female mosquitoes 6–10 days old.

Solutions

The Malpighian tubule saline contained the following (in mM): 156 NaCl, 6.4 KCl, 1 CaCl₂, 1 MgCl₂, 25 HEPES, and 5 glucose. To induce an acute acid load in the Malpighian tubules, 20 mM NaCl was replaced with 20 mM NH₄Cl at a pH of 7.0. Sodium chloride was replaced by an equimolar concentration of N-methyl-D-glucamine chloride (NMDG⁺) adjusted to pH 7.0 with 2.5 M HCl. The pH of all solutions was adjusted to 7.0 with 2.5 M NaOH. The pH of the solutions was measured with an Orion Research (Boston, MA) SA 520 pH meter, using an Orion pH electrode (model 91-04BN) and an Orion automatic temperature compensation probe (model 917002). The pH meter was autocalibrated with two certified buffer solutions, pH 7.0 and 10.0 (Fisher Scientific, Fairlawn, NJ). All experiments were performed at room temperature (22–25°C). The final Na⁺ and K⁺ concentrations of the saline were determined with flame photometry and the Cl⁻ concentrations with chloridrometry. Intracellular calibration of the pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) was performed by using the K⁺/H⁺ exchanger nigericin (Sigma, St. Louis, MO) at a final concentration of 7.25 µg/l in a high-K⁺ saline (75 mM)/low-NaCl (75 mM) saline to mimic the intracellular potassium concentrations found in mosquito Malpighian tubules (22). The pH of the calibration solutions was adjusted to at least three pH values between 6.5 and 8.0 with 2.5 M KOH. The osmolality of all solutions was 320 mosmol/kgH₂O as determined by vapor pressure osmometry using a Wescor osmometer (Logan, UT).

Fluid Secretion

Rates of fluid secretion were measured as previously described (22). Briefly, adult female mosquitoes were anesthetized at 4°C and placed on ice. The animals were decapitated, and the alimentary canal was removed with forceps and placed in saline at room temperature. Individual Malpighian tubules were severed from their attachment to the pylorus of the midgut-hindgut. The Malpighian tubules were transferred to a drop of saline that previously had been placed under a layer of light white paraffin oil (Fisher, St. Louis, MO) contained in a glass petri dish. The severed end of each tubule was drawn out of the saline drop into the oil phase with the aid of a glass hook fabricated on a microforge. After several minutes, the Malpighian tubules began to secrete fluid into the oil phase. The volume of the secreted fluid droplet was determined by optically measuring the diameter of the secreted fluid droplet and assuming the secreted fluid droplet was in the shape of a prolate spheroid. The diameter of the secreted fluid droplet was determined by optically measuring the diameter of the secreted fluid droplet and assuming the diameter of the droplet was again measured for an additional 30 min at 5-min intervals.

Microfluorometry

Individual Malpighian tubules were prepared for microfluorometric determination of pH as described previously (22). Briefly, Malpighian tubules were dissected as described for the fluid secretion experiments, except that the blind end of the tubule was severed with watchmaker’s forceps. The tubule was placed in a 200-µl bath placed on the stage of a Diaphot inverted microscope (Nikon, New York, NY) and suspended between two holding pipettes without perfusion of the tubular lumen. The fluorescence of BCECF-AM was measured with a dual-excitation, single-emission spectrofluorometer (SPEX, Edison, NJ) attached via a fiber-optic cable to the microscope. The fluorescent excitation light (500 and 440 nm) generated by the spectrofluorometer was reflected via a 515-nm dichroic mirror (Omega Optics, Brattleboro, VT) through a Fluor ×40 oil-immersion lens (Nikon) to the Malpighian tubule. The emitted light passed through the dichroic mirror and then through a 535-nm barrier filter to a photomultiplier tube. A circular aperture in front of the photomultiplier tube limited the emitted light to a 150-µm length of the Malpighian tubule. The fluorescence signal was averaged every 2 s at each excitation wavelength. The ratio of the number of photons emitted at 500 nm/440 nm used to determine the pH was calculated every 5 s. The ratio of photon counts of the emitted light at 500 nm/440 nm at known extracellular pH values was used to determine intracellular pH by using first-order linear regression analysis. Background photon counts, constant throughout the experiment, amounted to <5% of the signal counts and were subtracted from the signal counts.

Malpighian tubules were loaded for 30 min with 5 µM BCECF-AM dissolved in saline. The BCECF-AM was replaced with saline, and the experiment was conducted. Incubation of mosquito Malpighian tubules with BCECF-AM was found to have no effect on tubular fluid secretion or on transepithelial voltage, in contrast to previous reports (22). The Malpighian tubule was acid loaded by using the NH₄Cl pulse technique (3). The duration of the NH₄Cl pulse was 2 min. Switching between bath solutions was accomplished with a Teflon zero-dead-volume rotary valve (Rheodyne, Cotati, CA). The flow rate of saline through the bath was 1.5 ml/min, controlled by a peristaltic pump (Cole-Parmer, Chicago, IL). At the end of the experiment, the 500 nm/440 nm ratio was used to calibrate the intracellular BCECF, using the high-K⁺/nigericin method (26). Nigericin was eliminated from the system after every calibration procedure by passing 1 mg/100 ml of BSA through the bath lines.

Chemicals

BCECF-AM was dissolved in 100% DMSO and diluted to a final concentration of 0.1% DMSO in saline. Nigericin, purchased from Sigma, was dissolved in 100% ethanol and diluted to a final concentration of 0.1% ethanol. Amiloride [3,5-diamino-N-(aminomethyl)-6-chloropyrazinocarboxamide hydrochloride], benzamil [3,5-diamino-[amino-(benzlamino) methylene]-6-chloropyrazinocarboxamide hydrochloride], and clonidine [2-(2,6-dichloroaniline)-2-imidazoline hydrochloride] were purchased from Research Biochemicals International (Natick, MA) and dissolved in saline. EIPA and 5-(N-methyl-N-isobutyl)-amiloride (MIA) from Research Biochemicals International were dissolved in DMSO and diluted in saline with a final concentration of DMSO of 0.1%. Harmaline (1-methyl-7-methoxy-3,4-dihydro-β-carboline hydrochloride), purchased from Sigma, was dissolved in saline.

Analysis of Changes in pH

Calculation of slopes of pH vs. time were determined by performing a first-order linear regression analysis of the initial data points 20 s after the attainment of a new steady-
state pH. The slope from the linear regression analysis is expressed as the change in pH units per second after the change in extracellular solution. The slope was multiplied by 1,000 to result in an integer number as reported in previous studies (4, 8).

Statistics

The differences between control and experimental periods were tested for significance at the $P < 0.05$ level with paired or unpaired Student's $t$-tests. The IC$_{50}$ was determined from fluid secretion rates vs. dose of inhibitor with a program for nonlinear regression analysis of sigmoidal dose-response (GraphPad Prism, San Diego, CA).

RESULTS

Effects of Inhibitors of Na$^+$/H$^+$ Exchange on Rate of Fluid Secretion

Dose-response curves for the effects of inhibitors of Na$^+$/H$^+$ exchange on the rate of fluid secretion by individual isolated mosquito Malpighian tubules are shown in Fig. 1. The IC$_{50}$ rank order of potency was EIPA (7 μM) > MIA (11 μM) > amiloride (89 μM) > harmaline (129 μM) > clonidine (234 μM). Of the six compounds tested, EIPA, MIA, and harmaline were complete antagonists of fluid secretion. The degree of inhibition of fluid secretion by EIPA and MIA were two orders of magnitude greater than that evoked by harmaline. The incomplete antagonists of fluid secretion included amiloride (the parent compound of EIPA and MIA), clonidine, and benzamil. The dose-response curves for amiloride and benzamil were not extended to 10 mM because the compounds were insoluble at these concentrations. The results indicate that EIPA is the most potent antagonist of fluid secretion by mosquito Malpighian tubules, with an IC$_{50}$ of 7 μM. In subsequent pH$_i$ experiments, a dose of 100 μM EIPA was chosen because the dose is above the IC$_{50}$ for fluid secretion yet below the maximal inhibition of fluid secretion in mosquito Malpighian tubules. Additionally, the N-substituted analogs of amiloride are known to have the highest binding affinity to the Na$^+$/H$^+$ exchanger (4, 14).

Response of Steady-State pH$_i$ to Low Na and EIPA

The steady-state pH$_i$ values for the mosquito Malpighian tubule of ~7.0 presented in the tables and figures are not significantly different from the pH$_i$ value of 7.03 ± 0.05 previously reported (22). Replacement of bath saline Na$^+$ ions with NMDG$^+$ ions or addition of 100 μM EIPA to the bathing saline for 5 min had similar effects on steady-state pH$_i$, as shown in Table 1 and Figs. 2 and 3. On replacement of saline with either of these reagents, the pH$_i$ became more acidic by an average of 0.4–0.5 pH units. The time courses of both responses were similar, as reflected in similar initial acidification rates and in relation to the nadir of the response. After the pH$_i$ reached a new steady state, the recovery was not significantly different from zero, indicating the dependency of steady-state pH$_i$ on Na$^+$/H$^+$ exchange. The small recovery of pH$_i$ before the removal of 100 μM EIPA between 850 and 950 s may reflect acid extrusion from the cell by the apical H$^+$-ATPase, as this acid-extrusion mechanism was not inhibited. The rates of recovery of pH$_i$ after replacement of NMDG$^+$ ions with Na$^+$ ions or removal of 100 μM EIPA were also similar. The similarity of these responses indicates that the Na$^+$/H$^+$ exchanger is active at the steady-state pH$_i$ and is thus a major determinant of the steady-state pH$_i$.

Response of Steady-State pH$_i$ to Exposure to NH$_4$Cl Pulse

The Na$^+$/H$^+$ exchanger in mosquito Malpighian tubules was activated by using the NH$_4$Cl pulse technique (3). Typical pH$_i$ responses are shown in Table 2 and Figs. 4 and 5. The data from 21 mosquito Malpighian tubules exposed to NH$_4$Cl for 2 min followed by recovery in control saline are summarized in the control column in Table 2. On bath replacement of 20 mM NaCl with 20 mM NH$_4$Cl, the pH$_i$ increased from point A to point B (see also the first or 1° NH$_4$Cl exposure in Figs. 4 and 5, left) due to an influx of NH$_3$ across the basolateral membrane, which, on buffering by intracellular H$^+$, leads to the alkalinization at point B. The modest drop in pH$_i$ from point B to point C before the removal of the NH$_4$Cl from the bath is a result of the slow entry of extracellular NH$_3$ during the pulse. The pronounced acidification at point D on removal of NH$_4$Cl from the bath is a result of the rapid efflux of NH$_3$ from the cell, leaving H$^+$ in the cell. If an acid-extrusion mechanism is present at point D, the pH$_i$ will recover to pre-NH$_4$Cl addition pH$_i$ values at point E. These experiments indicate that the NH$_4$Cl pulse technique may be used to acidify the cells of the mosquito Malpighian tubule and that, on acidification resulting from the removal of NH$_4$Cl, some mechanism is responsible for the extrusion of H$^+$ ions from the cells. The possibility that the extrusion of acid from the cells was mediated by Na$^+$/H$^+$ exchange was explored.
Malpighian tubules is in parentheses. 

On replacement of the 0 mM Na and 100 mM EIPA, the dependence of EIPA-sensitive mechanism accounts for the was also observed (Fig. 3) in the presence of 100 mM EIPA-sensitive mechanism accounts for the was also observed (Fig. 3) in the presence of 100 mM EIPA, the 

<table>
<thead>
<tr>
<th>Replacement</th>
<th>Control pH$_i$</th>
<th>Slope to Acidification, $\times 10^{-3}$ pH/s</th>
<th>Minimum pH$_i$</th>
<th>Recovery Slope, $\times 10^{-3}$ pH/s</th>
<th>Slope After Replacement, $\times 10^{-3}$ pH/s</th>
<th>Control pH$_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM Na (7)</td>
<td>7.09 ± 0.11</td>
<td>$-14.2 \pm 5.3$</td>
<td>6.47 ± 0.14*</td>
<td>0.4 ± 0.2</td>
<td>6.32 ± 0.13</td>
<td>7.09 ± 0.12</td>
</tr>
<tr>
<td>EIPA (4)</td>
<td>7.01 ± 0.03</td>
<td>$-14.0 \pm 3.1$</td>
<td>6.60 ± 0.09*</td>
<td>0.1 ± 0.5</td>
<td>10.42 ± 2.9</td>
<td>7.04 ± 0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE. NMDG$^+$, N-methyl-D-glucamine chloride; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; pH$_i$, intracellular pH. No. of Malpighian tubules is in parentheses. *P < 0.05 compared with pH$_i$ values.

**Effect of 0 mM Na and 100 μM EIPA on Rate of Recovery From Acute Acid Load**

To provide evidence that a Na$^+$/H$^+$ exchanger is present in mosquito Malpighian tubules and that the exchanger is responsible for acid extrusion after a NH$_4$Cl pulse, paired experiments were performed in the absence and presence of 0 mM Na (Fig. 4) or 100 μM EIPA (Fig. 5). The results indicate that a Na$^+$-dependent EIPA-sensitive mechanism accounts for the acid extrusion (points D-E) after the NH$_4$Cl pulse. The rates of recovery after the NH$_4$Cl pulse in the absence of extracellular Na$^+$ or in the presence of 100 μM EIPA are significantly different from the control recovery from the NH$_4$Cl pulse (Table 2; compare 1° pulse vs. 2° pulse slopes from point D to point D’ or E). A modest recovery of pH$_i$ from point D to point D’ compared with those from D’ to E was observed in 8 of the 13 Malpighian tubules studied and may indicate acid extrusion by the apical H$^+$/ATPase. This modest recovery was also observed (Fig. 3) in the presence of 100 μM EIPA. On replacement of the 0 mM Na and 100 μM EIPA with saline, pH$_i$ recovered at rates comparable to pH$_i$ recovery after the first NH$_4$Cl pulse (points D’-E), suggesting that the Na$^+$/H$^+$ exchanger remains active after replacement of 0 mM Na and 100 μM EIPA. The degrees of acidification from point C to point D in the absence of extracellular Na$^+$ (6.29 ± 0.12) and in the presence of 100 μM EIPA (6.34 ± 0.07) were significantly greater than in the control pulses (6.84 ± 0.12 and 6.65 ± 0.06, respectively). The reason for this enhanced acidification is presently unknown, but the results support the Na$^+$/H$^+$ exchanger as an important mechanism for acid extrusion. Additionally, the rates of pH$_i$ recovery (points D’-E) after inhibition by 0 mM Na (16.8 ± 0.4 × 10$^{-3}$ pH/s) and 100 μM EIPA (19.4 ± 0.5 × 10$^{-3}$ pH/s) were greater than those in control pulses (points D’-E, 6.9 ± 0.8 and 9.6 ± 1.6 × 10$^{-3}$ pH/s, respectively). The increased rate of recovery may be related to the enhanced extrusion of H$^+$ by the Na$^+$/H$^+$ exchanger at the more acidic pH (3).

**DISCUSSION**

In the present study, the rate of fluid secretion and the steady-state pH$_i$ of mosquito Malpighian tubules were affected by inhibitors of Na$^+$/H$^+$ exchange. The results indicate that a Na$^+$/H$^+$ exchange mechanism is a major determinant of the rate of fluid secretion by mosquito Malpighian tubules. The Na$^+$/H$^+$ exchange mechanism determines the steady-state pH$_i$ and is also active during an acid load induced by NH$_4$Cl, as may be found during metabolic acidosis.

Most of the previous reports of the effects of Na$^+$/H$^+$ exchange inhibitors on fluid secretion by Malpighian tubules were affected by inhibitors of Na$^+$/H$^+$ exchange. The results indicate that a Na$^+$/H$^+$ exchange mechanism is a major determinant of the rate of fluid secretion by mosquito Malpighian tubules. The Na$^+$/H$^+$ exchange mechanism determines the steady-state pH$_i$ and is also active during an acid load induced by NH$_4$Cl, as may be found during metabolic acidosis.

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**Table 1. Effects of extracellular 0 mM Na (replaced with NMDG$^+$) and 100 μM EIPA on steady-state pH$_i$**

- **Replacement**
  - Control pH$_i$
  - Slope to Acidification, $\times 10^{-3}$ pH/s
  - Minimum pH$_i$
  - Recovery Slope, $\times 10^{-3}$ pH/s
  - Slope After Replacement, $\times 10^{-3}$ pH/s
  - Control pH$_i$

- **0 mM Na (7)**
  - 7.09 ± 0.11
  - $-14.2 \pm 5.3$
  - 6.47 ± 0.14*
  - 0.4 ± 0.2
  - 6.32 ± 0.13
  - 7.09 ± 0.12

- **EIPA (4)**
  - 7.01 ± 0.03
  - $-14.0 \pm 3.1$
  - 6.60 ± 0.09*
  - 0.1 ± 0.5
  - 10.42 ± 2.9
  - 7.04 ± 0.07

Values are means ± SE. NMDG$^+$, N-methyl-D-glucamine chloride; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; pH$_i$, intracellular pH. No. of Malpighian tubules is in parentheses. *P < 0.05 compared with pH$_i$ values.

**Fig. 2. Intracellular pH in a single Malpighian tubule with basolateral saline (S) substitutions of 0 mM Na$^+$ with N-methyl-D-glucamine chloride (NMDG$^+$) in saline.**

**Fig. 3. Intracellular pH in a single Malpighian tubule with basolateral saline substitutions containing 100 μM EIPA in saline.**
tubules have not presented dose-response curves for their effects, nor have the previous studies tested the effects of N-substituted analogs of amiloride or nonamiloride compounds. The use of dose-response curves of amiloride analogs may aid in the identification of specific Na\(^+\)-coupled transport mechanisms in tissues. Amiloride and its analogs have been shown to have dose-, structural-, and species-dependent effects on Na\(^+\) transport systems (14) and, specifically, on the Na\(^+\)/H\(^+\) exchanger (19). At doses <1 μM, the effects of amiloride are on the Na channel; at 100 μM doses, the effects are on Na\(^+\)/H\(^+\) exchange; and at 1 mM doses, the effects are relatively nonspecific, including the effects on the Na channel, the Na\(^+\)/H\(^+\) exchanger, the Na\(^+\)/Ca\(^{2+}\) exchanger, and the Na\(^+\)-K\(^+\)-ATPase. Three different structural amiloride analogs were used in the present study; EIPA and MIA are examples of 5-amino substitutions, and benzamil containing a benzyl subst-

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**Table 2. Characteristics of 1-min 20 mM NH\(_4\)Cl pulse to induce an acute acid load in Malpighian tubules of Aedes aegypti**

<table>
<thead>
<tr>
<th>Phase of NH(_4)Cl Pulse</th>
<th>Control (21)</th>
<th>1° Pulse (7)</th>
<th>2° Pulse with 0 mM Na</th>
<th>1° Pulse (6)</th>
<th>2° Pulse with 100 μM EIPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pH(_i) before NH(_4)Cl pulse, point A</td>
<td>7.07 ± 0.05</td>
<td>7.05 ± 0.10</td>
<td>7.06 ± 0.09</td>
<td>7.04 ± 0.03</td>
<td>7.02 ± 0.04</td>
</tr>
<tr>
<td>Slope, ×10(^{-3}) pH/s, from point A to point B</td>
<td>68.4 ± 36.8</td>
<td>35.2 ± 6.8</td>
<td>32.3 ± 0.7</td>
<td>81.7 ± 4.8</td>
<td>72.0 ± 5.9</td>
</tr>
<tr>
<td>Maximum pH(_i) in NH(_4)Cl, point B</td>
<td>7.41 ± 0.05*</td>
<td>7.37 ± 0.8*</td>
<td>7.36 ± 0.05*</td>
<td>7.44 ± 0.01*</td>
<td>7.39 ± 0.01*</td>
</tr>
<tr>
<td>Resting pH(_i) before NH(_4)Cl removal, point C</td>
<td>7.24 ± 0.04*</td>
<td>7.24 ± 0.06*</td>
<td>7.23 ± 0.04*</td>
<td>7.23 ± 0.01*</td>
<td>7.23 ± 0.02*</td>
</tr>
<tr>
<td>Slope, ×10(^{-3}) pH/s, from point C to point D</td>
<td>29.5 ± 4.2</td>
<td>24.3 ± 4.8</td>
<td>25.2 ± 2.6</td>
<td>51.5 ± 8.7</td>
<td>49.5 ± 4.2</td>
</tr>
<tr>
<td>Minimum after NH(_4)Cl removal, point D</td>
<td>6.79 ± 0.04*</td>
<td>6.84 ± 0.12*</td>
<td>6.29 ± 0.12‡</td>
<td>6.65 ± 0.06*</td>
<td>6.34 ± 0.07‡</td>
</tr>
<tr>
<td>Slope, ×10(^{-3}) pH/s, after inhibitor washout, from point D to point E</td>
<td>8.7 ± 1.0</td>
<td>6.9 ± 0.8</td>
<td>0.7 ± 0.6‡</td>
<td>9.6 ± 1.6</td>
<td>0.3 ± 0.3†</td>
</tr>
<tr>
<td>Control pH(_i) after NH(_4)Cl pulse, point E</td>
<td>16.8 ± 8.4‡</td>
<td>19.4 ± 0.5‡</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. No. of Malpighian tubules is in parentheses. See Figs. 4 and 5 for reference to time points. *P < 0.05 compared with control pH\(_i\) before NH\(_4\)Cl pulse. †P < 0.05 compared with control (1 or 2°) NH\(_4\)Cl pulse. ‡P < 0.05 compared with slope D to point E of control (1 or 2°) NH\(_4\)Cl pulse.

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**Fig. 4. Intracellular pH in a single Malpighian tubule with basolateral saline substitutions containing 20 mM NH\(_4\)Cl (1° AC) and 20 mM NH\(_4\)Cl (2° AC) followed by saline containing 0 mM Na\(^+\) substituted with NMDG\(^+\).** The points labeled on the intracellular pH profile are as follows: A: steady-state intracellular pH before the 20 mM NH\(_4\)Cl pulse. B: alkalinization of intracellular pH on influx of NH\(_3\). C: slow and small acidification of intracellular pH due to the slow influx of NH\(_4\). D: rapid and large acidification of intracellular pH due to the rapid efflux of NH\(_3\) from the cell leaving H\(^+\) in the cell. E: steady-state pH after the 20 mM NH\(_4\)Cl pulse. In the second NH\(_4\)Cl pulse, D* refers to new steady-state pH in the presence of 0 mM Na.

**Fig. 5. Intracellular pH in a single Malpighian tubule with basolateral saline (S) substitutions of saline containing 20 mM NH\(_4\)Cl (1° AC) and saline containing 20 mM NH\(_4\)Cl (2° AC) followed by saline containing 100 μM EIPA (see Fig. 4 for explanation of points A-E).**
stition of the terminal nitrogen of the guanidino moiety of amiloride. The 5-amino-substituted analogs are the most potent inhibitors of Na\(^{+}/H^{+}\) exchange in mammals, whereas benzamil is a less potent inhibitor of the Na\(^{+}/H^{+}\) exchanger compared with the Na\(^{+}\) channel. The amiloride analogs were dissolved in saline at a pH of 7.0, which is well below its acid dissociation constant value of 8.7, thus resulting in protonated amiloride. Because Na\(^{+}\) competes for the amiloride site on the Na\(^{+}/H^{+}\) exchanger, studies with the protonated form of amiloride should be performed in a low-Na\(^{+}\) saline (3). However, fluid secretion in mosquito Malpighian tubules is dependent on the Na\(^{+}\) content of the saline, thereby precluding similar experiments. Hence, the measurements made in this study are probably overestimates of the IC\(_{50}\) for the compounds when compared with results from similar experiments conducted in saline with a low concentration of Na\(^{+}\). Nonamiloride-derived compounds used in this study include harmaline and clonidine, which contain an N-containing heterocyclic aromatic ring structures similar to the structure of amiloride. These compounds have been found to be one to two orders of magnitude less effective in inhibiting Na\(^{+}/H^{+}\) exchange than the amiloride analogs used by Orlowski (19) and in the present study.

In insects, amiloride has been studied extensively for its effects on fluid secretion by Malpighian tubules at doses of 10 \(\mu\)M in *Hemideina maori* (17), 20 \(\mu\)M in *Drosophila* adults (6), 100 \(\mu\)M in *Locusta migratoria* (7), 500 \(\mu\)M in *Rhodinus prolixus* (15) and 1 mM in *Drosophila* larva (1, 28) and *Aedes aegypti* (11). A dose-response curve (0.1 \(\mu\)M–1 mM) was performed for fluid secretion in Malpighian tubules of the tsetse fly, *Glossina morsitans* (9). In every insect studied, amiloride inhibited fluid secretion. Most of the studies have concluded that the effects of amiloride were on the Na\(^{+}\) channel, whereas two studies (11, 15) have suggested that amiloride may affect Na\(^{+}/H^{+}\) exchange or K\(^{+}/H^{+}\) exchange. None of these amiloride-based studies has used analogs of amiloride to more specifically probe the respective insect Malpighian tubule for different mechanisms of Na\(^{+}\) transport, including Na\(^{+}\) channels in the form of benzamil or Na\(^{+}/H^{+}\) exchangers in the form of EIPA and MIA. In mammals, N-substituted amiloride analogs including EIPA and MIA have been the primary tools for investigating the “housekeeping” Na\(^{+}/H^{+}\) exchangers (NHE or NHE1) and the epithelial or apical NHE3 (8, 19). More recently, the bismethacyloyl guanidine derivative 3-[(3-guanidino-2-methyl-3-oxo-propyl)-5-methyl-phenyl]-N-isopropylide dihydrochloride (S3226) has been found to discriminate between NHE1 and NHE3 activity (23); hence, S3226 could be useful in identifying the isofoms of the exchanger present in insect Malpighian tubules. Measurements of \(^{22}\text{Na}\) influx in NHE-deficient Chinese hamster ovary cells transfected with the isoforms of NHE1 and NHE3 (19) revealed that the rank order of potency for NHE3 to be EIPA > amiloride = benzamil, which is similar to our findings with respect to rates of fluid secretion in mosquito Malpighian tubules. Although this similarity in rank order of potency may suggest that the mosquito Malpighian tubule contains an NHE3, the nature of fluid secretion by Malpighian tubules is quite complex, involving many ions, ion transporters, and second messengers (2, 18) that may also be inhibited by amiloride (1) and its analogs.

The second series of experiments was performed to probe the cells of mosquito Malpighian tubules for changes in steady-state pH\(_{i}\) by replacing extracellular Na\(^{+}\) with NMDG\(^{+}\) or by inhibition with 100 \(\mu\)M EIPA. If Na\(^{+}/H^{+}\) exchange is present, then removal of extracellular Na\(^{+}\), a cofactor for the exchanger, and inhibition with EIPA should decrease the efflux of H\(^{+}\), increase cellular H\(^{+}\) concentration, and decrease pH\(_{i}\). This was shown to be the case for the mosquito Malpighian tubule. A decrease in steady-state pH\(_{i}\) indicates that the Na\(^{+}/H^{+}\) exchanger is active in steady-state conditions to balance acid-loading processes. A decrease in steady-state pH in the absence of extracellular Na\(^{+}\) is absent in some cells containing a Na\(^{+}/H^{+}\) exchanger (5, 27), reflecting low rates of acid loading; yet, a decrease in steady-state pH\(_{i}\) is found in many cells containing a Na\(^{+}/H^{+}\) exchanger (13, 24), reflecting high rates of acid loading. The source of the acid-loading processes balanced by the Na\(^{+}/H^{+}\) exchange is unknown in mosquito Malpighian tubules but may include apical membrane Na\(^{+}/H^{+}\) or K\(^{+}/H^{+}\) exchange coupled to the H\(^{+}\)-ATPase extrusion of H\(^{+}\) (29).

With the use of pH-selective microelectrodes, the effects of extracellular Na\(^{+}\) replacement and 1 mM amiloride on intracellular pH have been investigated in *Drosophila* larval proximal segments of the anterior Malpighian tube (1, 28). Wessing et al. (28) showed that bathing Malpighian tubules with Na\(^{+}\)-free saline significantly increased pH\(_{i}\) by 0.06, whereas a K\(^{+}\)-free saline significantly decreased pH\(_{i}\) by 0.07; 1 mM amiloride did not significantly affect pH\(_{i}\). These changes coupled with measurement of luminal pH, intracellular K\(^{+}\), and potential differences led Wessing et al. to conclude that a K\(^{+}/H^{+}\) exchanger is located on the luminal membrane of *Drosophila* larval Malpighian tubules. The discrepancy between the results of replacement of Na\(^{+}\) in mosquito vs. *Drosophila* Malpighian tubules may reflect the degree of acid loading found in the steady state or differences in H\(^{+}\) -coupled transport, including apical membrane Na\(^{+}/H^{+}\) vs. K\(^{+}/H^{+}\) exchange. The differences in results between amiloride and EIPA may again reflect degrees of acid loading or differences in affinity of the cation exchanger for the exchange inhibitor.

In the third series of experiments, the effects of an acute acid load on steady-state pH\(_{i}\) were examined to reveal the mechanism whereby mosquito Malpighian tubules extrude H\(^{+}\). Addition of NH\(_{4}\)Cl resulted in typical (3, 5, 13, 24, 27) changes in pH\(_{i}\), reflecting basolateral membrane permeability to NH\(_{3}\) and in acid loading of the cell, as indicated by the prominent acidification after removal of the NH\(_{4}\)Cl. The recovery from the acid load in mosquito Malpighian tubules was found to be dependent on a Na\(^{+}\)-coupled mechanism and an EIPA-sensitive mechanism. Taken together,
these results point to the involvement of a Na\(^+\)/H\(^+\) exchange mechanism in regulating pH. In larval *Drosophila* Malpighian tubules, the NH\(_4\)Cl pulse-pH\(_i\) profile was not as typical as that observed in mosquito Malpighian tubules, at times lacking the initial alkalization and also lacking a rapid acidification after removal of NH\(_4\)Cl, which may reflect a difference in membrane permeability for NH\(_4\) (1). Despite these differences, recovery of pH\(_i\) after an acid load in larval *Drosophila* Malpighian tubules occurs in the absence of Na\(^+\) and is inhibited 33% by 1 mM amiloride.

In summary, fluid secretion and pH\(_i\) measurements in mosquito Malpighian tubules indicate a role for a Na\(^+\)/H\(^+\) exchange mechanism in maintaining the steady-state pH\(_i\) and extruding acid after an acute acid load. Inhibition of fluid secretion in mosquito Malpighian tubules by EIPA having a IC\(_{50}\) of 7 \(\mu\)M suggests the presence of Na\(^+\)/H\(^+\) exchange. The role of other acid-extruding mechanisms, including the apical H\(^+\)-ATPase, remains to be determined in the regulation of steady-state pH\(_i\) in mosquito Malpighian tubules, as do the role of acid-loading mechanisms and the buffering capacity of the cell. The nature of the possible coupling of a Na\(^+\)/H\(^+\) exchanger with the apical H\(^+\)-ATPase to drive Na\(^+\) secretion is also unknown. Similarly, the location of the Na\(^+\)/H\(^+\) exchanger, apical vs. basolateral (10), and the isoform of the Na\(^+\)/H\(^+\) exchanger (31), needs to be determined pharmacologically and immunohistochemically. The location of the Na\(^+\)/H\(^+\) exchanger was not pursued in the present study; however, it is probably on the basolateral membrane and functions to regulate steady-state pH\(_i\), as pH\(_i\) responds rapidly to a manipulations of the bathing medium. If the Na\(^+\)/H\(^+\) exchanger is located on the apical membrane and regulates Na\(^+\) secretion into the lumen, then reduction of extracellular Na\(^+\) would perhaps alkalize the cell if the apical membrane H\(^+\)-ATPase maintains its activity. Resolution of these questions awaits further studies, particularly luminal perfusion of the Malpighian tubules with low-Na\(^+\) saline and 100 \(\mu\)M EIPA alone and in concert with similar basolateral substitutions presented in this study. Whether the Na\(^+\)/H\(^+\) exchanger described in the mosquito Malpighian tubules is important in ameliorating the effects of acidosis, as has been suggested for the locust (25), also remains to be determined.

**Perspectives**

The nature of the Na\(^+\)/H\(^+\) exchanger found in Malpighian tubules has yet to be described at the nucleotide and amino acid level. The similarity of the Malpighian tubule Na\(^+\)/H\(^+\) exchanger to NHE3 in terms of inhibitor sensitivity is suggestive of a role in Na\(^+\) transport; however, further molecular biological studies to elucidate Na\(^+\), amiloride, and regulatory binding sites need to be performed. In the intact mosquito, the role of the Na\(^+\)/H\(^+\) exchanger in regulating fluid excretion is beyond the scope of the present investigations but may be quite substantial. Studies by Williams et al. (30) revealed that a female mosquito excretes 40% of the water and Na\(^+\) contained in the plasma portion of the blood meal within 2 h of feeding. As the Malpighian tubule is primarily responsible for secretion of water and Na\(^+\) leading to the post-blood meal diuresis and natuuriuresis, the role of the pH\(_i\) in regulating fluid secretion may be twofold. First, as shown in the present study, fluid secretion is dependent on Na\(^+\)/H\(^+\) exchange, which may be activated in response to the increased Na\(^+\) load from the blood meal. Second, fluid secretion in Malpighian tubules is driven by the H\(^+\)-ATPase (20), which is dependent on the pH\(_i\) of the cells, which is dependent on the activity of the Na\(^+\)/H\(^+\) exchanger. In the future, the precise contribution of the Na\(^+\)/H\(^+\) exchanger may be assessed by determining the impact of the blood meal pH on hemolymph pH with measurements of the excreted fluid pH.

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