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Mechanisms of K^+ transport across basolateral membranes of principal cells in Malpighian tubules of the yellow fever mosquito, *Aedes aegypti*

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Summary

The mechanisms of K^+ entry from the hemolymph into principal cells of Malpighian tubules were investigated in the yellow fever mosquito, *Aedes aegypti*. The K^+ channel blocker Ba^{2+} (5 mmol l^{-1}) significantly decreased transepithelial (TEP) fluid secretion (V_s) from 0.84 nl min^{-1} to 0.37 nl min^{-1} and decreased the K^+ concentration in secreted fluid from 119.0 mmol l^{-1} to 54.3 mmol l^{-1} with no change in the Cl^- concentration. Even though the Na^+ concentration increased significantly from 116.8 mmol l^{-1} to 144.6 mmol l^{-1}, rates of TEP ion secretion significantly decreased for all three ions. In addition, Ba^{2+} had the following significant electrophysiological effects: it depolarized the TEP voltage (V_t) from 19.4 mV to 17.2 mV, increased the TEP resistance (R_t) from 6.4 kΩcm to 6.9 kΩcm, hyperpolarized the basolateral membrane voltage of principal cells (V_{bl}) from –75.2 mV to –88.2 mV and increased the cell input resistance from 363.7 kΩ to 516.3 kΩ. These effects of Ba^{2+} reflect the block of K^+ channels that, apparently, are also permeable to Na^+. Bumetanide (100 μmol l^{-1}) had no effect on TEP fluid secretion and electrical resistance but significantly decreased TEP K^+ secretion, consistent with the inhibition of electroneutral $Na^+/K^+/2Cl^-$ cotransport. TEP Na^+ secretion significantly increased because other Na^+ entry pathways remained active. Bumetanide plus Ba^{2+} completely inhibited TEP electrolyte and fluid secretion, with fast and slow kinetics reflecting the Ba^{2+} block of basolateral membrane K^+ channels and the inhibition of $Na^+/K^+/2Cl^-$ cotransport, respectively. The single and combined effects of Ba^{2+} and bumetanide suggest that (1) K^+ channels and $Na^+/K^+/2Cl^-$ cotransport are the primary mechanisms for bringing K^+ into cells, (2) K^+ channels mediate a significant Na^+ influx, (3) Na^+ has as many as four entry pathways and (4) the mechanisms of TEP K^+ and Na^+ secretion are coupled such that complete block of TEP K^+ renders the epithelium unable to secrete Na^+.

Key words: transepithelial Na^+ secretion, transepithelial K^+ secretion, transepithelial Cl^- secretion, barium block, K^+ channel, bumetanide, $Na^+/K^+/2Cl^-$ cotransport, basolateral membrane voltage, cell input resistance, transepithelial voltage, transepithelial resistance.

Introduction

In the past two decades, our laboratory has investigated ion secretion in Malpighian tubules of the female yellow fever mosquito, *Aedes aegypti* (Beyenbach, 1995, 2001; Beyenbach and Petzel, 1987). Our attention has focused on the mechanisms and regulation of transepithelial Na^+, K^+ and Cl^- secretion from the hemolymph into the tubule lumen. In brief, transepithelial Na^+ and K^+ secretion is active, passing through principal cells of the tubule that supply the energy for active transport. Transepithelial Cl^- transport is passive through the paracellular pathway and/or stellate cells.

The present study is concerned with identifying the transport pathways that mediate the entry of K^+ from the hemolymph into principal cells. The K^+ conductance of the basolateral membrane, which accounts for 64% of the total membrane conductance, is one pathway for K^+ entry (Beyenbach and Masia, 2002; Masia et al., 2000). Although this K^+ conductance can be blocked by barium, the blockade does not inhibit transepithelial K^+ secretion completely (Masia et al., 2000). Thus, it was of interest to identify the K^+ entry mechanism remaining in the presence of Ba^{2+}. Transport *via* the $Na^+/K^+/2Cl^-$ cotransporter first came to mind in view of significant effects of bumetanide on transepithelial secretion of Na^+ and K^+ (Hegarty et al., 1991).

In the present study, we have used three experimental methods to probe the mechanism of K^+ entry from hemolymph into principal cells. Using the fluid secretion assay of Ramsay (1953), we evaluated the effects of Ba^{2+} and bumetanide on the transepithelial secretion of K^+, Na^+, Cl^- and water. In isolated perfused Malpighian tubules, we studied the effects of Ba^{2+} and bumetanide on transepithelial voltage and resistance. Using the methods of two-electrode voltage clamp (TEVC), we examined the effects of Ba^{2+} and bumetanide on the basolateral

membrane voltage and input resistance of principal cells (Masia et al., 2000). We found two equally important routes for the entry of K^+ into principal cells: (1) an electroconductive route that can be blocked by Ba^{2+} and (2) an electroneutral route *via* $Na^+/K^+/2Cl^-$ cotransport that can be inhibited by bumetanide. The significant inhibition of transepithelial Na^+ secretion by Ba^{2+} suggests that basolateral K^+ channels are permeable to Na^+. The stimulation of transepithelial Na^+ secretion by bumetanide and reciprocal changes in the concentrations of Na^+ and K^+ in secreted fluid indicate that Na^+ can replace K^+. However, the tubules cease epithelial transport and fluid secretion altogether if they are prevented from secreting K^+.

Materials and methods

Mosquitoes and Malpighian tubules

The mosquito colony was maintained as described by Pannabecker et al. (1993). Malpighian tubules were dissected from cold-anesthetized and decapitated female mosquitoes (*Aedes aegypti* L.) 3–7 days post-eclosion. A light tug on the rectum under Ringer solution (see below for composition) freed the intestine and Malpighian tubules from the abdomen. A single Malpighian tubule was then separated from the intestine using fine forceps (Dumont #5; Fine Science Tools, Foster City, CA, USA). The pinched proximal end of the tubule was subsequently removed with a pair of sharp jeweler's broaches.

When effects on transepithelial Na^+, K^+, Cl^- and water secretion were of interest, the tubule was studied by the methods of Ramsay (1953) and wavelength dispersive spectroscopy (Williams and Beyenbach, 1983). When effects on transepithelial voltage and resistance were of interest, the tubule was microperfused *in vitro* and studied by the method of Helman (1972). When effects on the basolateral membrane voltage and input resistance of principal cells were of interest, the tubule was studied by the method of TEVC (Masia et al., 2000).

Ringer solution and drugs

Ringer solution contained the following: 150 mmol l^{-1} NaCl, 25 mmol l^{-1} Hepes, 3.4 mmol l^{-1} KCl, 1.8 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $MgCl_2$, 1.7 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} glucose. The pH was adjusted to 7.1 with NaOH. The osmolality was 320 mosmol kg^{-1} H_2O. Ba^{2+} was used as $BaCl_2$ at a concentration of 5 mmol l^{-1}. In these experiments, the control Ringer solution was supplemented with 15 mmol l^{-1} mannitol for osmotic balance with the experimental solution containing 5 mmol l^{-1} $BaCl_2$. In a previous study, we determined that 5 mmol l^{-1} Ba^{2+} is a saturating dose for blocking K^+ channels in the basolateral membrane of principal cells (Masia et al., 2000).

Bumetanide (Sigma, St Louis, MO, USA) was dissolved in Ringer solution and used at a concentration of 100 μmol l^{-1}. Previous attempts to obtain a dose–response curve of the effects of bumetanide were unsuccessful because the effects of bumetanide are cumulative and irreversible. For this reason, we used the bumetanide concentration (0.1 mmol l^{-1}) employed in a previous study (Hegarty et al., 1991).

The Ramsay assay

Each Malpighian tubule served as its own control, first under control and then under experimental conditions. Rates of transepithelial fluid secretion were measured as described previously (Hegarty et al., 1991) with the following modifications. With about 80% of the tubule length remaining in a 40 μl droplet of Ringer solution, the open end of the tubule was pulled into the surrounding oil and gently draped around a small steel broach. Thus, fluid secreted by the tubule exited into oil, forming a droplet. The dimensions of this droplet were measured over time in order to determine volume. Cumulative secreted volume was measured every 5 min for an initial control period (see Fig. 1). Secreted volume was then removed, and the experimental agent was added to the peritubular Ringer solution. Thereafter, cumulative volume was measured again every 5 min for at least 30 min. Secreted volume was collected at the end of the experimental period. $BaCl_2$ was added to the peritubular Ringer solution by replacing 10 μl with Ringer solution containing 20 mmol l^{-1} $BaCl_2$. Due to the limited water solubility of bumetanide, it was necessary to exchange 20 μl of Ringer solution with an equal volume containing 200 μmol l^{-1} bumetanide. The transepithelial fluid secretion rate (V_s) was calculated as the slope of a least-squares regression line fitted to the plot of time *versus* volume secreted by the tubule over at least 30 min control and experimental periods (Fig. 1A). In the Ba^{2+} plus bumetanide study, a quadratic polynomial was fitted to the data (Fig. 1B). Here, the rate of fluid secretion was calculated by taking the derivative of the polynomial and calculating V_s at a specific time.

The concentrations of Na^+, K^+ and Cl^- in fluid secreted by Malpighian tubules and in peritubular Ringer solutions were measured against appropriate standards using the methods of wavelength dispersive spectroscopy (electron probe), as described previously (Williams and Beyenbach, 1983), with the following modifications. Dried sample spots of 30–50 pl volumes were analyzed using a JEOL 8900 electron microprobe at a beam current of 50 nA. X-rays emitted at the wavelengths of K^+ and Cl^- were quantified using a pentaerythritol high-intensity crystal while those emitted at the wavelength of Na^+ were measured using a thallium acid phthalate crystal. A set of standard curves was constructed by plotting known concentrations of Na^+, K^+ and Cl^-, each in a series of six standard solutions, against the number of X-ray counts per second.

In vitro microperfusion of Malpighian tubules

Malpighian tubules were perfused *in vitro* for the measurement of the transepithelial voltage (V_t) and resistance (R_t), as described previously (Yu and Beyenbach, 2002). The peritubular bath (500 μl) was perfused with Ringer solution at a rate of 5.6 ml min^{-1}. V_t was measured and recorded continuously, and R_t was measured periodically when of

interest. Values of V_t and R_t are steady-state values taken between 10 min and 60 min of control and experimental periods.

Two-electrode voltage clamp

Measurements of the basolateral membrane voltage (V_{bl}) and principal cell input resistance (R_{pc}) were obtained using the methods of TEVC, as described by Masia et al. (2000) and Wu and Beyenbach (2003). Again, values of V_{bl} and R_{pc} are steady-state values taken between 10 min and 60 min of control and experimental periods. The peritubular bath (500 µl) was perfused with Ringer solution at a rate of 2.6 ml min^{-1}. To prevent the movement of the tubule, the bottom of the lucite bath was coated with poly-L-lysine (evaporation of 0.125 mg ml^{-1} poly-L-lysine).

Statistical treatment of the data

Data are summarized as means ± S.E.M. (*N*, number of observations). Since each Malpighian tubule served as its own control, experiments were analyzed with the Student's paired *t*-test. Significance is defined as $P<0.05$.

Results

Effects of barium and barium plus bumetanide on transepithelial electrolyte and fluid secretion

Isolated Malpighian tubules of the female yellow fever mosquito secreted fluid spontaneously when bathed in Ringer solution. The mean rate of fluid secretion (V_s) was 0.84 nl min^{-1} (Fig. 1A) under control conditions, with a range from 0.71 nl min^{-1} to 0.94 nl min^{-1}. Fluid secretion was constant for the initial 30-min control period (Fig. 1A) when Malpighian tubules secreted a fluid with approximately equimolar concentrations of Na^+ and K^+ (Table 1). The Na^+ concentration in secreted fluid was 116.8 mmol l^{-1}, i.e. 35 mmol l^{-1} less than the Na^+ concentration in the peritubular Ringer solution. The K^+ concentration in secreted fluid was 119.0 mmol l^{-1}, i.e. 115.6 mmol l^{-1} more than the peritubular concentration (Table 1). Chloride was the dominant anion in secreted fluid with a concentration of 197.7 mmol l^{-1}, i.e. 38.9 mmol l^{-1} higher than the peritubular Cl^- concentration (Table 1).

The product of V_s and the ion concentration in secreted fluid yields the rate of transepithelial ion secretion (Fig. 2B–D). The mean rate of transepithelial Na^+ secretion was 98.1±15.6 pmol min^{-1}, the rate of K^+ secretion was 99.9±21.9 pmol min^{-1}, and the rate of Cl^- secretion was 166.6±9.5 pmol min^{-1} (*N*=7 tubules; Fig. 2).

The addition of 5 mmol l^{-1} $BaCl_2$ to the peritubular Ringer bath significantly reduced V_s to 44% of control values, from 0.84 nl min^{-1} (control) to 0.37 nl min^{-1}, with a range from 0.28 nl min^{-1} to 0.44 nl min^{-1} (Figs 1A, 2A; Table 1). The reduced rate of V_s was constant for as long as it was studied, i.e. 60 min, with negligible fluctuations in V_s in the presence of $BaCl_2$ (Fig. 1A).

Barium affected the ionic composition of secreted fluid

Table 1. *Single and combined effects of barium and bumetanide on transepithelial electrolyte and fluid secretion in isolated Malpighian tubules of the yellow fever mosquito*, Aedes aegypti

	Electrolytes in secreted fluid				Transepithelial		Principal cell	
	Fluid secretion (nl min^{-1})	$[Na^+]$ (mmol l^{-1})	$[K^+]$ (mmol l^{-1})	$[Cl^-]$ (mmol l^{-1})	Voltage (mV)	Resistance (kΩcm)	Basolateral membrane voltage (mV)	Input resistance (kΩ)
Control	0.84±0.03 (7)	116.8±16.2 (7)	119.0±27.2 (7)	197.7±8.3 (7)	19.4±8.3 (11)	6.37±0.64 (11)	−75.2±4.7 (12)	363.7±18.4 (12)
Ba^{2+} (0.5 mmol l^{-1})	0.37±0.03* (7)	144.6±7.3* (7)	54.3±28.7* (7)	192.4±10.3 (7)	17.2±3.6* (11)	6.87±0.81* (11)	−88.2±5.2* (12)	516.3±29.9* (12)
Control[1]	1.15±0.08 (14)	110.4±6.8 (14)	88.0±6.8 (14)	221.1±5.2 (14)	52.1±8.1 (8)	11.6±2.0 (8)	NM	NM
Bumetanide (0.1 mol l^{-1})[1]	1.01±0.14 (14)	169.5±9.2* (14)	33.2±7.1* (14)	223.0±5.0 (14)	64.6±7.3* (8)	12.4±1.8* (8)	NM	NM
Control	0.61±0.02 (6)	93.8±8.8 (6)	55.1±8.7 (6)	150.3±9.9 (6)	30.4±8.1 (7)	11.0±2.1 (7)	−78.4±4.6 (6)	314.9±27.9 (6)
Ba^{2+} plus bumetanide	0.06±0.02* (6)	120.4±5.1* (6)	25.3±2.4* (6)	140.5±8.7 (6)	38.2±3.0* (7)	13.6±2.1* (7)	−59.3±6.5* (6)	464.9±39.0* (6)

Values are means ± S.E.M. (*N*). For fluid and electrolyte data collected by the method of Ramsay, each tubule served as its own control. Secreted fluid was collected after the usual 30 min control period and then again 20–70 min later after barium and/or bumetanide had been added to the peritubular Ringer. Voltage is measured with respect to ground in the peritubular bath.

[1]Data from Hegarty et al. (1991); * $P<0.05$; NM, not measured.

(Table 1). The Na^+ concentration in secreted fluid significantly increased from 116.8 mmol l^{-1} (control) to 144.6 mmol l^{-1}, and the K^+ concentration significantly decreased from 119.0 mmol l^{-1} (control) to 54.3 mmol l^{-1}. Ba^{2+} had no significant effect on the Cl^- concentration in secreted fluid (Table 1).

The product of V_s and ion concentration revealed significant effects of Ba^{2+} on transepithelial secretion rates of all three ions (Fig. 2B–D). The rate of transepithelial Na^+ secretion decreased to 55% of control values, from 98.1 pmol min^{-1} to 53.8±4.4 pmol min^{-1}. The rate of K^+ secretion decreased even more, to 20% of control values, from 99.9 pmol min^{-1} to 19.5±10.6 pmol min^{-1}, and the rate of Cl^- secretion decreased to 43% of control values, from 166.6 pmol min^{-1} to 71.4±6.0 pmol min^{-1} (Fig. 2B–D).

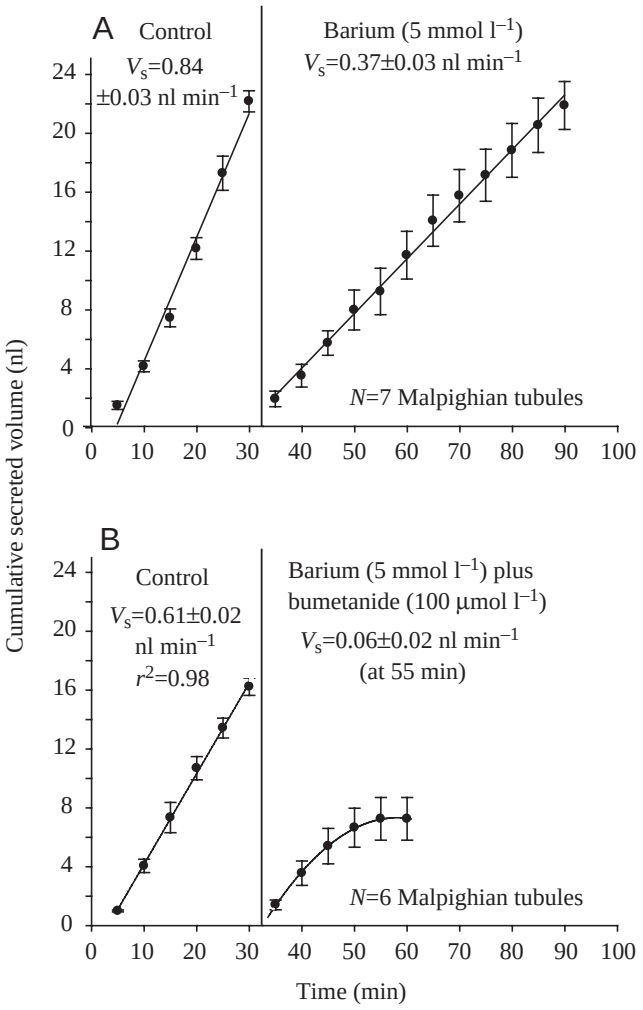

Fig. 1. Transepithelial fluid secretion in isolated Malpighian tubules of female yellow fever mosquitoes studied by the method of Ramsay (1953). Cumulative secreted volume is plotted against time first under control conditions and then in the presence of (A) Ba^{2+} and (B) Ba^{2+} plus bumetanide. After the usual initial 30 min control period, fluid secreted by the tubules was removed for analysis with an electron microprobe, and 5 mmol l^{-1} $BaCl_2$ (A) or 5 mmol l^{-1} $BaCl_2$ plus 100 µmol l^{-1} bumetanide (B) was added to the peritubular Ringer solution. Fluid secreted under experimental conditions was collected at the end of the experiment, 25–70 min after introducing the agent of interest to the peritubular Ringer bath. The slope of regression lines yields the flow rate from the open end of the tubule, which equals the rate of transepithelial fluid secretion. Each tubule is used as its own control. Values are means ± S.E.M.

Although the Ba^{2+} block of K^+ channels in the basolateral membrane of principal cells substantially reduced transepithelial K^+ secretion, it did not completely inhibit K^+ secretion (Fig. 2C). For this reason, we searched for the transepithelial K^+ transport pathway remaining in the presence of Ba^{2+}. In these experiments, the control rate of fluid secretion was 0.61 nl min^{-1} with a range from 0.56 nl min^{-1} to 0.68 nl min^{-1} (Fig. 1B; Table 1). The addition of 5 mmol l^{-1} Ba^{2+} plus bumetanide (100 µmol l^{-1}) led V_s to decrease progressively, reaching the lowest detectable rate of fluid secretion, 0.06 nl min^{-1}, about 25 min after adding these two agents to the peritubular bath (Table 1). After 30 min, V_s had decreased to zero for all six Malpighian tubules tested (Fig. 1B). Since a new steady state was never obtained, we estimated V_s as the derivative of the secreted volume that had accumulated 25 min after the addition of barium and bumetanide to the peritubular bath (Fig. 1B; Table 1).

In the presence of barium plus bumetanide, the Na^+ concentration in secreted fluid significantly increased from 93.8 mmol l^{-1} to 120.4 mmol l^{-1}, and the K^+ concentration significantly decreased from 55.1 mmol l^{-1} to 25.3 mmol l^{-1} (Table 1). The concentration of Cl^- in secreted fluid did not change significantly: 150.3 mmol l^{-1} *versus* 140.5 mmol l^{-1} (Table 1). These data are qualitatively similar to those observed in the presence of Ba^{2+} alone.

The product of V_s and ion concentration in the presence of Ba^{2+} plus bumetanide showed profound effects on transepithelial ion secretion rates (Fig. 2). After 25 min of the experimental period, transepithelial Na^+ secretion had dropped to values 12% of control, from 57.8±5.5 pmol min^{-1} (control) to 7.1±2.9 pmol min^{-1} (N=6; Fig. 2B). At the same time, transepithelial K^+ secretion had fallen to 4% of control, from 34.3±5.9 pmol min^{-1} (control) to 1.36±0.5 pmol min^{-1} (N=6; Fig. 2C) and Cl^- secretion had decreased to 9% of control values, from 92.8±6.3 pmol min^{-1} (control) to 8.1±3.0 pmol min^{-1} (N=6; Fig. 2C). After 30 min in the presence of Ba^{2+} plus bumetanide, transepithelial transport rates for all three ions were reduced to zero.

Effect of barium and barium plus bumetanide on tubule electrophysiology

In isolated perfused Malpighian tubules, the control transepithelial voltage (V_t) was 19.4 mV (lumen-positive), and the transepithelial resistance (R_t) was 6.37 kΩcm (Table 1). In principal cells studied by the methods of TEVC, the control basolateral membrane voltage (V_{bl}) was –75.2 mV, and the input resistance of principal cells (R_{pc}) was 363.7 kΩ (Table 1). The difference between V_t and V_{bl} is the apical membrane voltage, 94.6 mV.

Peritubular Ba^{2+} significantly depolarized V_t from 19.4 mV

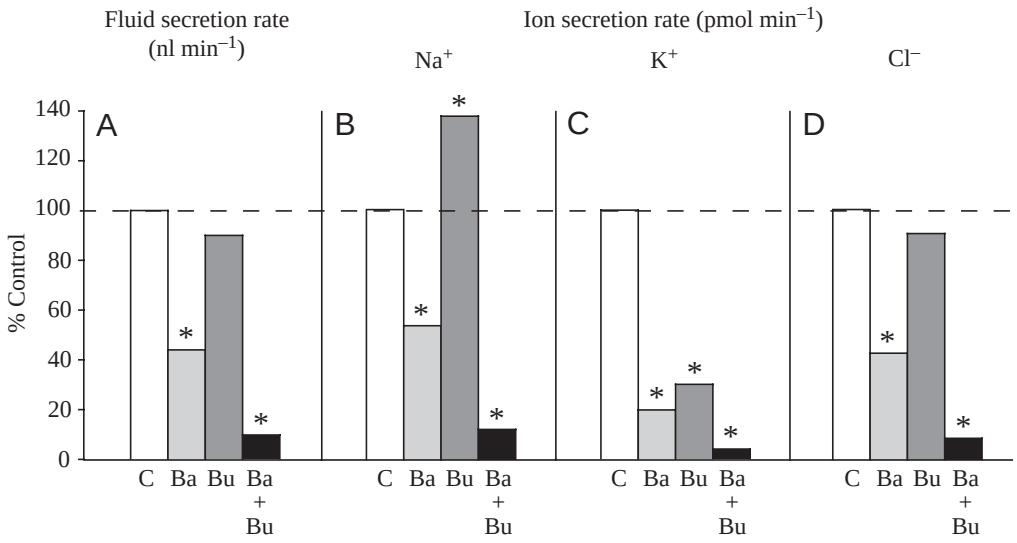

Fig. 2. Single and combined effects of barium (Ba; 5 mmol l^{-1}) and bumetanide (Bu; 100 μmol l^{-1}) on transepithelial ion secretion in isolated Malpighian tubules of the yellow fever mosquito. After the usual initial 30 min control period, fluid secreted by the tubules was removed for analysis with an electron microprobe, and barium and/or bumetanide was added to the peritubular Ringer solution. Fluid secreted under experimental conditions was collected at the end of the experiment, 25–70 min after introducing the agent of interest to the peritubular Ringer bath. Data are expressed as % of control. N=7 for C and Ba; N=6 for Bu and Ba+Bu. For numeric data, see Table 1. Asterisks indicate significant differences (P<0.05). Effects of bumetanide alone are previously published data (Hegarty et al., 1991).

to 17.2 mV and significantly increased R_t from 6.37 kΩcm to 6.87 kΩcm (Table 1). In addition, 5 mmol l^{-1} Ba^{2+} significantly hyperpolarized V_{bl} from –75.2 mV to –88.2 mV and significantly increased R_{pc} from 363.7 kΩ to 516.3 kΩ.

The effects of barium on V_t, V_{bl}, R_t and R_{pc} were immediate, as rapid as the peritubular batch could be changed to include Ba^{2+}. Moreover, Ba^{2+} elicited these effects in a single step, i.e. there were no secondary time-dependent effects. Likewise, the off-effects upon Ba^{2+} washout were immediate and complete, displaying simple kinetics of an open channel block, as in previous studies (Beyenbach and Masia, 2002; Masia et al., 2000; Wu and Beyenbach, 2003).

Peritubular Ba^{2+} plus bumetanide had the following effects measured after 25 min of treatment: in isolated perfused Malpighian tubules, the control V_t was 30.4 mV and the control R_t was 11.0 kΩcm (Table 1). In principal cells studied by the methods of TEVC, the control V_{bl} was –78.4 mV while the control R_{pc} was 314.9 kΩ (Table 1). The addition of Ba^{2+} plus bumetanide to the peritubular bath significantly hyperpolarized V_t from 30.4 mV to 38.2 mV and significantly increased R_t from 11.0 kΩcm to 13.6 kΩcm (Table 1). V_{bl} significantly depolarized from –78.4 mV to –59.3 mV and significantly increased R_{pc} from 314.9 kΩ to 464.9 kΩ (Table 1). The time course of the electrophysiological effects of Ba^{2+} and bumetanide mirrored the time course of the effects on electrolyte and fluid secretion.

Discussion

The present study identifies two pathways for the entry of K^+ into principal cells across the basolateral membrane: one electroconductive, the other electroneutral. K^+ channels are identified on the basis of channel block by barium (Fig. 3), which inhibits 80% of transepithelial K^+ secretion and has significant effects on voltage and resistance (Table 1). The electroneutral transport step is identified by the effects of bumetanide, a known blocker of $Na^+/K^+/2Cl^-$ cotransport. In a previous study, bumetanide inhibited 70% of transepithelial K^+ secretion without significant effects on transepithelial electrical resistance (Hegarty et al., 1991). In the present study, the co-administration of barium and bumetanide completely inhibited transepithelial K^+ secretion, revealing no other transepithelial K^+ transport mechanisms.

The inhibitory effects of barium on insect Malpighian tubules have been observed in the locust (Hyde et al., 2001), ant (Leyssens et al., 1994), fruitfly (Wessing et al., 1993), beetle (Nicoloson and Isaacson, 1987), mealworm (Wiehart et al., 2003a), cricket (Xu and Marshall, 1999a) and weta (Neufeld and Leader, 1998). The present study corroborates the consensus that Ba^{2+}-sensitive K^+ channels mediate the entry of K^+ across the basolateral membrane of principal cells in Malpighian tubules. The K^+ conductance of the basolateral membrane is so large that intracellular K^+ is at or near electrochemical equilibrium with extracellular K^+ in the hemolymph or peritubular Ringer solution (Ianowski et al., 2001; Leyssens et al., 1993).

Effects of the loop diuretics bumetanide and furosemide on insect Malpighian tubules have been reported in the mealworm (Wiehart et al., 2003b), blowfly (O'Donnell and Maddrell, 1984), fruit fly (Linton and O'Donnell, 1999), tobacco hornworm (Audsley et al., 1993; Reagan, 1995), ant (Leyssens et al., 1994), locust (Baldrick et al., 1988) and cricket (Xu and Marshall, 1999b). The consensus of these studies is that bumetanide and furosemide inhibit $Na^+/K^+/2Cl^-$ and/or K^+/Cl^- cotransport systems (Baldrick et al., 1988; Gillen and Bowles, 2001; Leyssens et al., 1994) in the basolateral membrane, thereby preventing the entry of NaCl and/or KCl into the cell. The present study is consistent with this conclusion. However, the evidence for $Na^+/K^+/2Cl^-$ cotransport is merely pharmacological because this transporter has not been isolated

from and/or cloned in any insect Malpighian tubule. In Malpighian tubules of *Aedes aegypti*, bumetanide was found to significantly inhibit transepithelial K^+ secretion without affecting transepithelial resistance and the fractional resistance of the basolateral membrane, which is consistent with an effect on a non-conductive transport pathway such as that of an electroneutral transport system (Hegarty et al., 1991).

More importantly, the present study illustrates that inhibitors of K^+ transport significantly increase the transepithelial secretion of Na^+ and Cl^-. In what follows, we will seek explanations for the effects of barium and bumetanide on transepithelial Na^+, K^+ and Cl^- secretion that are consistent with the present data and present models of transepithelial electrolyte transport in Malpighian tubules of *Aedes aegypti*.

Inhibition of transepithelial K^+ and fluid secretion by barium

The addition of the K^+ channel blocker Ba^{2+} to the peritubular bath promptly reduced the rate of fluid secretion to 44% of control values together with significant reductions in the rates of transepithelial Na^+, K^+ and Cl^- secretion

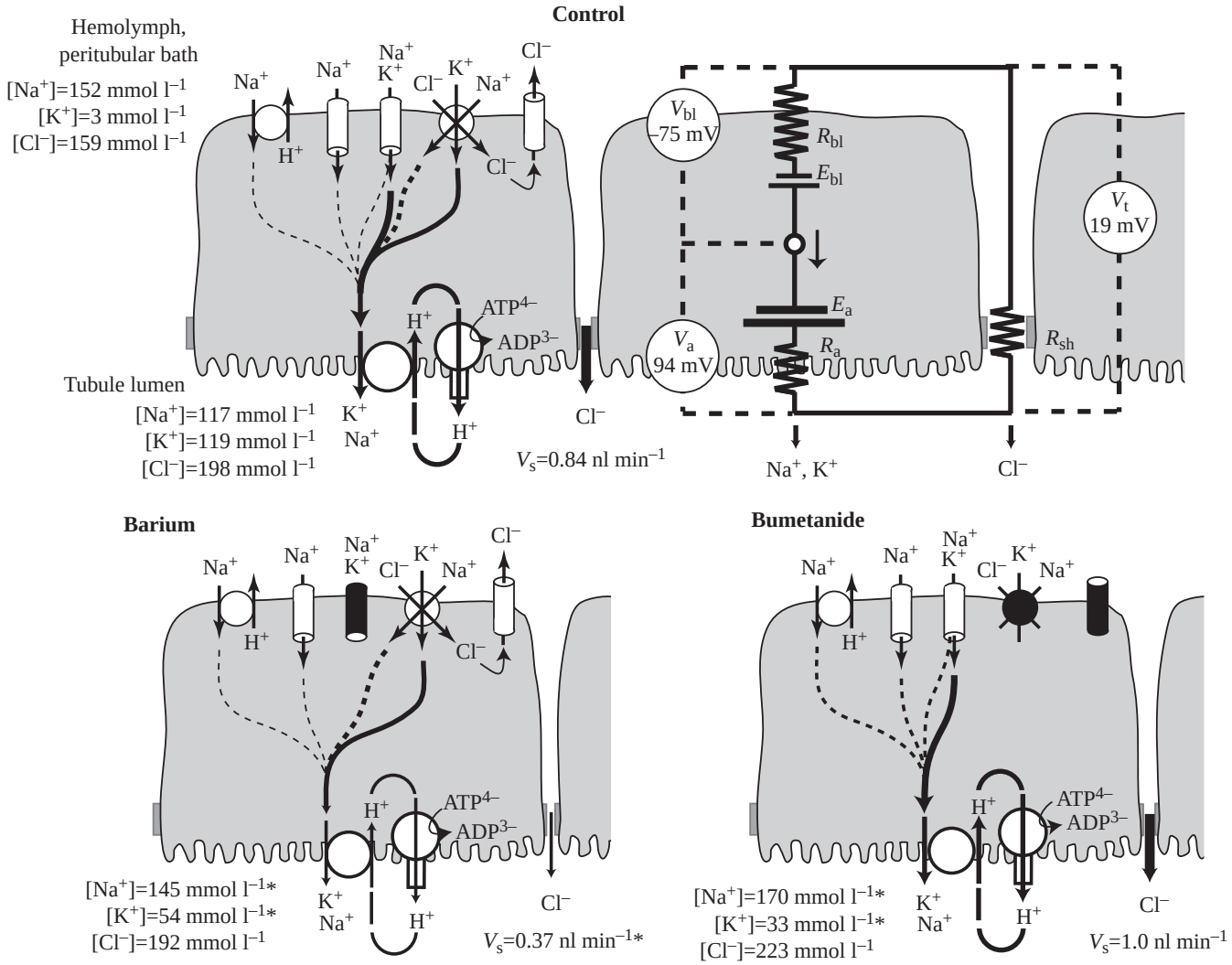

Fig. 3. Models of transepithelial NaCl and KCl secretion in Malpighian tubules of *Aedes aegypti*. The cations Na^+ and K^+ take a transcellular pathway and Cl^- takes a paracellular shunt pathway. Transcellular and paracellular pathways are electrically coupled, forming an intraepithelial current loop. The current generator is the V-type H^+-ATPase located in the apical membrane of principal cells (Beyenbach, 2001). Current across the apical membrane is carried by H^+. Current returning to the cytoplasmic face of the V-type H^+-ATPase is carried by Cl^- ions passing from the hemolymph to the tubule lumen through the paracellular pathway, by K^+ and Na^+ entering the cell across the basolateral membrane and by Cl^- leaving the cell through Cl^- channels (Yu et al., 2003). Barium blocks basolateral membrane K^+ channels, thereby increasing the resistance of the basolateral membrane (R_{bl}) and decreasing the loop current. The net effect is the inhibition of transepithelial NaCl, KCl and fluid secretion. Bumetanide blocks $Na^+/K^+/2Cl^-$ cotransport across the basolateral membrane, leaving electroconductive pathways largely intact. As a result, intraepithelial current flow is not affected and rates of transepithelial cation, anion and fluid secretion remain unchanged. However, rates of transepithelial K^+ secretion decrease and Na^+ secretion increase because one major pathway for K^+ entry is blocked, leaving three other Na^+ entry pathways open. V_s, rate of fluid secretion; E, electromotive force; V, voltage; R, resistance; a, apical membrane; bl, basolateral membrane; sh, epithelial shunt; t, transepithelial.

(Fig. 2). The block of basolateral membrane K^+ channels is expected to reduce the entry of K^+ into principal cells, thereby limiting intracellular K^+ for secretion into the tubule lumen (Fig. 3). Accordingly, the rate of transcellular K^+ transport is inhibited by 80%. Clearly, K^+ channels provide a major route for K^+ entry into the cell. They account for 64% of the total conductance of the basolateral membrane (Beyenbach and Masia, 2002). When these K^+ channels are blocked by Ba^{2+} in Malpighian tubules of ants, the intracellular K^+ concentration drops from 88 mmol l^{-1} to 73 mmol l^{-1} (Leyssens et al., 1993). A similar drop in the intracellular K^+ concentration of *Aedes* Malpighian tubules reduces the driving force for K^+ extrusion across the apical membrane, explaining in part why the K^+ concentration in secreted fluid drops from 119 mmol l^{-1} to 54 mmol l^{-1} in the presence of Ba^{2+} (Table 1).

Inhibition of transepithelial K^+ secretion by bumetanide

In a previous study, we saw no effect of bumetanide (0.1 mmol l^{-1}) on transepithelial fluid secretion (Hegarty et al., 1991). However, an examination of secreted fluid revealed significant effects on the concentrations of Na^+ and K^+ in secreted fluid (Table 1). Bumetanide decreased the K^+ concentration by 60 mmol l^{-1} but increased the Na^+ concentration by a similar amount with no change in total cation concentration and hence no change in the concentration of secreted Cl^-. Thus, measures of fluid secretion as the only bioassay can be misleading. For this reason, the parallel study of ion concentrations and electrophysiology offers details that otherwise would be missed in the Ramsay assay.

Since the K^+ concentration in secreted fluid decreased and the Na^+ concentration increased, it follows that bumetanide decreased the rate of transepithelial K^+ secretion and increased the rate of Na^+ secretion (Table 1; Fig. 2). Significantly, bumetanide inhibited K^+ secretion by 70%, similar to the inhibition measured in the presence of Ba^{2+} (Fig. 2). Bumetanide is known to inhibit $Na^+/K^+/2Cl^-$ cotransport in Malpighian tubules (Baldrick et al., 1988; Hegarty et al., 1991; Ianowski et al., 2001; Leyssens et al., 1994; Reagan, 1995; Wiehart et al., 2003a). Hence, the inhibition of K^+ secretion to levels similar to those observed in the presence of Ba^{2+} reveals that $Na^+/K^+/2Cl^-$ transport is as important a route for K^+ entry as are K^+ channels. Moreover, the complete inhibition of transepithelial K^+ secretion by the co-administration of Ba^{2+} and bumetanide identifies channel- and carrier-mediated transport as the two major, if not exclusive, mechanisms for bringing K^+ into the cell across the basolateral membrane.

The inhibition of transepithelial K^+ secretion by 80% in the presence of barium and by 70% in the presence of bumetanide suggests that carrier- and channel-mediated K^+ entry pathways are functionally coupled, where effects on K^+ channels affect $Na^+/K^+/2Cl^-$ cotransport and *vice versa*.

Inhibition of transepithelial Na^+ secretion by barium

The significant inhibition of transepithelial Na^+ secretion by barium can be explained by (1) the general reduction in transepithelial electrogenic ion transport as barium blocks a major conductive pathway and (2) K^+ channels that permit the passage of Na^+.

As shown in Fig. 3, transepithelial secretion of NaCl and KCl can be modeled with an electrical circuit consisting of a transcellular pathway that mediates active transport of K^+ and Na^+ and a paracellular pathway that mediates passive transport of Cl^-. Transcellular and paracellular pathways are electrically coupled, forming an intraepithelial circuit where cationic current (Na^+ and K^+) passing through principal cells is the same as anionic current (Cl^-) through the paracellular pathway. The Ba^{2+} block of K^+ channels significantly increases the input resistance of principal cells from 363.7 kΩ to 516.3 kΩ, reflecting the substantial increase in the resistance of the basolateral membrane (Table 1). The large increase in basolateral membrane resistance is expected to decrease transcellular cationic current and, consequently, paracellular current (Fig. 3). Indeed, estimates of transcellular and paracellular currents, or the intraepithelial loop current, show significant reductions in the presence of Ba^{2+} (Wu and Beyenbach, 2003). Thus, as loop current decreases, the rate of K^+ and Na^+ transport through principal cells decreases and the rate of Cl^- transport decreases. Direct measurements of transepithelial Na^+, K^+ and Cl^- secretion confirm this to be the case (Fig. 2). Moreover, significant reductions in transepithelial NaCl and KCl secretion have the effect of bringing less water into the tubule lumen, hence the decrease in fluid secretion in the presence of Ba^{2+} (Figs 1, 2).

Ba^{2+} may also affect transepithelial Na^+ secretion directly. Ion channels are never perfectly ion-selective. Some epithelial K^+ channels can be highly selective for K^+ with K^+/Na^+ permeability ratios as high as 100 (Hurst et al., 1992). Other K^+ channels admit Na^+ more readily, with K^+/Na^+ permeability ratios as low as 13 (Teulon et al., 1994), 10 (Suzuki et al., 1994) or 5 (Labarca et al., 1996). A similar low K^+/Na^+ permeability ratio of K^+ channels in the basolateral membrane of *Aedes* Malpighian tubules would allow a substantial Na^+ influx in view of the high electrochemical driving force (~100 mV), supporting the entry of Na^+ into the cell. The Ba^{2+} block of basolateral membrane K^+ channels would then be expected to inhibit not only K^+ entry and transepithelial K^+ secretion but also Na^+ entry and transepithelial Na^+ secretion. Accordingly, the significant inhibition of transepithelial Na^+ secretion by Ba^{2+} suggests that K^+ channels in the basolateral membrane of principal cells offer some permeability to Na^+ (Figs 2B, 3).

Stimulation of transepithelial Na^+ secretion by bumetanide

The inhibition of $Na^+/K^+/2Cl^-$ cotransport across the basolateral membrane is expected to decrease Na^+ entry into the cell and to decrease transepithelial Na^+ secretion, like K^+ (Fig. 3). Paradoxically, the opposite is observed. Bumetanide significantly stimulates transepithelial Na^+ secretion, from 122 pmol min^{-1} to 169 pmol min^{-1} (Hegarty et al., 1991). Hypothetical solutions to this paradox give a glimpse at the

relative roles of several pathways available to Na^+ for entering the cell.

Since $Na^+/K^+/2Cl^-$ cotransport is a major pathway for K^+ entry into the cell (Fig. 3), bumetanide inhibition is expected to lower the intracellular K^+ concentration. Consistent with this hypothesis is the significant depolarization of the basolateral membrane voltage from 63 mV to 51 mV, reflecting the drop in the K^+-diffusion potential across the basolateral membrane (Hegarty et al., 1991). Direct measurements of intracellular K^+ concentrations in Malpighian tubules of the locust show that furosemide, which blocks $Na^+/K^+/2Cl^-$ cotransport like bumetanide, causes intracellular K^+ concentration to fall and to be replaced by Na^+ (Hopkin et al., 2001). Likewise, intracellular Na^+ replaces K^+ in Malpighian tubules of *Rhodnius* that are stimulated to secrete Na^+ in the presence of serotonin (Ianowski et al., 2001). Similar reciprocal changes in intracellular K^+ and Na^+ concentrations in the presence of bumetanide in Malpighian tubules of *Aedes aegypti* would be expected to lead to the observed decrease in the K^+ concentration and the increase in the Na^+ concentration in secreted fluid (Fig. 2). Even though bumetanide blocks the entry of both K^+ and Na^+ into the cell, the intracellular Na^+ can still rise because three other pathways for Na entry remain open: (1) Na^+/H^+ exchange transport (Hegarty et al., 1992; Petzel, 2000), (2) an Na^+ conductance that accounts for 16% of the total conductance of the basolateral membrane (Beyenbach and Masia, 2002) and (3) K^+ channels that apparently allow the passage of some Na^+ (Fig. 3). Of these three, Na^+ entry *via* Na^+/H^+ exchange is probably the most important pathway because amiloride, a blocker of Na^+/H^+ exchange, inhibits transepithelial Na^+ secretion by 70% (Hegarty et al., 1992).

Summary and remaining questions

Ba^{2+} inhibits not only transepithelial K^+ secretion but also transepithelial Na^+ and Cl^- secretion. By blocking K^+ channels that offer some permeability to Na^+, barium reduces the transcellular cationic current from hemolymph to tubule lumen (Fig. 3, circuit diagram). Since the return current is carried by Cl^-, also passing from hemolymph to tubule lumen, it follows that barium reduces transepithelial Cl^- secretion.

In as much as basolateral membrane K^+ channels are more permeable to K^+ than to Na^+, barium inhibits transepithelial K^+ secretion to a greater degree than Na^+ secretion (Fig. 2). Nevertheless, the Na^+ concentration in secreted fluid rises because K^+ channels provide but one minor pathway for transepithelial Na^+ secretion in the presence of multiple other Na^+ entry mechanisms across the basolateral membrane (Table 1; Fig. 2). In spite of the increase in the luminal Na^+ concentration, transepithelial Na^+ secretion decreases because of the overriding reduction in the intraepithelial loop current by barium (Fig. 3, circuit diagram).

By contrast, bumetanide leaves electroconductive pathways intact. As a result, bumetanide has no effect on transepithelial resistance, no effect on the fractional resistance of the basolateral membrane (Hegarty et al., 1991), no effect on intraepithelial loop current, no effect on transepithelial total cation secretion and no effect on Cl^- and fluid secretion. Total transepithelial cation secretion did not change, but transepithelial Na^+ secretion significantly increased with an equivalent decrease in K^+ secretion. Bumetanide blocks one of two major K^+ entry pathways, but only one of four Na^+ entry pathways, bringing about reciprocal changes in intracellular and luminal K^+ and Na^+ concentrations and, consequently, the inhibition of K^+ secretion and the stimulation of Na^+ secretion.

Finally, the electrophysiological data are consistent with Ba^{2+} blocking an electroconductive pathway such as that provided by K^+ channels in the basolateral membrane of principal cells. Upon the addition of Ba^{2+} to the peritubular medium, the observed changes in transepithelial voltage and resistance and basolateral membrane voltage and resistance are internally consistent with channel block. By contrast, bumetanide had no effect on epithelial or membrane resistance, and the small changes in transepithelial and membrane voltage can be accounted for by changing intracellular K^+ concentrations. Moreover, the kinetics of the inhibitions suggest two distinct mechanisms of action. Rapid kinetics of the effects of Ba^{2+} evince channel block, and slow kinetics of the effects of bumetanide are consistent with the inhibition of a carrier.

One question that this study leaves open is how bumetanide blocks the cAMP stimulation of transepithelial Na^+ secretion, which we have observed in a previous study (Hegarty et al., 1991). In *Aedes* Malpighian tubules, corticotropin-releasing factor (CRF)-like diuretic peptides and their second messenger cAMP selectively increase transepithelial NaCl secretion by activating an Na^+ conductance in the basolateral membrane of principal cells (Beyenbach, 2001; Petzel et al., 1985, 1987; Williams and Beyenbach, 1983, 1984). How the blockade of the $Na^+/K^+/2Cl^-$ cotransporter brings about the blockade of Na^+ channels is unclear at present. However, it is known that the $Na^+/K^+/2Cl^-$ cotransporter interacts with channels, such that effects on one will also affect the other (O'Neill and Steinberg, 1995; Brzuszczak et al., 1996; Marunaka et al., 1999; Huang et al., 2000; Singh et al., 2001; Walter et al., 2001; Wang, 2003). It is conceivable that a similar coupling of carrier and channels prevents the activation of Na^+ channels when bumetanide has blocked the $Na^+/K^+/2Cl^-$ cotransporter.

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