Voltage clamping single cells in intact Malpighian tubules of mosquitoes

R. Masia, D. Aneshansley, W. Nagel, R. J. Nachman, and K. W. Beyenbach. Voltage clamping single cells in intact Malpighian tubules of mosquitoes. Am J Physiol Renal Physiol 279: F747–F754, 2000.—Principal cells of the Malpighian tubule of the yellow fever mosquito were studied with the methods of two-electrode voltage clamp (TEVC). Intracellular voltage (V(_{pc})) was −86.7 mV, and input resistance (R(_{in})) was 388.5 kΩ (n = 49 cells). In six cells, Ba^{2+} (15 mM) had negligible effects on V(_{pc}), but it increased R(_{in}) from 325.3 to 684.5 kΩ (P < 0.001). In the presence of Ba^{2+}, leucokinin-VIII (1 μM) increased V(_{pc}) by 10.2 ± 0.3 mV (P < 0.001) and reduced R(_{in}) to 340.2 kΩ (P < 0.002). Circuit analysis yields the following: basolateral membrane resistance, 652.0 kΩ; apical membrane resistance, 340.2 kΩ; shunt resistance (R(sh)), 344.3 kΩ; transcellular resistance, 992.2 kΩ. The fractional resistance of the apical membrane (0.35) and the ratio of transcellular resistance and R(sh), (3.53) agree closely with values obtained by cable analysis in isolated perfused tubules and confirm the usefulness of TEVC methods in single principal cells of the intact Malpighian tubule. Dinitrophenol (0.1 mM) reversibly depolarized V(_{pc}) from −94.3 to −10.7 mV (P < 0.001) and reversibly increased R(_{in}) from 412 to 2,879 kΩ (P < 0.001), effects that were duplicated by cyanide (0.3 mM). Significant effects of metabolic inhibition on voltage and resistance suggest a role of ATP in electrogensis and the maintenance of conductive transport pathways.

yellow fever mosquito; shunt resistance; potassium channel; barium; leucokinin

MATERIALS AND METHODS

Mosquitoes and Malpighian tubules. The mosquito colony was maintained as described by Pannabecker et al. (18). On the day of the experiment a female mosquito (3–7 days post-eclosion) was cold anesthetized and decapitated. A Malpighian tubule was removed under Ringer solution from its attachment to the gut and transferred to a Lucite perfusion chamber with a filling volume of 0.5 ml. The bottom of the chamber was covered with a thin layer of black dissecting wax, to which Malpighian tubules readily adhere. The tubules were viewed from above with a stereoscope microscope at ×50 magnification (Wild, Heerbrugg, Switzerland). A principal cell was selected near the blind end of the tubule and impaled with two conventional microelectrodes for TEVC studies.

Ringer solution and drugs. Ringer solution contained the following (in mM): 150 NaCl, 25 HEPES, 3.4 KCl, 1.8
NaHCO$_3$, 1 MCl$_2$, 1.7 CaCl$_2$, and 5 glucose. The pH was adjusted to 7.1 with NaOH. The osmolality of Ringer was 320 mosmol/kgH$_2$O. High-K$^+$ solution contained 34 mM K$^+$, substituting K$^+$ for Na$^+$ in equimolar quantities.

To block the K$^+$ conductance of the basolateral membrane of principal cells, we used BaCl$_2$ at concentrations up to 15 mM. In these experiments, the control Ringer solution contained a concentration of mannitol equivalent to the osmolalities of BaCl$_2$.

Synthetic leucokinin-VIII was a gift from Mark Holman and Ron Nachman (Texas A&M University).

All agents were added to the peritubular Ringer solution. Normally, the bath volume was ~250 μl, as Ringer solution flowed through the perfusion chamber at a rate of 6.5 ml/min. On the assumption that the bath change can be described by first order kinetics, it takes only 10 s to replace 99% of the bath volume. Rapid bath changes were desirable in evaluations of the effects of barium, DNP, and KCN on voltage and resistance. However, bath flow was stopped before adding leucokinin-VIII to conserve peptide.

Electrophysiological studies. All electrophysiological measurements were made in nonperfused tubules resting on the bottom of the perfusion bath (Fig. 1A). Tubes were ~3.5 mm long. A principal cell, ~0.5 mm from the blind end of the tubule, was selected for impalement with current and voltage electrodes. The placement of microelectrodes ~10 length constants from the open end of the tubule, reduced short-circuiting into the bath of the current injected into the cell (1, 19). Impalement of the principal cell near its center yielded the most stable current and voltage recordings.

Microelectrodes (Kwik-Fil, Borosilicate Glass Capillaries, TW 100f-4; World Precision Instruments, Sarasota, FL) were pulled on a programmable puller (model P-87; Sutter Instruments, Novato, CA) to yield resistances between 20 and 30 MΩ when filled with 3 M KCl. One electrode served to record the $V_{pc}$ and the other served to inject current when measuring $R_{pc}$. The electrodes were bridged to the measuring and clamp circuits using Ag/AgCl junctions that were prepared by first degreasing the Ag wire with alcohol, and then by Cl-plating it in 0.1 M HCl for 20 min at a current of 50 μA. The bath was grounded with a 4% agar bridge containing Ringer solution.

For voltage and current recording from principal cells, we used the GeneClamp model 500 voltage and patch clamp amplifier (Axon Instruments, Foster City, CA) equipped with head stages for TEVC experiments in oocytes: head stage HS-2A gain 10MGU for current injection and head stage HS-2A gain 1LU for voltage recording. The voltage clamp was only engaged for measurements of $R_{pc}$, which was evaluated from the current changes accompanying four or more 11-ms hyperpolarizing voltage clamp steps of 10–30 mV each. Clampfit (pClamp 6, Axon Instruments, Foster City, CA) was used to produce current-voltage (I-V) plots from which resistance was determined from linear fits to the data.

All current and voltage data were displayed on an oscilloscope (Iwatsu, Japan) and on a strip chart recorder (model BD 64; Kipp and Zonen, Crown Graphic). Data were also collected in digital form with the aid of a Macintosh computer (7300/200) equipped with data acquisition hardware and software (Multifunction I/O Board PCI-1200 and Signal Conditioning and Termination Board Model SC-2071; LabView for Macintosh, version 4.1; National Instruments Manufacturer, Austin, TX).

Circuit analysis. Since transepithelial electrolyte secretion in Malpigian tubules of Aedes aegypti is electrogenic (4, 5, 28), transepithelial transport of ions can be modeled with the electrical equivalent circuit shown in Fig. 1B. In brief, the active transport pathway, taken by Na$^+$ and K$^+$ through principal cells, consists of electromotive forces ($E$) and the resistance ($R$) of the basolateral (bl) and apical (a) membranes. A shunt pathway for Cl$^-$ consists of the single resistance, $R_{sh}$, located outside principal cells. Transcellular and paracellular pathways are electrically coupled such that cat-

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\text{Fig. 1. Two-electrode voltage clamp (TEVC) method in a single principal cell of an isolated Malpighian tubule of the yellow fever mosquito, Aedes aegypti. A: a principal cell near the blind end of the tubule is impaled with current and voltage electrodes. B: electrical circuit of transepithelial active transport of Na$^+$ and K$^+$ through principal cell in parallel with transepithelial passive transport of Cl$^-$. C: the electrical model circuit. D: reduced model circuit used for data analysis. V, voltage; R, resistance; E, electromotive force; pc, principal cell; bl, basolateral membrane of principal cell; a, apical membrane of principal cell; sh, shunt; and t, transepithelial.}$
ionic current through principal cells equals anionic current through the shunt (Fig. 1B).

Figure 1C illustrates the measuring circuit together with the epithelial circuit model. The resistance of the agar bridge ($R_{\text{bridge}}$) in the peritubular Ringer bath, 7.5 kΩ, is constant and subtracted in all resistance measurements. The resistance of fluid secreted into the tubule lumen ($R_{\text{lumen}}$) is in contact with the peritubular Ringer bath via the open end of the tubule (Fig. 1A). Since $R_{\text{lumen}}$ is about 2,000 times greater than the $R_{\text{sh}}$ (1, 19), the fraction of current that crosses the apical membrane into the tubule lumen does not short-circuit through the open end of the tubule, but returns to ground via the nearest shunt ($R_{\text{sh}}$). Hence, $R_{\text{lumen}}$ can be neglected in the circuit analysis, which reduces the electrical model to two parallel pathways for injected current: one pathway across the basolateral membrane of the principal cell, the other across the apical membrane and the shunt (Fig. 1D).

We used two assumptions to obtain numerical estimates for the resistances of cell membranes and the shunt. The first assumption, the “barium assumption,” states that $Ba^{2+}$ increases the resistance of the basolateral membrane to such high values that measurements of $R_{\text{pc}}(\text{Ba}^{2+})$ approach the sum of the apical membrane resistance and the $R_{\text{sh}}$. The second assumption, the “leukokinin assumption,” states that in the presence of $Ba^{2+}$ the application of leukokinin-VIII lowers the $R_{\text{sh}}$ resistance to such low values that measurements of $R_{\text{pc}}(\text{Ba}^{2+}+\text{LK})$ approach the resistance of the apical membrane (Eq. 1). Thus

$$R_a = R_{\text{pc(Ba}^{2+}+\text{LK})}$$

$$R_{sh} = R_{\text{pc(Ba}^{2+})} - R_a$$

$$R_{bl} = \frac{R_p R_{\text{pc(Ba}^{2+})}}{R_{\text{pc(Ba}^{2+})} - R_p}$$

The limitations of these assumptions are treated in the DISCUSSION.

**Statistical evaluation of data.** Each cell was used as its own control so that the data could be analyzed for the difference between paired samples, control vs. experimental (paired Student’s t-test).

**RESULTS**

Control values of basolateral membrane voltage and input resistance of principal cells. Under control conditions, the average $V_{\text{pc}}$ was $-86.7 \pm 1.2$ mV in 49 principal cells, each from a different Malpighian tubule. The $R_{\text{pc}}$ was determined by voltage clamping of $V_{\text{pc}}$ to a series of potentials by current injection into the cell. Figure 2 shows representative current traces and the corresponding I-V relationships for control conditions and after addition of 5 mM $Ba^{2+}$ to the peritubular Ringer bath. Charging of membrane capacitance was essentially complete within the pulse duration of 11 ms (Fig. 2). The current overshoots, lasting for about 2 ms at the onset and termination of the pulse, reflect mostly the time constants of the clamp circuit. A plot of clamp voltage vs. current yields I-V relationships that reveal small deviations from linearity with increasing conductance at hyperpolarizing voltages. For the principal cell shown in Fig. 2, $R_{\text{pc}}$ estimated in the vicinity of zero current (open-circuit voltage) was 371.4 kΩ under control conditions. $Ba^{2+}$ did not alter the deviation from linearity of the I-V relationship. It did, however, decrease the slope of the I-V plot to reflect the increase in $R_{\text{pc}}$ to 740.4 kΩ. In subsequent experiments, $R_{\text{pc}}$ was measured from a linear fit to four hyperpolarizing clamp steps of 10–20 mV each. $R_{\text{pc}}$ was on average 388.5 ± 13.4 kΩ for 49 principal cells (tubules).

**Effects of $K^+$ and $Ba^{2+}$ on voltage and resistance.** Previous studies suggested that $K^+$ uptake across the basolateral membrane of principal cells is passive, presumably through $K^+$ channels (4, 5). Thus it was of interest to analyze the response of the basolateral membrane to elevations of bath $K^+$ concentration and to blockage of $K^+$ channels with $Ba^{2+}$. Figure 3 shows a typical experiment. After electrode impalements, $V_{\text{pc}}$ stabilized at a value of −98 mV; $R_{\text{pc}}$ at that time was 270 kΩ. Increasing bath $K^+$ concentration from 3.4 to 34 mM decreased $V_{\text{pc}}$ and $R_{\text{pc}}$ to −52 mV and 180 kΩ, respectively. Both $V_{\text{pc}}$ and $R_{\text{pc}}$ returned to control values upon returning bath $[K^+]$ to the normal concentration of 3.4 mM. Depolarization and repolarization of $V_{\text{pc}}$ were as fast as the rate of bath change. On average, a 10-fold increase in peritubular [K+] caused the significant ($P < 0.001$) depolarization of $V_{\text{pc}}$ from $-86.7 \pm 4.5$ to $-47.0 \pm 3.2$ mV and a significant ($P < 0.001$) decrease in $R_{\text{pc}}$ from 416.2 ± 40.1 to 257.2 ± 21.8 kΩ (mean ± SE, $n = 6$ principal cells; 6 tubules).

Also shown in Fig. 3 is the response of $V_{\text{pc}}$ and $R_{\text{pc}}$ to $Ba^{2+}$. Each $Ba^{2+}$ concentration between 0.05 and 15 mM hyperpolarized $V_{\text{pc}}$ and increased $R_{\text{pc}}$. Effects on
$V_{pc}$ and $R_{pc}$ appeared to saturate between 1.5 and 5.0 mM (Fig. 3). The changes of $V_{pc}$ developed with speed comparable to those observed after increasing bath K$^+$ concentration. Reversibility was always complete after washout of Ba$^{2+}$.

The excellent stability of the impalement shown in Fig. 3 enabled a second elevation of K$^+$ to 34 mM, which exerted essentially the same response as in the initial test period. In the presence of high-K$^+$ Ringer (34 mM K$^+$), the effect of Ba$^{2+}$ was incomplete at the concentration of 5 mM, as shown by the additional increase in $R_{pc}$ after elevation of Ba$^{2+}$ to 15 mM. Similar results were observed in each of three other cells where the effects of Ba$^{2+}$ in high-K$^+$ Ringer was tested.

Dose-response curves were determined in five additional cells, each from a different tubule (and mosquito) in normal Ringer solution. The results are depicted in Fig. 4, which illustrates the consistent increase of $R_{pc}$ in the presence of Ba$^{2+}$, even at the lowest concentration of 0.05 mM. $R_{pc}$ increased sharply between 0 and 0.5 mM Ba$^{2+}$ with an IC$_{50}$ of 0.076 mM. The effect of Ba$^{2+}$ on $V_{pc}$ was on average negligible (Fig. 4), although the cell illustrated in Fig. 3 shows clear hyperpolarizations in the presence of Ba$^{2+}$.

Estimates of membrane and shunt resistances. As shown in Fig. 1D, the input resistance $R_{pc}$ is comprised of three components, $R_{bl}$, $R_a$, and $R_{sh}$, which can be estimated using Ba$^{2+}$ and leucokinin-VIII, as described in the MATERIALS AND METHODS. Data from six cells are summarized in Fig. 5. Under control conditions, $V_{pc}$ was $-73.2 \pm 5.6$ mV, and the $R_{pc}$ was $325.3 \pm 31.0$ k$\Omega$ (Table 1). The addition of 15 mM Ba$^{2+}$ to the peritubular bath solution had little effect on $V_{pc}$, but it significantly increased $R_{pc}$ to $684.5 \pm 46.5$ k$\Omega$ ($P < 0.001$). The subsequent application of leucokinin-VIII (10$^{-6}$ M) in the presence of Ba$^{2+}$ significantly ($P < 0.002$) hyperpolarized the cell to $-101.8 \pm 6.9$ mV, whereas $R_{pc}$ significantly ($P < 0.003$) decreased to $340.2 \pm 46.6$ k$\Omega$. These changes in $V_{pc}$ and $R_{pc}$ induced by Ba$^{2+}$ and leucokinin-VIII were reversible in the stepwise return to control Ringer solution, consistent with specific as well as independent effects of Ba$^{2+}$ and leucokinin-VIII on the basolateral K$^+$ channels and the shunt, respectively (Fig. 5; 14).

 Resistances determined from the data in Fig. 5 yielded 340.2 k$\Omega$ for the apical membrane, 652.0 k$\Omega$ for the basolateral membrane, and 344.3 k$\Omega$ for the shunt (Eqs. 1–3). In Table 1 these resistances are compared with those determined by cable analysis (CA) in isolated perfused Malpighian tubules. The comparison must be relative because resistances determined by CA are normalized to tubule length, which cannot be done in TEVC studies of a principal cell. However, taking ratios cancels normalization, thereby allowing a quantitative comparison of the two methods (Table 1). Accordingly, the ratio of transcellular and shunt resis-
DISCUSSION

TEVC experiments in principal cells of the Malpighian tubule. TEVC is a valuable method for the study of ion movement across the Xenopus oocyte membrane. For other systems, in particular epithelial cells in situ, this technique has hitherto not been applied. The major obstacle is the commonly small size of cells which precludes impalement with two electrodes. Size is not a limitation in principal cells of the Malpighian tubule of A. aegypti. We found that impalements by two electrodes were durable for several hours, in one case over 4 h; they survived repeated solution changes and repeated voltage perturbations for the examination of I-V relationships (Figs. 2 and 3).

Current-voltage relationships in principal cells. The average voltage measured in principal cells, $V_{pc} = -86.7 \, \text{mV}$, is the voltage across the basolateral membrane ($V_{bl}$). It is also the voltage across the apical membrane in series with the shunt pathway ($V_a + V_b$; Fig. 1). In the present study, $V_{bl}$ was significantly greater than $-66.2 \, \text{mV}$, measured previously in non-perfused Malpighian tubules (23), and significantly greater than $-58.0 \, \text{mV}$, measured in isolated perfused tubules (18). The basolateral membrane voltage of Aedes Malpighian tubules is known to vary widely with rates of transepithelial transport. For example, $V_{bl}$ is $-77 \, \text{mV}$ under control conditions when tubules secrete fluid at a rate 0.8 nl/min; but in the presence of cAMP, $V_{bl}$ is only $-24 \, \text{mV}$ when fluid secretion rises 250% to 2.8 nl (5, 27). Thus variations of $V_{bl}$ reflect the functional range of the basolateral membrane between antidiuretic and diuretic transport rates. Furthermore, the resistance of the basolateral membrane is twice as great as that of the apical membrane, yielding voltage drops (current times resistance) across the basolateral membrane twice as great as those across the apical membrane (Table 1; Ref. 4, 23).

I-V relationships in principal cells of A. aegypti Malpighian tubules were not perfectly linear (Fig. 2). Slopes increased in the direction of hyperpolarizing clamp voltages, where the chord conductance increased $\sim 20\%$ between 0 and $-140 \, \text{mV}$. A systematic analysis, attempting to associate this increase with a particular ion pathway, was not the object of the present study exploring the feasibility of TEVC methods in principal cells of the Malpighian tubule. For this reason we confined measures of resistances to the immediate neighborhood of open-circuit voltages, which in 49

Table 1. Resistances measured by cable analysis in isolated perfused Malpighian tubules of Aedes aegypti and by 2-electrode voltage clamp (TEVC) methods in principal cells

<table>
<thead>
<tr>
<th></th>
<th>$R_a$</th>
<th>$R_{sh}$</th>
<th>$R_{bl}$</th>
<th>$R_{tc}$</th>
<th>$R_{tc}/R_{sh}$</th>
<th>$R_{f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA, kΩ-cm (7 tubules)</td>
<td>11.4 ± 1.7</td>
<td>16.8 ± 3.0</td>
<td>29.9 ± 6.7</td>
<td>14.1 ± 2.9</td>
<td>3.33 ± 1.18</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>TEVC, kΩ (6 cells)</td>
<td>325.3 ± 31.0</td>
<td>344.3 ± 65.1</td>
<td>652.0 ± 101.0</td>
<td>340.2 ± 46.6</td>
<td>3.53 ± 0.97</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE. $R_a$, transepithelial resistance; $R_{pc}$, input resistance, principal cell; $R_{sh}$, shunt resistance; $R_{bl}$, basolateral membrane resistance; $R_{ap}$, apical membrane resistance; $R_{tc}$, sum of $R_a$ and $R_{pc}$; $R_{f}$, fractional resistance, apical membrane; CA, cable analysis; and TEVC, 2-electrode voltage clamp. CA data are from Pannabecker et al. (19).
principal cells yielded an average input resistance of 388.5 kΩ under control conditions.

Estimates of membrane and shunt resistances. In the present study, membrane and shunt resistances were estimated with the aid of two assumptions, the barium assumption, and the leucokinin assumption (see Materials and Methods). In the strictest sense, the barium assumption is not fulfilled since Ba\(^{2+}\) may not block all conductances of the basolateral membrane, but it must block the major, dominant conductance of the basolateral membrane. Since \(R_{bl}\) is the parallel resistance of the basolateral membrane and the sum of the resistances of the shunt and apical membrane (Fig. 1D), the basolateral membrane resistance must increase 43-fold in order for \(R_{bl}\) to increase from 321.3 to 682.3 kΩ in the presence of 15 mM Ba\(^{2+}\) (Fig. 4). Thus a 43-fold increase in basolateral membrane resistance meets the barium assumption (Fig. 1D).

In the strictest sense, the leucokinin assumption is not fulfilled because the \(R_{sh}\) does not reduce to zero in the presence of leucokinin-VIII. However, leucokinin-VIII reduces the \(R_{sh}\) resistance nearly ninefold, from 16.8 to 1.9 kΩ cm in isolated perfused Malpighian tubules (18, 19). Such a large decrease in the \(R_{sh}\) meets the leucokinin assumption for estimates of the apical membrane resistance. Accordingly, \(R_{pc}\) in the presence of both Ba\(^{2+}\) and leucokinin-VIII approaches the resistance of the apical membrane: 340.2 kΩ. It follows that the \(R_{pc}\) is 344.3 kΩ, and the \(R_{bl}\) is 652.0 kΩ (Table 1, Eqs. 1–3, Fig. 1D). The ratio of the transcellular and shunt resistance is 3.53, and the fractional resistance of the apical membrane is 0.35 (Table 1). Remarkably similar ratios, 3.33 and 0.32, respectively, were obtained using 1) a different experimental method, CA in isolated perfused Malpighian tubules (19); 2) a variation of the Yonath-Civan method to estimate the shunt resistance (30); and 3) a different agent, DNP, to distinguish between transcellular and paracellular pathways (Table 1). The excellent agreement between resistance ratios measured by TEVC methods (and the use of Ba\(^{2+}\) and leucokinin-VIII) and CA of in vitro perfused Malpighian tubules (and the use of DNP) confirms the validity of voltage clamp studies in single principal cells of Malpighian tubules and bolsters confidence in both methods.

Electrical coupling of principal cells. Validation of the methods of TEVC in principal cells of Malpighian tubules allowed a preliminary estimate of the degree of electrical coupling (Table 1). The transepithelial conductance in the isolated perfused Malpighian tubule is the sum of the shunt conductance (1/\(R_{sh}\)) and the transcellular conductance (1/\(R_{cell}\)), where \(R_{cell}\) is the sum of \(R_{a}\) and \(R_{bl}\). Using the data of Table 1, the transepithelial conductance in isolated perfused tubules is 87.7 μS/cm tubule length. TEVC studies (Table 1) yield a transepithelial conductance of 3.91 μS/principal cell, or 22.4 cells for a tubule 1 cm long (Table 1). An actual count yields ~125 principal cells/cm tubule length (21). It follows that the input conductance of a principal cell does not reflect a single cell but several principal cells, 5.6 cells on average, that must be electrically coupled.

The basolateral membrane of principal cells. The present study confirms previous data (23), indicating a dominant K\(^+\)-conductive pathway in the basolateral membrane of principal cells. With the possible exception of Drosophila (12), most Malpighian tubules offer such a K\(^+\) conductance in membranes facing the hemolymph (10, 11, 13, 15, 26). Moreover, intracellular K\(^+\) is close to electrochemical equilibrium with extra-

Table 2. Reversible effects of metabolic inhibitors on intracellular voltage (\(V_{pc}\)) and input resistance (\(R_{pc}\)) in Malpighian tubules of Aedes aegypti

<table>
<thead>
<tr>
<th></th>
<th>(n)</th>
<th>Control</th>
<th>Washout</th>
<th>(R_{pc}, \text{k}\Omega)</th>
<th>Control</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery</td>
<td>3</td>
<td>94.3 ± 1.1</td>
<td>-10.7 ± 2.5</td>
<td>340.2 ± 2.2</td>
<td>411.7 ± 26.7</td>
<td>2,879.0 ± 210.9*</td>
</tr>
<tr>
<td>Recovery</td>
<td>7</td>
<td>85.6 ± 2.3</td>
<td>-10.0 ± 1.5</td>
<td>90.6 ± 1.5</td>
<td>389.0 ± 24.9</td>
<td>3,226.9 ± 304.8*</td>
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</table>

Values are means ± SE; \(n\) = number of cells. \(V_{pc}\), basolateral membrane voltage of principal cells. *\(P < 0.001\).

Fig. 6. Conductive pathways for transepithelial NaCl and KCl secretion in Malpighian tubules of A. aegypti. The cations Na\(^+\) and K\(^+\) are moved into the tubule lumen through principal cells by active transport mechanisms residing in the apical membrane. Cl\(^-\) moves passively into the tubule lumen, presumably via the paracellular pathway defined by septate and/or continuous junctions. The basolateral membrane offers conductive pathways for K\(^+\) and Na\(^+\). Na\(^+\) and K\(^+\) diffusion potentials suggest a K\(^+\) conductance approximately four times greater than the Na\(^+\) conductance (23). Next to conductive Na\(^+\) entry, Na\(^+\) may enter principal cells via cotransport and antiport systems (20). The apical membrane houses an electrogenic, bafilomycin-sensitive V-type H\(^+\)-ATPase (6) that extrudes H\(^+\) into the tubule lumen, generating membrane voltages in excess of ~100 mV. The proton electrochemical potential created by the V-type H\(^+\)-ATPase powers the extrusion of K\(^+\) and Na\(^+\) by secondary active transport via hypothetical antiporters with a H\(^+\)/cation stoichiometry greater than 1 (electrogenic).
cellular K\(^+\) in Malpighian tubules of A. aegypti and other insects (10, 13, 20, 26). In the present study, the maximal inhibition of the basolateral membrane K\(^+\) conductance in control Ringer solution occurs at a Ba\(^{2+}\) concentration of 1.5 mM (Fig. 4). The basolateral membrane voltage may hyperpolarize in the presence of Ba\(^{2+}\), but it never depolarizes (Figs. 2–4), which is opposite to the Ba\(^{2+}\) response of most other cells of vertebrate origin, where the block of K\(^+\) conductance depolarizes membrane voltages toward zero (2). The effect of Ba\(^{2+}\) hyperpolarizing membrane voltages is not uncommon in cells of insect origin. Ba\(^{2+}\) hyperpolarizes the basolateral membrane voltage of locust hindgut epithelial cells (7) and midgut epithelial cells of the larval tobacco hornworm (13).

In the case of principal cells of Aedes Malpighian tubules, the essential polarizing component of the basolateral membrane is not a K\(^+\) diffusion potential, but rather the electrogenic proton pump located in the apical membrane (6, 8). As illustrated in the transport model of Fig. 6, this proton pump is a V-type ATPase that extrudes H\(^+\) into the tubule lumen (or the microenvironment of apical microvilli) at the expense of metabolic energy. Active H\(^+\) transport into the tubule lumen polarizes the apical membrane (cell negative) with respect to the luminal fluid to values in excess of 110 mV (Fig. 6, Ref. 4, 5). Current returning to the cytoplasmic side of the pump, via the shunt and the basolateral membrane, couples the electrical potential of the apical membrane to the basolateral membrane, where it provides the electromotive force for the entry of K\(^+\) into the cell. K\(^+\) entry into the cell depolarizes the basolateral membrane. Thus, blocking K\(^+\) influx with Ba\(^{2+}\) hyperpolarizes the membrane (Fig. 3). The opposite is observed after increasing K\(^+\) flux into the cell by elevating bath K\(^+\) concentration, together with a decrease in input resistance (Fig. 3). The reduced efficacy of Ba\(^{2+}\) to block basolateral membrane conductance in the presence of high K\(^+\) concentrations suggests competition between K\(^+\) and Ba\(^{2+}\) for K\(^+\) channels, as in neural and epithelial membranes of vertebrate origin (2, 25).

In addition to energizing transport across the basolateral membrane, the electrical potential generated at the apical membrane by the V-type H\(^+\)-ATPase serves as driving force for the transport of 1) Na\(^+\) and K\(^+\) from the cell to lumen via putative electrogenic H\(^+\)/Na\(^+\) and H\(^+\)/K\(^+\) antiporters in the apical membrane (4, 6, 8, 12, 20, 26) and 2) anions, in particular Cl\(^-\), from the peritubular medium to the lumen through the shunt (Fig. 6).

**Metabolic inhibitors.** In a previous study of isolated perfused Malpighian tubules (19), we learned that DNP reversibly abolished basolateral and apical membrane voltages of principal cells to values close to 0 mV while increasing the transepithelial resistance (R\(_t\)) by 47% (Fig. 1B). We interpreted these changes to indicate the inhibition of active transport through principal cells. Moreover, we assumed that this inhibition increases the transcellular resistance (R\(_{\text{trans}}\) + R\(_s\)) such that measurements of the R\(_t\) approach the R\(_{\text{sh}}\) (Table 1, Fig. 1). The present study using TEVC allowed a test of this assumption.

Both DNP and KCN depolarized the intracellular voltage of principal cells to ~10 mV while increasing the R\(_{\text{pc}}\) seven- to eightfold (Table 2). Such a large increase in R\(_{\text{pc}}\) confirms our previous assumption regarding the increase in transepithelial resistance after metabolic inhibition by DNP (19). Moreover, in the present study, R\(_{\text{pc}}\) in the presence of DNP is largely the parallel resistance of basolateral and apical cell membranes (Fig. 1D). Thus the resistances of both basolateral and apical membranes during metabolic inhibition can be calculated from R\(_{\text{pc}}\) in the presence of DNP or KCN, since it is known that DNP increases the fractional resistance of the apical membrane from 0.32 to 0.57 (19). These calculations show that DNP causes the basolateral membrane resistance to increase from 652 to 5,038 k\(\Omega\), and the apical membrane to increase from 340 to 6,718 k\(\Omega\). Similar increases take place in the presence of KCN. These large increases in basolateral and apical membrane resistance suggest that conductive pathways in both membranes are shutting down during metabolic inhibition, reducing or preventing movement of ions into and out of the cell. Intracellular ion homeostasis would thus be preserved, allowing transport to spring back again after metabolism is restored. Full recovery of voltage and conductance after washout of metabolic inhibitors is consistent with this interpretation of the data (Table 2). DNP is known to collapse proton gradients across mitochondrial membranes, and cyanide is an inhibitor of cytochrome c oxidase. Since both DNP and KCN inhibit ATP synthesis at different sites of oxidative metabolism, their effects on voltage and resistance most likely reflect the inhibition of ATP synthesis and intracellular ATP depletion. Accordingly, the effects of metabolic inhibition on voltage and resistance suggest high turnover rates of ATP and a role of ATP in maintaining conductive transport pathways in principal cells.

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**REFERENCES**


