J. Membrane Biol. 132, 63–76 (1993)

The Journal of
Membrane Biology

Regulation of Epithelial Shunt Conductance by the Peptide Leucokinin

Thomas L. Pannabecker, Timothy K. Hayes†, and Klaus W. Beyenbach
Department of Physiology, VRT 826, Cornell University, Ithaca, New York 14853, and, †Laboratories for Invertebrate Neuroendocrine Research, Texas A&M University, College Station, Texas 77483

Summary. Isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti* spontaneously secrete NaCl, KCl and water across an epithelium of modest transepithelial resistance (40–80 Ωcm^2) and high transepithelial voltage (30–70 mV, lumen positive). Transepithelial electrochemical potentials indicate that Na and K are secreted by active and Cl by passive transport mechanisms. The addition of synthetic leucokinin-VIII (LK-VIII, insect myotropic peptide) to the peritubular bath significantly increases the rates of transepithelial NaCl, KCl and water secretion. In parallel, LK-VIII depolarizes the transepithelial voltage from 59.3 to 5.7 mV, decreases the transepithelial resistance from 57.7 to 9.9 Ωcm^2, and renders the basolateral and apical membrane voltages nearly equipotential (~ -90 mV). Unilateral step changes of the [Cl] in the peritubular bath or tubule lumen elicit small transepithelial Cl diffusion potentials in the absence of LK-VIII but large transepithelial Cl diffusion potentials, up to 85% of Nernst equilibrium potentials, in the presence of LK-VIII. In Malpighian tubules treated with dinitrophenol for estimates of the shunt resistance R_{sh}, LK-VIII reduces R_{sh} from 52.5 to 5.8 Ωcm^2. Bilateral reductions of the Cl concentration in tubule lumen and peritubular bath fully restore R_{sh} to 55.8 Ωcm^2 in the presence of LK-VIII. LK-VIII has no effects when presented from the luminal side. These results suggest that LK-VIII increases the Cl conductance of the epithelial shunt via a receptor located at the basolateral side of the epithelium.

Key Words Malpighian tubule · myotropic and diuretic peptides · leucokinin-VIII · epithelial shunt pathway · chloride conductance · dinitrophenol

Introduction

Malpighian tubules, the organs of salt and water excretion in insects, initiate renal regulation of extracellular fluid homeostasis by transepithelial secretion rather than glomerular filtration. The tubules are suspended in the hemolymph (extracellular fluid) of the insect abdomen and are anchored to the hindgut into which they empty their secretions (Fig. 1). Malpighian tubules of the yellow fever mosquito *Aedes aegypti* secrete a nearly isosmotic fluid containing NaCl and KCl as the principal electrolytes and osmolytes (Williams & Beyenbach, 1983; Beyenbach & Petzel, 1987). Secretion of KCl and NaCl takes place across a single layer of epithelial cells of modest transepithelial electrical resistance (40–80 Ωcm^2) and large transepithelial lumen-positive voltage (Williams & Beyenbach, 1984). Evaluation of electrochemical potentials in the peritubular fluid and the tubule lumen show that Na and K are secreted by active transport mechanisms (against the electrochemical potential) and that secretion of Cl is passive and driven by electrochemical potentials (Williams & Beyenbach, 1984). Current models of transepithelial transport across Malpighian tubules view Na and K taking active transport pathway(s) through the principal cells (Maddrell, 1978; Phillips, 1981, 1983; Beyenbach & Petzel, 1987; Wieczorek et al., 1989; Bertram et al., 1991). The transepithelial pathway(s) taken by the counterion Cl during secretion into the tubule lumen are unknown, even though the removal of Cl from the peritubular bathing Ringer solution brings the secretion of NaCl, KCl and water to a halt (*unpublished observations*).

The leucokinins are a family of invertebrate neuropeptides isolated from the head and ganglia of the cockroach *Leucophaea maderae* (Holman et al., 1986*a*,*b*, 1987*a*,*b*). They are all octapeptides which share a common C-terminal pentapeptide amide which is required for biological activity. This structural feature is shared with the tachykinin family of vertebrate peptides (substance P), which also exhibit a common C-terminal pentapeptide amide and a highly variable amino acid composition towards the N-terminal end. While tachykinins are known to (i) lower blood pressure, (ii) stimulate intestinal and genito-urinary smooth muscle, (iii) increase salivary, lachrymal and pancreatic secretion, and (iv) stimulate ileal electrolyte transport (Erspamer, 1981), only two functions are known for the leucokinins: the increase of hindgut motility in the cockroach (Holman et al., 1991), and the increase of

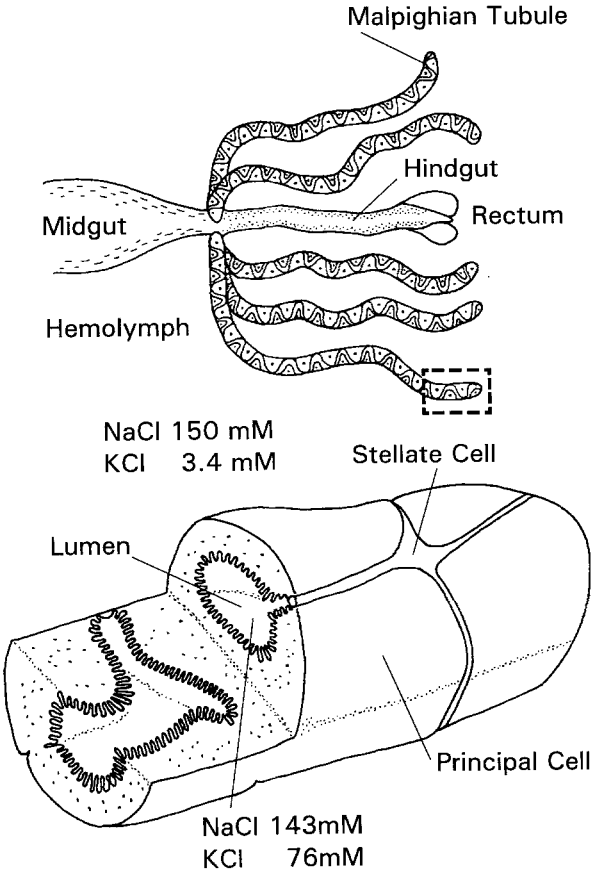

Fig. 1. Schematic diagram of the excretory system in the yellow fever mosquito, *A. aegypti*. Urine formation is initiated by tubular secretion in five Malpighian tubules suspended in the hemolymph of the abdominal cavity. Secreted fluid empties into the gut at the junction of the midgut and hindgut. The cutout shows a distal segment of the blind-ended Malpighian tubule and its two cell types, principal and stellate cells. When bathed in Ringer solution containing, among other solutes, 150 mM NaCl and 3.4 mM KCl, isolated Malpighian tubules secrete a fluid into the lumen containing 143 mM NaCl and 76 mM KCl as the principal osmolytes (Williams & Beyenbach, 1983).

transepithelial fluid secretion in isolated Malpighian tubules of the yellow fever mosquito (Hayes et al., 1989).

Preliminary studies in our laboratory have shown that one of the leucokinins, leucokinin-VIII, increases the rates of transepithelial NaCl, KCl and fluid secretion. Furthermore, leucokinin-VIII depolarizes the transepithelial voltage n a dose-dependent manner to values close to 0 mV, an effect which is diminished when the Cl concentration in the peritubular Ringer bath is reduced (Hayes et al., 1989). These observations suggest that leucokinin-VIII increases the Cl permeability of the epithelium thereby short-circuiting the voltage across the epithelium. The experimental evidence in the present study supports this hypothesis. Furthermore, principal cells, which provide the pathways for active transepithelial Na and K transport, are not the site of the increase in epithelial Cl conductance. Instead leucokinin-VIII increases the Cl conductance of a shunt pathway leading to the observed effects on tubule electrophysiology and the stimulation of transepithelial secretion of NaCl and KCl.

Materials and Methods

Mosquitoes and Isolation of Malpighian Tubules

Mosquitoes of the species *Aedes aegypti* (derived from the NIH strain) were reared and maintained in a controlled environment at 26°C and a light : dark cycle of 12 : 12 hr. Approximately 200 eggs were hatched by placing them in a flat pan (30 × 22 cm) containing 1.1 liter tap water. After hatching (1 day) the larvae were fed a liquid diet consisting of lactalbumin hydrolysate (Sigma, St. Louis, MO), yeast hydrolysate (ICN Biochemicals, Cleveland, OH), and ground Rat-Mouse- and Hamster chow (RMH 3200 chow, Agway, Syracuse, NY). These dry ingredients were mixed (equal parts by volume), and 4 g of this mixture was added to 30 ml water to yield a suspension that was stored under refrigeration. Every other day 2 ml of the suspension was added to the rearing pan and gently stirred. It took between five and seven days for the larvae to metamorphose to pupae. One day after pupation the pupae were transferred to an Erlenmeyer flask containing 300 ml fresh tap water. The opening of the flask was connected to a mosquito-net-covered plastic bucket to provide a crawl surface for adults which usually emerge 2 days later. Adult mosquitoes were fed a 3% sucrose diet provided ad lib from an inverted test tube plugged with cotton. To initiate reproduction, female mosquitoes were allowed to feed on bovine blood (Na-citrate 3 g/liter, at 37°C) offered through a taut sheet of parafilm. Gravid mosquitoes deposited their eggs on the moist, rough surfaces of 6 × 1 cm paper towel strips which lined the inside surface of a 500-ml plastic beaker holding 100 ml tap water.

Experiments were conducted during the light phase and at room temperature. Since Malpighian tubules of female mosquitoes are larger in size and therefore easier to manipulate than tubules of male mosquitoes, we used only Malpighian tubules of female adults ranging in age from three to six days.

On the day of the experiment, a female mosquito was cold-anesthetized, decapitated and then submerged in Ringer solution. Securing the thorax with one pair of forceps and pulling on the rectum with a second pair freed the gastrointestinal tract to which Malpighian tubules are attached from the abdominal cavity (Fig. 1). The Malpighian tubules were then separated from the gastrointestinal tract using forceps and dissection needles. Tubule segments measuring 0.8 to 1 mm in length were dissected from the distal (blind) end of the tubule and teased open at both ends for in vitro microperfusion by the method of Burg et al. (1966).

Ringer Solution

Mosquito Ringer solution was prepared fresh on the day of the experiment and contained in mM: NaCl 150, HEPES 25, KCl 3.4,

$CaCl_2$ 1.7, $NaHCO_3$ 1.8, $MgCl_2$ 0.6, and glucose 5. The pH was adjusted to 7.1 using NaOH. Cl was replaced isosmotically with isethionate. In solutions containing isethionate the $CaCl_2$ concentration was raised to 2.3 mM to keep the free Ca concentration unchanged (Pollard et al., 1977). Leucokinin-VIII was synthesized by one of us (Hayes) and used at a concentration of 10^{-6} M. The uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP), was used at a concentration of 10^{-4} M.

Synthesis of Leucokinin-VIII

Leucokinin-VIII is one of eight octapeptides isolated from head extracts of the cockroach *L. maderae*. Its primary structure is shown below (Holman et al., 1987*b*). The peptide sequence common to all eight leucokinins and necessary for biological activity is in boldface.

Gly-Ala-Asp-**Phe**-Tyr-**Ser-Trp-Gly-NH$_2$**

1 2 3 4 5 6 7 8

Leucokinin-VIII was synthesized by solid-phase peptide synthesis that utilized a tert-butyloxycarbony (tBoc) protection-deprotection strategy designed for the Biosearch 9500 peptide synthesizer (Now Milligen Corp., Milford, MA) according to the method of Hudson et al. (1986). Reactive side chains of select amino acids were protected as follows: Asp, O-benzyl ester; Ser, benzyl ether; and Tyr, 2-bromobenzyloxycarbonyl derivative. The side chains of the remaining amino acids did not require protection. The peptide was assembled on a 4-methyl benzhydrylamine resin, and amino acid coupling was activated by diisopropylcarbodiimide. The initial Gly was coupled to the resin two times before the initial tBoc protection was removed for the addition of the second amino acid. All other amino acids were coupled one time. The peptide was cleaved from the resin with a cleavage solution consisting of anisole (1.5 ml/g resin), ethanedithiol (0.5 ml/g resin), and hydrogen fluoride (10 ml/g resin). Cleavage was accomplished at −15°C for 30 min and then at 0°C for an additional 30 min to produce the peptide as C-terminal amide. The resin was extensively washed with diethylether; and the peptide was extracted with 40% acetic acid and lyophilized to dryness.

The crude lyophilized peptide residue was dissolved in 50% acetic acid, clarified and injected for HPLC purification. A BioRad™ (Richmond, CA) HiPore C-18 semipreparative column (250 × 10 mm) was developed with a 0–70% acetonitrile (MeCN) gradient in 5 mM trifluoroacetic acid over 49 min at 2 ml/min and 31°C. The fractions at 32–33 min contained the major peak of UV absorbance at 214 nm. Samples of these fractions when injected for HPLC analysis (250 × 4.6 mm Vydac™, analytical C-18 HPLC column, Hesparia, CA; a gradient of 0–70% MeCN over 49 min; 1 ml/min) gave a sharp symmetrical peak. The retention time of 24.2 min on the analytical system was short enough to indicate that the peptide product was completely deblocked and had not been alkylated during the cleavage process.

The peptide was tested for amino acid composition, homogeneity and quantity by PicoTag™ amino acid analysis supplied by Waters Associates (Milford, MA) and described by Cohen et al. (1984). Samples were hydrolyzed at 105°C for 24 hr by vapor phase HCl prior to derivatization. The molar ratios of amino acids were consistent with the target structure (GADFYSWGamide: Asx, 1.2; Ser, 0.9; Gly, 2.0; Ala, 1.0; Tyr, 0.9; Phe, 1.0). Trp was not observed in the analysis since this particular amino acid is always destroyed by the hydrolysis conditions. The intact peptide absorbed UV well at 280 nm indicating that Trp was indeed present in the intact peptide.

Measurements of Transepithelial Fluid Secretion by the Method of Ramsay

The Ramsay method (Ramsay, 1954) was used to test the effects of leucokinin-VIII on (i) transepithelial fluid secretion rate and (ii) the concentrations of Na, K, and Cl in secreted fluid. Briefly, two to four Malpighian tubules were placed in a 40 μl droplet of Ringer solution. The droplet was then covered with light paraffin oil and the open end of each tubule was pulled from the Ringer solution into the oil so that secreted fluid exited from the tubule as a droplet separate from the bathing Ringer. The volume of the secreted droplet was calculated from its dimensions measured via an ocular micrometer (Petzel, Berg & Beyenbach, 1987).

Control rates of fluid secretion were measured by averaging the changes in secreted droplet volume over six 5-min intervals. Subsequently, 20 μl of the bathing Ringer was replaced with Ringer containing leucokinin-VIII, and experimental rates were measured as the average change in secreted volume over the next six 5-min intervals (Williams & Beyenbach, 1983).

Electron Probe Analysis of Fluid Secreted by Isolated Malpighian Tubules

The elemental composition of secreted fluid was measured by electron probe microdroplet analysis as described previously (Williams & Beyenbach, 1983). Briefly, five approximately 200-pl replicates of each collected fluid and corresponding bathing Ringer sample were crystallized on a polished beryllium surface and analyzed against Na, K, and Cl standards on the same surface. The analysis was done with a JOEL 733 superprobe using wavelength-dispersive spectroscopy. All standard solutions were prepared to resemble Ringer solution as much as possible in order to minimize measurement artifacts due to different crystal sizes in the dehydrated fluid samples. The isosmotic replacement of Na with K in Ringer solution (in 25 mM increments) yielded Na and K standards. The isosmotic replacement of Cl with isethionate yielded a series of Cl standards. Standard curves had good linearities with correlation coefficients of 0.99, 0.97, and 0.99 for, respectively, Na, K, and Cl.

Electrophysiological Measurements in Isolated Perfused Malpighian Tubules

Malpighian tubules were perfused in vitro as described previously by Williams and Beyenbach, 1984 (Fig. 2*a*). After attaching the tubule to the holding pipettes, the lumen was cannulated with a double-barreled perfusion pipette (Theta-Borosilicate glass, 1402401, Hilgenberg, Malsfeld, Germany). One barrel was used to perfuse the tubule lumen with Ringer solution and to measure the transepithelial voltage (V_T) via custom-made high impedance amplifiers ($10^{11}\Omega$) with respect to ground in the peritubular Ringer bath (Fig. 2*a*). The other barrel, also filled with Ringer solution, was used to inject current I_T (200–350 nA) for measurements of the transepithelial electrical resistance (R_T) by cable analysis. The cable analysis yields the tubule length constant, the transepithelial resistance (R_T) and the tubule axial resistance (core resistance). The analysis and the cable equations have been

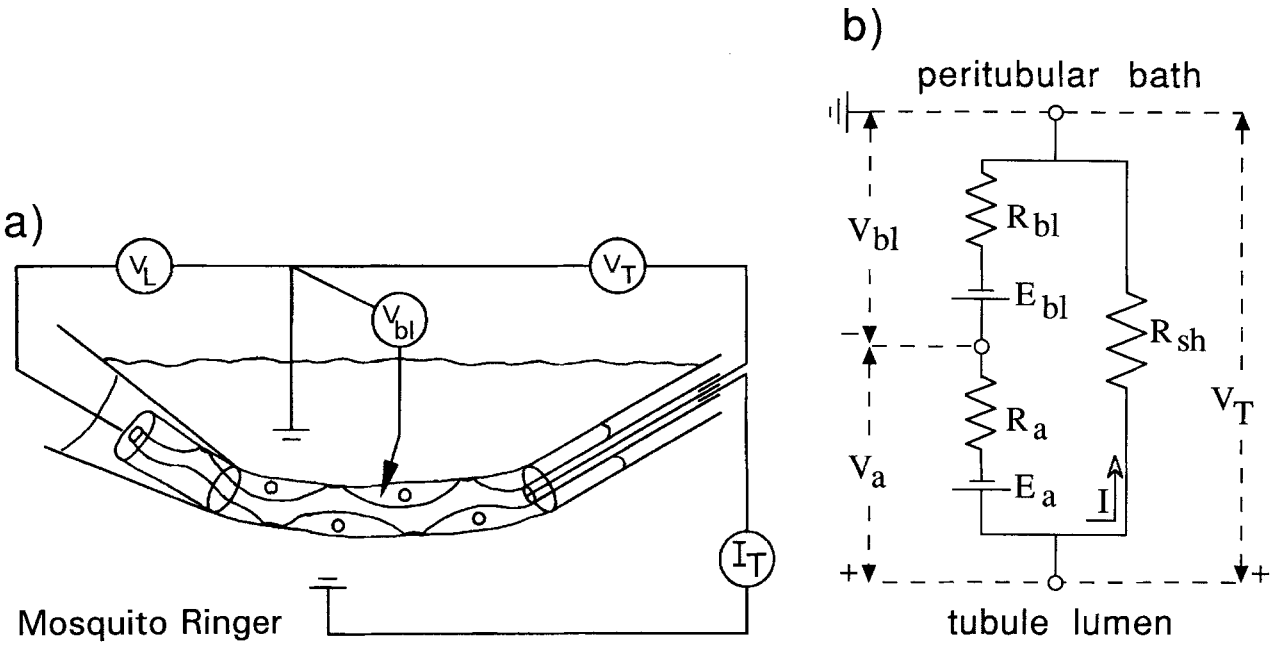

Fig. 2. (*a*) Preparation of Malpighian tubules of *A. aegypti* for in vitro microperfusion by the method of Burg et al. (1966). Experiments were always started by perfusing the tubule lumen with the same mosquito Ringer solution present in the peritubular bath. The transepithelial voltage V_T was measured via one barrel of a double-barreled perfusion pipette which also served to perfuse the tubule lumen. Transepithelial current (I_T) was injected into the tubule lumen via the other barrel of the perfusion pipette to measure the transepithelial resistance from the consequent voltage deflections ΔV_T and ΔV_L at, respectively, the proximal and distal end of the perfused segment. The basolateral membrane voltage (V_{bl}) of principal cells (with large central nuclei) was measured with a conventional microelectrode. Stellate cells are indicated as the short and thin structures between principal cells. (*b*) Electrical analog model of transepithelial electrolyte transport across Malpighian tubules of *A. aegypti* (after Ussing and Windhager, 1964). Active transepithelial secretion of Na and K through principal cells is represented by the transcellular pathway consisting of the resistances (R) and electromotive forces (E) of the basolateral (*bl*) and apical (*a*) membranes of principal cells. Passive transepithelial transport of Cl is represented by the shunt pathway (*sh*) located outside the principal cell. I is intraepithelial current under open-circuit conditions. The shunt pathway has no emf when symmetrical Ringer solution is present in the peritubular bath and tubule lumen. The transepithelial voltage V_T and the basolateral membrane voltage V_{bl} are measured experimentally with respect to ground in the peritubular bath, and the apical membrane voltage V_a is calculated as the difference between these two measured voltages.

described previously by Helman (1972) and by Beyenbach and Frömter (1985).

For measurements of the basolateral membrane voltage (V_{bl}) and the fractional resistance of the apical membrane (f_{R_a}) a principal cell was impaled with a conventional microelectrode at a site near the midpoint of the isolated perfused segment (Fig. 2*a*). The microelectrodes were pulled from borosilicate glass capillaries (1.0 mm OD, with fiber, WPI, Sarasota, FL) on a Flaming/Brown Micropipette Puller (Model P-87, Sutter Instrument, Novato, CA) and filled with 850 mM K-acetate and 150 mM KCl. Microelectrode resistances ranged between 90 and 140 MΩ. Microelectrode measurements were only accepted if (i) the voltage measurement did not drift towards zero mV by more than 1 mV/min, and (ii) the voltage returned to ±3 mV of ground potential after withdrawal from the cell. The fractional resistance of the apical membrane (f_{R_a}) was calculated using the cable equations derived previously (Beyenbach & Frömter, 1985).

The tubule lumen was perfused through the voltage-sensing barrel at a rate less than 5 nl/min (Fig. 2*a*). The tubule was superfused (bath flow) at a rate of 4 ml/min. Bath volume was 500 μl. A rotary valve system allowed for switching between control and experimental peritubular bathing media. In experiments requiring rapid changes of the Cl concentrations the speed of the extracellular solution change was compromised by the need to maintain the microelectrode impalement of principal cells. The Ringer solution perfusing the tubule lumen could be changed in less than 2 sec without jeopardizing the microelectrode impalement. Ringer solution changes in the peritubular bath took longer, but usually less than 10 sec. These precautions allowed us to hold a single microelectrode impalement for up to 2 hr in some experiments (*see* Fig. 5) while the solutions in the peritubular bath and tubule lumen were repeatedly changed.

Ag/AgCl bridges were used at all fluid/wire interfaces. In addition a 4% agar-Ringer bridge was used at the electrode measuring transepithelial voltage (V_T, lumen) and at the ground electrode (peritubular bath). In experiments involving Cl concentration changes in the peritubular bath or tubule lumen, all voltages were corrected for electrode potentials measured separately with free-flowing (broken-tip) microelectrodes containing 1 M KCl.

All voltages were acquired on line, digitized, and stored on an IBM-XT computer. A custom-designed software program solved the cable equations yielding a printout of raw and analyzed data while the experiment was in progress. In addition, transepithelial and membrane voltages were monitored continuously on a Gould Brush Strip Chart recorder (Model Mark 220, Gould, Cleveland, OH). Transepithelial resistance and membrane fractional resistance were measured at least every other minute.

The electrophysiological data were analyzed according to the analog electrical circuit shown in Fig. 2*b*. The analog model is an extension of the model proposed by Ussing and Windhager (1964) which considers parallel active and passive transport pathways across the epithelium. In the case of the Malpighian tubule the active transport pathway through the principal cell consists of (i) a basolateral membrane with resistance R_{bl} in series with the electromotive force E_{bl}, and (ii) an apical (luminal, brush border) membrane (Fig. 1) with resistance R_a in series with the electromotive force E_a. In parallel to this transcellular pathway is the shunt pathway with resistance R_{sh}. The transcellular resistance R_{cell} is the sum of the basolateral and apical membrane resistances ($R_a + R_{bl}$), and the transepithelial resistance R_T is the parallel resistance of the R_{cell} and R_{sh}. It follows that R_T approaches R_{sh} as R_{cell} increases to high values during inhibition of active transport.

Statistical Analysis

Each tubule was used as its own control. Accordingly, the data were analyzed by the paired Student's *t*-test. When paired comparisons were not possible the *t*-test for the significance of the difference of sample means was used. When several experimental treatments were compared to a single control, one-way analysis of variance was applied, and the significance of the differences of the means was determined with Duncan's multiple range test.

Results

Effects of Leucokinin-VIII on Transepithelial Secretion of Fluid and Electrolytes

Isolated Malpighian tubules of *A. aegypti* spontaneously secrete fluid in vitro (Williams & Beyenbach, 1983). In the present study the tubules secreted fluid at a rate of 0.49 nl/min when bathed in Ringer solution containing 150 mM NaCl and 3.4 mM KCl (Fig. 1, Table 1). Under these conditions the secreted Na, K and Cl concentrations were, respectively, 141.8 mM, 64.9 mM, and 204.9 mM (Table 1). The addition of leucokinin-VIII (10^{-6} M) to the peritubular Ringer solution significantly increased the rate of transepithelial fluid secretion to 0.91 nl/min (Table 1). In parallel, the concentration of Na in secreted fluid significantly dropped to 100.5 mM, the K concentration significantly increased to 84.8 mM, and the Cl concentration remained unchanged (Table 1).

The product of the rate of fluid secretion and the ion concentration in secreted fluid is the ion secretion rate. Table 1 shows that leucokinin-VIII significantly increased the transepithelial secretion rates of all three ions.

Effects of Leucokinin-VIII on Tubule Electrophysiology

Effects from the Peritubular Side

Under control symmetrical perfusion of the tubule, i.e., with Ringer solution perfusing the peritubular bath and tubule lumen, Malpighian tubules of *A. aegypti* invariably generate lumen-positive, transepithelial voltages of appreciable magnitude (Williams & Beyenbach, 1984; Beyenbach & Petzel, 1987). In the single, representative tubule shown in Fig. 3 the transepithelial voltage was 60 mV (lumen positive) and the transepithelial resistance was 62 Ωcm^2. At the same time the basolateral membrane

Table 1. The effects of leucokinin-VIII (10^{-6} M) on transepithelial electrolyte and fluid secretion in isolated Malpighian tubules of *Aedes aegypti*

	Transepithelial fluid and electrolyte secretion				
	(*n*)	Fluid (nl/min)	Na (pmol/min)	K (pmol/min)	Cl (pmol/min)
Control	(10)	0.49 ± 0.04	69.9 ± 8.4	29.3 ± 3.3	100.1 ± 8.7
Leucokinin-VIII	(10)	0.91 ± 0.08	96.0 ± 16.2	72.5 ± 9.3	177.9 ± 16.8
P		<0.0001	<0.05	<0.001	<0.001

	Concentration in secreted fluid			
	(*n*)	Na (mM)	K (mM)	Cl (mM)
Control	(10)	141.8 ± 9.8	64.9 ± 9.1	204.9 ± 4.7
Leucokinin-VIII	(10)	100.5 ± 10.7	84.8 ± 11.2	193.5 ± 3.5
P		<0.0001	<0.002	NS

Mean ± SE; *n* = number of tubules; statistical significance was evaluated with the paired Student's *t*-test.

Table 2. The effects of leucokinin-VIII (10^{-6} M) on transepithelial electrophysiological variables in isolated perfused Malpighian tubules of *Aedes aegypti*

	V_T (mV)	R_T (Ωcm^2)	V_{bl} (mV)	V_a (mV)	f_{R_a}
Control	59.3 ± 3.0	57.8 ± 3.4	−64.2 ± 2.6	123.4 ± 2.8	0.28 ± 0.02
Leucokinin-VIII	5.7 ± 0.9	9.9 ± 1.3	−91.8 ± 2.1	96.6 ± 2.1	0.54 ± 0.03
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean ± SE; $n = 29$ tubules; V_T, transepithelial voltage; R_T, transepithelial resistance; V_{bl}, basolateral membrane voltage; V_a, apical membrane voltage; f_{R_a}, fractional resistance of the apical membrane; statistical significance was evaluated with the paired Student's *t*-test.

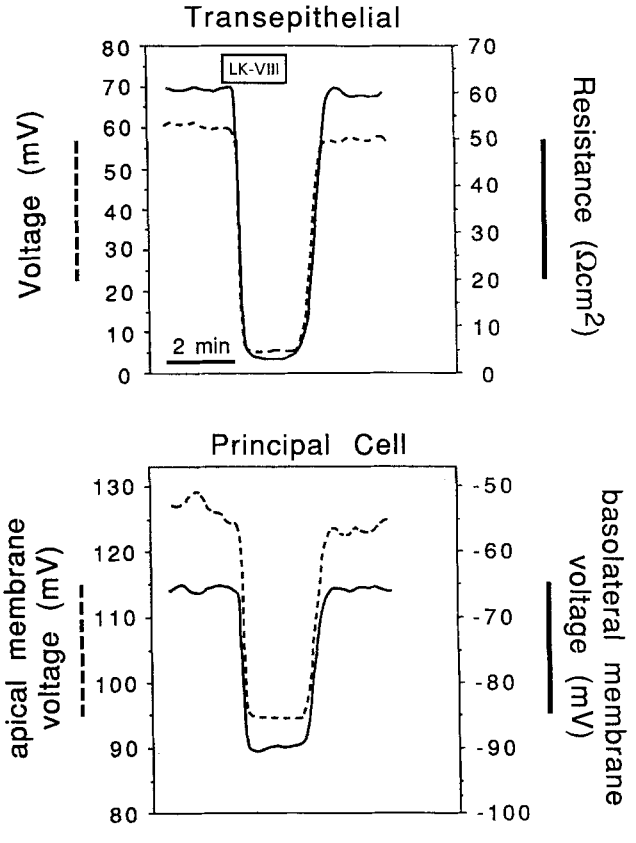

Fig. 3. The effects of leucokinin-VIII (LK-VIII, 10^{-6} M) on the electrophysiological variables of an isolated perfused Malpighian tubule of *A. aegypti*. The tubule was prepared for study as shown in Fig. 2 and perfused in vitro with Ringer solution in the peritubular bath and tubule lumen.

voltage was −65 mV and the apical membrane voltage was 125 mV. When the peritubular Ringer solution was changed to include leucokinin-VIII (10^{-6} M) all measured electrophysiological variables changed in parallel (Fig. 3). Most noticeable were the large changes of the transepithelial voltage and resistance. The transepithelial voltage depolarized from 60 to 5 mV, and the transepithelial resistance fell from 61 to 3 Ωcm^2. At the same time the basolateral membrane voltage hyperpolarized from 65 to 90 mV, and the apical membrane voltage depolarized from 126 to 95 mV. Transepithelial voltage and transepithelial resistance remained low as long as leucokinin-VIII was present in the peritubular bath. The effects of leucokinin-VIII were readily reversible upon washout of the octapeptide (Fig. 3). All measured variables returned to their control values when bath flow was changed to leucokinin-free Ringer (Fig. 3). No evidence for tachyphylaxis was observed; leucokinin-VIII could be repeatedly added and removed with no evidence for diminished responsiveness (*data not shown*).

What was observed in the single tubule shown in Fig. 3 was observed in every tubule treated with leucokinin-VIII (Table 2). Each measured electrophysiological variable changed with a high level of statistical significance after addition of leucokinin-VIII to the peritubular Ringer bath.

No Effects from the Luminal Side

Figure 4 illustrates the polarity of the leucokinin-VIII effect. Transepithelial voltage and resistance were measured in this experimental series using 13 Malpighian tubules. Throughout the experiment the tubules were perfused and bathed with Ringer solution. Under control conditions the transepithelial voltage measured 47.3 ± 5.9 mV, and the transepithelial resistance was 40.9 ± 8.5 Ωcm^2 (Fig. 4). When the Ringer solution perfusing the tubule lumen was changed to include leucokinin-VIII (10^{-6} M) the transepithelial voltage and resistance did not change and remained unchanged when the peritubular bath was returned to the control condition (Fig. 4). In contrast, when leucokinin-VIII was added to the peritubular bath, the transepithelial voltage significantly depolarized from 45.5 ± 5.9 to 2.7 ± 0.3 mV and the transepithelial resistance significantly decreased from 42.1 ± 5.9 to 2.6 ± 0.6 Ωcm^2.

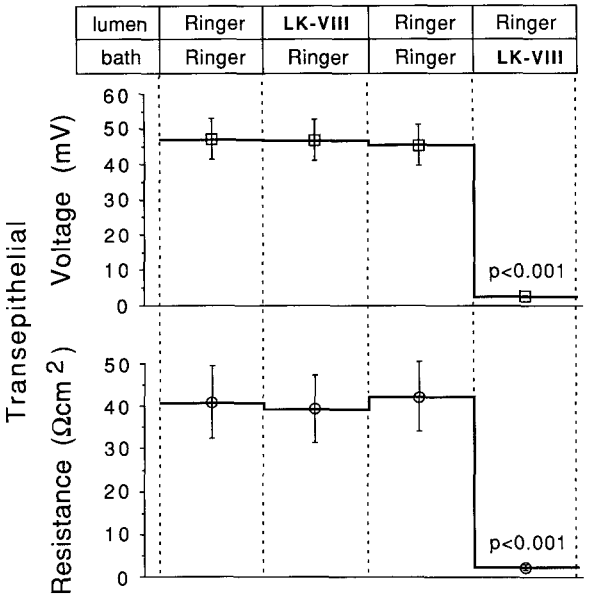

Fig. 4. The effects of leucokinin-VIII (LK-VIII) applied from the luminal side and peritubular side on the transepithelial voltage and resistance of Malpighian tubules perfused and bathed with Ringer solution. After control periods lasting up to 15 min LK-VIII (10^{-6} M) was added either to the perfusate flowing through the tubule lumen or to the peritubular bath. Mean ± SE of 13 Malpighian tubules.

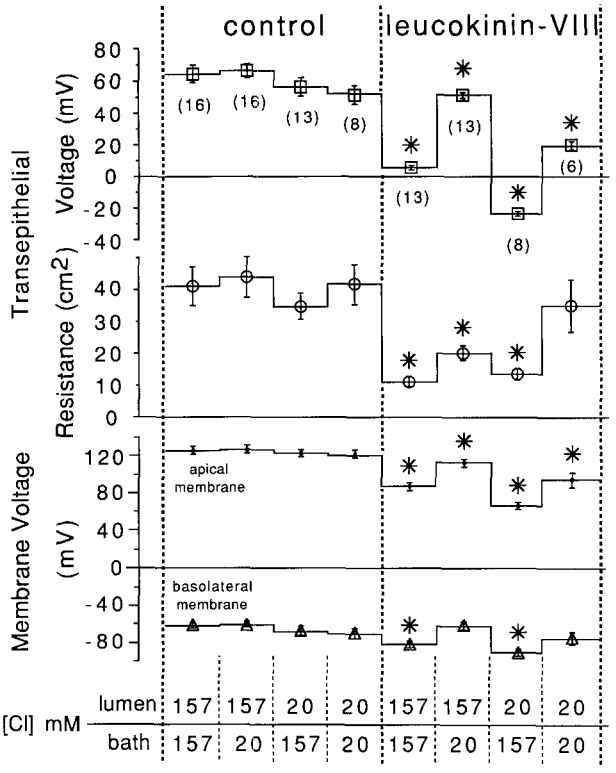

Fig. 5. Effects of extracellular Cl concentration on transepithelial voltage and resistance and on apical and basolateral membrane voltages of principal cells in isolated perfused Malpighian tubules in the absence and presence of leucokinin-VIII (10^{-6} M). Each of 16 Malpighian tubules was taken through the experimental sequence depicted on the abscissa from left to right. Of these, only six tubules were taken through all eight experimental steps, each lasting 7 min or less. Mean ± SE; (number of tubules); * $P <$ 0.05 or less, with reference to the same transepithelial Cl gradient under control conditions.

Transepithelial Cl Diffusion Potentials in the Intact Epithelium

Previous studies have shown that rapid depolarizations of the transepithelial voltage V_T like those observed after peritubular addition of leucokinin-VIII (Figs. 3,4; Hayes et al., 1989) or crude mosquito head extract (Williams & Beyenbach, 1984) were dependent on the Cl concentration in the peritubular bath. In brief, in the presence of low peritubular Cl concentrations (10 mM) the depolarization of the transepithelial voltage triggered by leucokinin-VIII was markedly blunted: ΔV_T was only 6 mV compared to 33 mV in the presence of normal bath Cl concentrations (Hayes et al., 1989). To further investigate the Cl dependence of the electrophysiological effects of leucokinin-VIII, we measured the effects of unilateral and bilateral reductions of the Cl concentration in the peritubular bath and tubule lumen on (i) transepithelial voltage and resistance and (ii) apical and basolateral membrane voltages of principal cells (Fig. 5).

Under control conditions, i.e., in the absence of leucokinin-VIII, unilateral and bilateral changes of the Cl concentration in the tubule lumen or the peritubular bath had negligible, statistically insignificant effects on tubule electrophysiology (Fig. 5). Reductions of the Cl concentration from 157 to 20 mM in the peritubular bath hyperpolarized the transepithelial voltage by only 2 mV, amounting to only 4% of the Cl equilibrium potential (Fig. 5). Reduction of the Cl concentration in the tubule lumen depolarized the transepithelial voltage by only 8 mV, amounting to 15% of the Nernst Cl equilibrium potential (Fig. 5). These effects are statistically insignificant as are the effects on transepithelial resistance and apical and basolateral membrane voltages (Fig. 5). Likewise, the reductions of the Cl concentration in the tubule lumen had insignificant effects on voltage and resistance. Furthermore, bilateral reductions of the Cl concentration in the peritubular Ringer and tubule lumen elicited no significant effects on the measured voltages and resistances (Fig. 5).

The addition of leucokinin-VIII (10^{-6} M) to the peritubular Ringer elicited major changes (Fig. 5):

the transepithelial voltage depolarized from 54.4 ± 5.5 to 5.7 ± 1.6 mV ($P < 0.001$), the transepithelial resistance dropped from 41.0 ± 6.0 to 11.2 ± 1.6 Ωcm^2 ($P < 0.001$), the apical membrane voltage depolarized from 125.8 ± 4.8 to 86.9 ± 4.5 mV ($P < 0.001$), and the basolateral membrane voltage hyperpolarized from −61.4 ± 3.4 to −81.2 ± 3.4 mV ($P < 0.001$). Again, basolateral and apical membrane voltages became nearly equipotential in the presence of leucokinin-VIII (Fig. 5).

In the presence of leucokinin-VIII, changes of the Cl concentration in the Ringer solutions now had significant effects on tubule electrophysiology (Fig. 5). As the bath Cl concentration was reduced from 157 to 20 mM the transepithelial voltage hyperpolarized from 5.7 ± 1.6 to 51.4 ± 1.8 mV, amounting to 85% of the transepithelial Cl equilibrium potential expected from a single barrier ideally permeable to Cl (Nernst equilibrium). In parallel with the hyperpolarization of the transepithelial voltage the apical membrane voltage significantly hyperpolarized with a ΔV_a of 25.6 mV (from 86.9 ± 4.5 to 112.5 ± 4.1 mV) and the basolateral membrane voltage significantly depolarized with a ΔV_{bl} of 20.1 mV from −81.2 ± 3.4 to −61.1 ± 4.0 mV (Fig. 5).

Reversal of the transepithelial Cl gradient produced by the unilateral reduction of the Cl concentration in the tubule lumen reversed the polarity of the transepithelial voltage to a lumen-negative transepithelial voltage of −23.5 ± 2.7 mV (Fig. 5). The significant change in voltage, ΔV_T −29.2 mV, amounts to 54% of the Cl Nernst equilibrium potential (Fig. 5). The apical and basolateral membrane voltages reversed their responses as well. The apical membrane voltage now depolarized with a ΔV_a of 18.2 mV and the basolateral membrane voltage now hyperpolarized with a ΔV_{bl} of 11.0 mV (Fig. 5).

In the presence of leucokinin-VIII, bilateral reductions of the Cl concentration in the tubule lumen and peritubular bath tended to move all measured variables towards control values observed in the absence of leucokinin-VIII. The transepithelial voltage repolarized to 19.7 ± 2.6 mV, the apical membrane voltage repolarized to 94.3 ± 7.8 mV, and the basolateral membrane voltage repolarized to −74.7 ± 6.4 mV. Most noticeable was the change in transepithelial resistance. Whereas unilateral reductions of the Cl concentration in either bath or lumen increased the transepithelial resistance from 11.2 ± 1.6 Ωcm^2 to, respectively, 20.1 ± 2.3 Ωcm^2 and 13.6 ± 1.9 Ωcm^2, the bilateral Cl reduction caused the transepithelial resistance to return to 35.1 ± 8.3 Ωcm^2 approaching values that are measured in the absence of leucokinin-VIII under control conditions (Fig. 5). Hence, the bilateral reduction of the Cl concentration to 20 mM fully reversed the effects of leucokinin-VIII on transepithelial resistance and partially reversed the effects of leucokinin-VIII on transepithelial voltage and the apical and basolateral membrane voltages of principal cells.

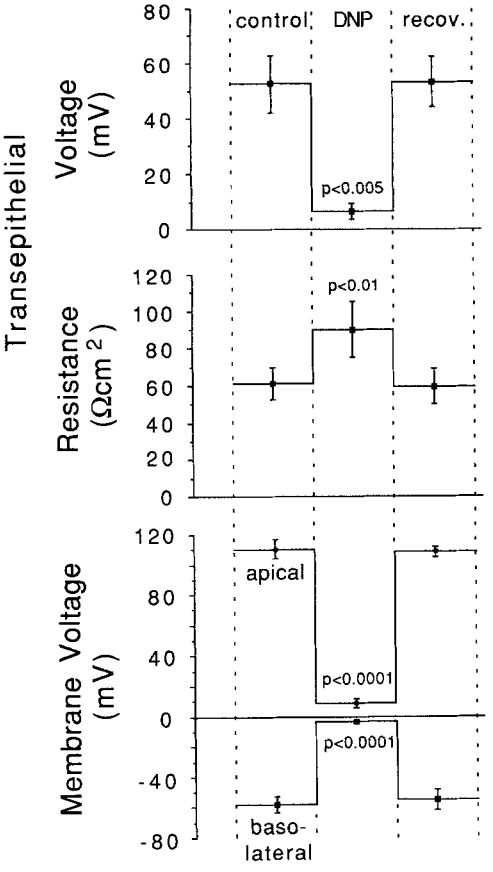

Fig. 6. The effects of dinitrophenol (DNP, 10^{-4} M) on transepithelial voltage and resistance and the apical and basolateral membrane voltages of isolated perfused Malpighian tubules. Mean ± SE of seven tubules. Measures of the transepithelial resistance in the presence of DNP are taken as estimates of the shunt resistance. Control, experimental, and recovery periods each lasted 10 min or less.

Effects of Dinitrophenol on Tubule Electrophysiology

The marked reduction of the transepithelial resistance, the depolarization of the transepithelial voltage to values close to 0 mV (Figs. 3–5; Table 2) and the large transepithelial Cl diffusion potentials in the presence of leucokinin-VIII (Fig. 5) suggested that leucokinin-VIII increased the Cl conductance of the shunt pathway (Fig. 2*b*). However, the effects of leucokinin-VIII on the fractional resistance of the apical membrane (which is independent of the

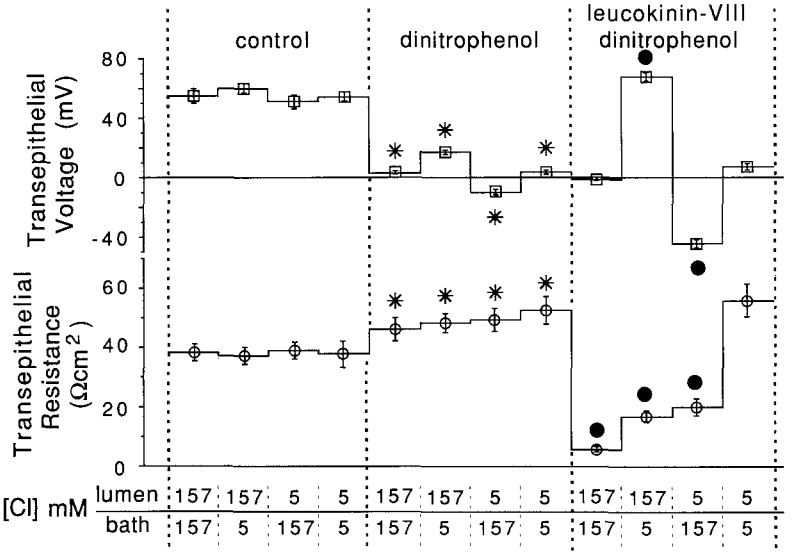

Fig. 7. Effects of extracellular Cl concentration on transepithelial voltage and resistance under control conditions and in the presence of dinitrophenol (DNP, 10^{-4} M) and dinitrophenol plus leucokinin-VIII (10^{-6} M). Each of 10 Malpighian tubules was taken through the experimental sequence indicated on the abscissa from left to right. Each experimental period lasted 7 min or less. Measures of the transepithelial resistance in the presence of DNP are taken as estimates of the shunt resistance. Mean ± SE; * $P < 0.05$ or less with reference to the same transepithelial Cl gradient under control conditions; • $P < 0.01$ or less with reference to the same Cl gradient in the presence of dinitrophenol.

shunt pathway) also indicated an effect on the membranes of principal cells (Table 2). To distinguish between effects of leucokinin-VIII on the shunt and principal cells, we sought ways to reduce the effects of leucokinin-VIII on the active transport pathway of principal cells. From the choices available to block active transport across principal cells we decided on the use of dinitrophenol. In addition to inhibiting transepithelial electrolyte and fluid secretion (Pannabecker, Aneshansley & Beyenbach, 1992) dinitrophenol (10^{-4} M) added to the peritubular bath nearly eliminated all voltages measured across the membranes of principal cells and across the whole epithelium (Fig. 6). The transepithelial voltage significantly depolarized from 52.6 ± 10.4 to 6.6 ± 2.9 mV ($P < 0.005$), the apical membrane voltage significantly depolarized from 110.6 ± 6.3 to 8.9 ± 3.1 mV ($P < 0.001$), and the basolateral membrane voltage significantly depolarized from −58.0 ± 5.2 to −3.3 ± 1.8 mV ($P < 0.0001$) in the seven tubules studied. In parallel, the transepithelial resistance significantly increased from 61.2 ± 8.6 to 90.3 ± 15.0 Ωcm² ($P < 0.01$). The effect of DNP on the fractional resistance of the apical membrane, which increased from 0.32 ± 0.04 to 0.57 ± 0.07 (*not shown* in Fig. 6) was also highly significant ($P < 0.001$). All effects of DNP were fully reversible upon washout of DNP from the peritubular bath: the transepithelial voltage repolarized to 53.2 ± 9.1 mV, the apical membrane voltage repolarized to 108.6 ± 3.5 mV, and the basolateral membrane voltage repolarized to −55.4 ± 6.8 mV (Fig. 6). In parallel, the transepithelial resistance returned to 59.6 ± 9.3 Ωcm², and the fractional resistance of the apical membrane returned to 0.24 ± 0.05.

Transepithelial Cl Diffusion Potentials in Dinitrophenol-Treated Tubules

The virtual absence of membrane voltages in principal cells treated with DNP suggest that active transport across these cells is inhibited. Inhibition of transcellular Na and K would be expected to increase the resistance of the transcellular pathway (R_{cell}) such that measures of the transepithelial resistance approach the shunt resistance R_{sh}. In previous studies we have shown that R_{cell} needs to increase only four times for measures of the transepithelial resistance to estimate R_{sh} (Pannabecker et al., 1992). In the present study we reasoned that during inhibition of active transport with DNP transepithelial Cl diffusion potentials reflect primarily the properties of the shunt pathway. Accordingly, transepithelial Cl diffusion potentials were measured in 10 isolated perfused Malpighian tubules, first under control conditions, then in the presence of dinitrophenol, and finally in the presence of dinitrophenol plus leucokinin-VIII (Fig. 7). As was observed in Fig. 5, reductions of the Cl concentration in the peritubular bath, the tubule lumen, or both, had negligible effects on transepithelial voltage and resistance under control conditions (Fig. 7). The addition of dinitrophenol (10^{-4} M) to the peritubular bath significantly depolarized the transepithelial voltage from 54.4 ± 3.1 to 3.3 ± 0.8 mV and increased the transepithelial resistance from 37.7 ± 4.5 to 46.2 ± 3.9 Ωcm² (Fig. 7). In the presence of dinitrophenol, reductions of the Cl concentration in the peritubular bath and tubule lumen now elicited transepithelial diffusion potentials that were larger than those observed in the absence of

dinitrophenol (Fig. 7). Cl diffusion gradients from lumen to bath significantly hyperpolarized the transepithelial voltage from 3.3 ± 0.8 to 17.1 ± 1.2 mV; the reversal of the transepithelial Cl diffusion gradient depolarized the transepithelial voltage from 3.6 ± 1.2 to −9.4 ± 1.8 mV (Fig. 7). These transepithelial Cl diffusion potentials were symmetrical: ΔV_T was 13.8 mV and 13.0 mV amounting to ~15% of the Cl equilibrium potential. Unilateral and bilateral reductions of the Cl concentrations tended to increase the transepithelial resistance in the presence of dinitrophenol but the changes were not statistically significant among DNP-treated tubules, though they were significantly different from their respective controls in the absence of DNP (Fig. 7).

Since the transepithelial voltage is already close to zero mV in DNP-treated tubules, the usual depolarization of the transepithelial voltage by leucokinin-VIII (Figs. 3–5) is not observed upon addition of leucokinin-VIII to the peritubular bath (Fig. 7). However, leucokinin-VIII still displayed a potent effect on transepithelial resistance which significantly dropped from 52.5 ± 4.7 to 5.8 ± 0.9 Ωcm^2 ($P < 0.001$; Fig. 7).

In the presence of DNP and leucokinin-VIII, transepithelial Cl diffusion gradients now elicited large transepithelial diffusion potentials (Fig. 7). Upon the unilateral reduction of the Cl concentration in the peritubular bath, the transepithelial voltage changed from −1.2 ± 1.0 to 67.8 ± 2.6 mV. The ΔV_T, 69 mV, came to 77% of the Cl equilibrium potential (Fig. 7). Reversal of the transepithelial Cl diffusion gradient depolarized the transepithelial voltage to −44.2 ± 3.1 mV. The ΔV_T, 51.4 mV, amounted to 57% of the Cl equilibrium potential (Fig. 7). The unilateral reduction of the Cl concentration in the peritubular bath significantly increased the transepithelial resistance from 5.8 ± 0.9 to 16.9 ± 1.9 Ωcm^2 (Fig. 7). Transepithelial resistance significantly increased to 20.1 ± 2.9 Ωcm^2 upon the unilateral reduction of the Cl concentration in the tubule lumen. However, the largest increase in transepithelial resistance was observed upon the bilateral reduction of the Cl concentration. Transepithelial resistance increased to 55.8 ± 5.5 Ωcm^2, which is not significantly different from the resistance measured in the absence of leucokinin-VIII (Fig. 7).

Discussion

Transepithelial Effects of Leucokinin-VIII

The leucokinins were first isolated in the laboratory of Holman who found that all octapeptides of this family increase the motility of the insect intestine (Holman et al., 1986*a*,*b*, 1987*a*,*b*). The leucokinins were subsequently found in our laboratory to stimulate the secretion of electrolytes and water in Malpighian tubules of the mosquito *A. aegypti* (Hayes et al., 1989). Isolated from the cockroach *Leucophaea,* the effects of natural and synthetic leucokinin in the mosquito *Aedes* suggest that the leucokinins are widely distributed in insects. Factor II, a peptide which we have partially isolated from mosquito heads (Petzel, Hagedorn & Beyenbach, 1985, 1986), may indeed contain the basic leucokinin structure because its effects on (i) transepithelial electrolyte and fluid secretion, and (ii) transepithelial voltage and resistance are identical to those of synthetic leucokinin-VIII observed in previous (Petzel et al., 1985) and present studies. Furthermore, the effects of Factor II are dependent on the Cl concentration in the peritubular medium like those of leucokinin-VIII (Hayes et al., 1989).

In the present study leucokinin-VIII is shown to increase the rate of transepithelial fluid secretion by increasing the rates of both NaCl and KCl secretion (Table 1). In contrast, cAMP (Williams & Beyenbach, 1983, 1984) and mosquito natriuretic factor (Beyenbach & Petzel, 1987; Petzel et al., 1987) increase the rate of only NaCl secretion. Hence, the stimulation of both NaCl and KCl secretion by leucokinin-VIII appears to be nonspecific when viewed from the perspective of active transport of Na and K across principal cells. However, viewed from the perspective of transepithelial Cl secretion leucokinin-VIII has a specific effect. By selectively increasing the Cl conductance of the shunt pathway, leucokinin-VIII makes Cl, the counterion of active Na and K transport, more readily available for secretion with the effect of increasing the rates of transepithelial secretion of both NaCl and KCl. This effect of leucokinin-VIII is predicted in the analysis of the electrical analog model of Malpighian tubules (Fig. 2*b*). Since active cation transport is electrically coupled to passive anion transport, an increase in the conductance of the anion pathway (shunt) increases current flowing through the circuit. Hence, the rates of transepithelial cation and anion transport increase (Fig. 2*b*).

The effect of leucokinin-VIII on the shunt pathway is supported by its effects on transepithelial voltage and resistance. The addition of leucokinin-VIII to the peritubular bath leads to the precipitous drop in transepithelial voltage and resistance (Fig. 3, Table 2). To our knowledge, there are no parallels in epithelial transport physiology where an extracellular messenger changes transepithelial voltage and resistance as dramatically as leucokinin-VIII. Analysis of the electrical analog circuit of the Malpighian tubule (Fig. 2*b*) predicts that a

large increase in the shunt conductance causes (i) large reductions of the transepithelial voltage and resistance, (ii) increased intraepithelial current flows, (iii) hyperpolarization of the basolateral membrane voltage, and (iv) depolarization of the apical membrane voltage. The experimental data match these predictions: In the presence of leucokinin transepithelial voltage drops from 60 to 6 mV, transepithelial resistance drops from 58 to 10 Ωcm^2, and increased intraepithelial currents hyperpolarize the basolateral membrane voltage from −64 to −92 mV and depolarize the apical membrane voltage from 123 to 97 mV (Fig. 3, Table 2).

Since low transepithelial voltage and resistance and nearly equipotential basolateral and apical membranes are the properties of leaky epithelia with low shunt resistances (Figs. 2*b*, 3, 5; Table 2), the experimental data suggest that leucokinin-VIII changes the Malpighian tubule from a moderately tight epithelium to a leaky, nearly self-shortcircuited epithelium by increasing the Cl conductance of the shunt pathway. The data also indicate effects on the active transport pathway since leucokinin-VIII increases the fractional resistance of the apical membrane (Table 2) which is independent of the shunt resistance. However, the changes in membrane fractional resistance cannot account for all changes in tubule electrophysiology which leaves an effect on the shunt conductance as the simplest hypothesis consistent with the data.

Searching for the Site of the Increase in Chloride Conductance

To test the hypothesis that leucokinin-VIII increases the Cl conductance of the shunt pathway direct measurements across the shunt pathway were desired. Unfortunately, measurements across the shunt pathway alone are not possible because every transepithelial measurement reflects the contributions of both cellular and shunt pathways in parallel to each other (Fig. 2*b*). We have attempted to deal with this problem by the process of elimination: if it can be shown that leucokinin-VIII does not increase the Cl conductance in the basolateral and apical membranes of principal cells, then an effect on the shunt pathway would be indicated. Accordingly, membrane Cl conductances were assessed in principal cells in the absence (control) and presence of leucokinin-VIII.

The absence of appreciable effects of unilateral and bilateral Cl concentration step changes on the apical and basolateral membrane voltages of principal cells indicate that the Cl conductance of these two membranes is very low (Fig. 5). Likewise, the effect of Cl concentration step changes on transepithelial voltage and resistance were small, statistically insignificant (Fig. 5), and consistent with a low Cl conductance of the shunt pathway. Treatment of the tubules with leucokinin-VIII markedly changed the response to transepithelial Cl gradients (Fig. 5). The unilateral reduction of the Cl concentration in the peritubular bath hyperpolarized the transepithelial voltage from 6 to 51 mV. The voltage change, 45 mV, is consistent with a transepithelial Cl diffusion potential across a single barrier (shunt?) reaching 84% of the Nernst equilibrium potential. The reversal of transepithelial Cl gradients yielded a lumen-negative voltage of −24 mV, consistent again with a transepithelial Cl diffusion potential across a single barrier. Significantly, the voltage changes taking place at the apical and basolateral membranes of principal cells are much smaller than the changes in transepithelial voltage (Fig. 5). Voltage changes across the whole epithelium that are much greater than those across principal cell membranes identify transepithelial Cl diffusion potentials across the shunt as the source of these changes.

According to the analog circuit model (Fig. 2*b*) a Cl diffusion potential of 45 mV across the shunt consequent to the reduction of the bath Cl concentration, would be expected to increase intraepithelial current flow leading to "*IR*" voltage changes that will depolarize the basolateral membrane voltage and hyperpolarize the apical membrane voltage, effects which are indeed observed (Fig. 5). Alternatively, the reversal of the Cl diffusion potential across the shunt, consequent to the unilateral reduction of the Cl concentration in the tubule lumen, will add a reverse component of intraepithelial current flow, leading to "*IR*" voltages that will now have opposite effects on membrane voltages: the hyperpolarization of the basolateral membrane voltage and the depolarization of the apical membrane voltage, two effects which again are supported by the data (Fig. 5).

Examining the electrical analog model (Fig. 2*b*) for the hypothetical leucokinin-induced increase of the Cl conductance of the basolateral membrane or apical membrane yields predictions that cannot be confirmed by the experimental data. The only electrical analog model which fits the experimental data in their entirety has leucokinin-VIII increasing the Cl conductance of the shunt. This interpretation of the data is supported by the effects of extracellular Cl concentrations on transepithelial resistance (Fig. 5). Unilateral reductions of the Cl concentrations have little effect on transepithelial resistance in the presence of leucokinin-VIII, but bilateral reductions cause transepithelial resistance to increase and return to values measured in the absence of leucokinin-VIII (Fig. 5). Apparently, diffusion of Cl through a highly Cl-permeable shunt adds sufficient

Cl to the shunt so that transepithelial resistance is not affected when the Cl concentration on just one side of the epithelium is lowered. In contrast, reductions of the Cl concentration on both sides of the epithelium appear to remove Cl from the shunt pathway in sufficient quantities to affect the increase in shunt resistance and transepithelial resistance (Fig. 5). The requirement for Cl reductions on both sides of the epithelium is additional evidence that leucokinin-VIII does not affect apical and basolateral membranes of principal cells in a major way.

Estimates of the Shunt Resistance in the Absence and Presence of Leucokinin

In the past, investigators of epithelial transport have estimated the resistance of the shunt pathway by selectively altering the active transport pathway with agents that are not expected to affect the shunt pathway. For example, Yonath and Civan (1971) used vasopressin to vary the short-circuit current (a measure of active transport) and transepithelial conductance across isolated toad bladders. The shunt resistance was then estimated as the transepithelial resistance at zero short-circuit current. Alternatively, we used amiloride to reduce the short-circuit current to zero in isolated perfused renal distal tubules and estimated the shunt resistance as the transepithelial resistance under this condition (Beyenbach et al., 1980). There we assumed that amiloride increases the resistance of the active transport pathway to a high value so that measures of the transepithelial resistance approach the resistance of the shunt (Beyenbach et al., 1980). Here we take a similar approach to estimate the shunt resistance in *Aedes* Malpighian tubules by using dinitrophenol (DNP) to inhibit active transport of Na and K through principal cells, thereby increasing the resistance of the active transport pathway such that measures of R_T approach R_{sh} (Fig. 2*b*).

Dinitrophenol is known to inhibit transepithelial electrolyte and fluid secretion in Malpighian tubules (Pilcher, 1970; Isaacson, Nicolson & Fisher, 1989). In Malpighian tubules of *A. aegypti* the effects of DNP on principal cells are particularly noteworthy: DNP reduces the basolateral membrane voltage from −52 to 3 mV and reduces the apical membrane voltage from 111 to 9 mV (Pannabecker et al., 1992). The virtual elimination of all membrane voltages in principal cells is indicative of the inhibition of active transport of Na and K as DNP collapses proton gradients needed for the synthesis of ATP and the fueling of active transport. Inhibition of active transport through principal cells is expected to increase the transcellular resistance (R_{cell}) as reflected in the increase of the transepithelial resistance (Fig. 6). Provided that R_{cell} increases by a factor of 4 or more, measures of the transepithelial resistance approach the resistance of the shunt (Pannabecker et al., 1992).

In the present study DNP significantly depolarizes the transepithelial voltage to values close to zero mV (Figs. 6, 7). In parallel, the transepithelial resistance significantly increases, revealing 46 Ωcm^2 as the estimate of the shunt resistance (Fig. 7). Since the transepithelial resistance in DNP-treated tubules is largely a measure of the shunt resistance, it follows that transepithelial Cl diffusion potentials measured in DNP-treated tubules reflect primarily the properties of the shunt pathway. Accordingly, symmetrical transepithelial Cl diffusion potentials that are measured across DNP-treated tubules reveal an appreciable shunt Cl conductance in the absence of leucokinin-VIII (Fig. 7). This finding raises the question why transepithelial Cl diffusion potentials are not observed under control conditions in the absence of DNP (Fig. 5). Apparently, Cl diffusion potentials across the shunt can be observed only when the resistance of the transcellular pathway (R_{cell}) is high as in DNP-treated tubules. The potentials appear to be shunted when R_{cell} is low in intact tubules under control conditions. Hence, DNP appears to unmask the normal Cl conductance of the shunt pathway in Malpighian tubules.

The addition of leucokinin-VIII to the peritubular bath of dinitrophenol-treated tubules drops the shunt resistance from 46 Ωcm^2 (control) to 6 Ωcm^2 and dramatically increases transepithelial Cl diffusion potentials (Fig. 7). Whereas in the absence of leucokinin-VIII transepithelial Cl diffusion potentials amount to only 15% of Cl equilibrium potentials, they reach 80% in the presence of leucokinin-VIII (Fig. 7) indicating a clear increase in transepithelial Cl conductance. Moreover, since this increase is observed in DNP-treated tubules the increase in Cl conductance is localized to the shunt. This conclusion is corroborated by estimates of the shunt resistance (Fig. 7). Leucokinin-VIII drops the shunt resistance from 46 to 6 Ωcm^2 in DNP-treated tubules (Fig. 7). This large drop in R_{sh} is reversed when the Cl concentration on both sides of the epithelium is reduced to 5 mM. The bilateral reduction of the Cl concentration is again a necessary condition to reverse the effects of leucokinin-VIII on resistance (Figs. 5, 7).

It is generally accepted that the shunt pathway is the transepithelial route of counter-ion transport. Shunt pathways are thought to have no active transport mechanisms, to merely provide a passive route for the counterion(s) of actively transported ions. Cl

appears to play this role of counterion in NaCl and KCl secretion in Malpighian tubules, where Na and K are moved through the active transport pathway of principal cells and where electrical coupling moves Cl through the passive transport pathway of the shunt (Fig. 2*b*). Accordingly, the data support the conclusion that leucokinin-VIII lowers the rate-limiting barrier to Cl transport in the shunt. Consistent with this conclusion the experimental evidence is summarized as follows: (i) Leucokinin-VIII decreases transepithelial resistance to 17% of control values (Table 2). Such a large drop in transepithelial resistance is expected from a decrease in shunt but not membrane resistance. (ii) Leucokinin-VIII increases the rates of transepithelial NaCl and KCl secretion (Table 1). The increase in the secretion rate of both salts is expected as the shunt increases its anionic permeability to the counterion of active Na and K transport. (iii) The effects of leucokinin-VIII on tubule electrophysiology are dependent on Cl, but Cl conductances in principal cells cannot be identified (Fig. 5). (iv) Transepithelial Cl gradients reveal large transepithelial diffusion potentials in the presence but not the absence of leucokinin-VIII (Figs. 5, 7). The near symmetry of these potentials would not be observed if leucokinin-VIII increased the Cl conductance at one of the membranes of principal cells. (v) Quantitative estimates of the shunt resistance in dinitrophenol-treated tubules show that leucokinin-VIII decreases the resistance of the shunt to 11% of control values (Fig. 7). (vi) In the presence of DNP and leucokinin-VIII high shunt resistances are recovered when Cl is removed from the extracellular solutions, indicating the increase of the shunt Cl conductance (Figs. 5, 7). (vii) Measurements of basolateral and apical membrane voltages of principal cells do not support the hypotheses that leucokinin-VIII increases the Cl conductance at one of these membranes (Fig. 5). While each of these arguments by itself is insufficient to prove the effect of leucokinin-VIII on shunt Cl conductance, together they constitute rather compelling evidence.

Possible Anatomical Sites of the Shunt

Since our studies fail to pinpoint the Cl shunt pathway in principal cells, the location of the shunt must be sought outside principal cells. Two anatomical sites are candidates: the stellate cells and the paracellular pathway. As shown in Fig. 1 the Malpighian tubule of *A. aegypti* consists of two cell types: principal and stellate cells. The principal cells are large, fusiform cells which fold upon themselves forming a lumen. At the points where the lateral edges of principal cells meet they may be joined and held together by small stellate cells (Fig. 1). Their cell height is not more than 5 μm compared to 50 μm as the center height of principal cells. When viewed through the stereo microscope stellate cells are highly transparent like "ghosts," revealing no intracellular structures. Their function and role in transepithelial transport are unknown. Conceivably, stellate cells could be the site where leucokinin-VIII regulates Cl shunt permeability. To do so, both basolateral and apical membranes of stellate cells must be equally permeable (or nearly so) to Cl to behave as the single Cl diffusion barrier evinced in the present study. Alternatively, leucokinin-VIII could regulate the Cl conductance at only one cell membrane while the opposite cell membrane is permanently permeable to Cl.

The paracellular pathway could also be a site where leucokinin-VIII regulates Cl shunt permeability. In the case of Malpighian tubules of *A. aegypti* this paracellular pathway is the extracellular pathway between (i) principal cells, and (ii) principal cells and stellate cells (Fig. 1). As shown in Fig. 1 the paracellular pathway is very short and limited to the septate junctions, the invertebrate analog of tight junctions (Lord & DiBona, 1976). Regulation of paracellular Cl permeability would therefore imply the regulation of septate junctional permeability. Further investigations are necessary to distinguish between stellate cells and the paracellular pathway as the site of the leucokinin-regulated shunt pathway.

This work was supported by a USDA grant 89-37250-4519 awarded to Dr. Pannabecker and by NSF grant DCB 8403305 awarded to Dr. Beyenbach. The experimental work is primarily the effort of Dr. Pannabecker over a three-year period of post-doctoral training in the laboratory of Dr. Beyenbach. The authors would like to thank Mr. David Hinckley, Ms. Faith Andrews, and Ms. Agueda Oviedo for their assistance in some of the experiments and Mr. F. Dräger (MPI, Dortmund, Germany) for assistance in graphical work. We thank Mr. John Hunt and the Department of Materials Sciences, College of Engineering, Cornell University, for the use of the JOEL 733 electron probe, and Dr. George Kidder for stimulating and fruitful discussions. The manuscript was written by K.W. Beyenbach at the Max Planck Institute für Systemphysiologie, Dortmund, Germany, and was supported by the Humboldt Stiftung of Germany.

References

Bertram, G., Schleithoff, L., Zimmermann, P., Wessing, A. 1991. BafilomycinA_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei:* Is a vacuolar type ATPase involved in ion and fluid secretion? *J. Insect Physiol.* **37**:201–209

Beyenbach, K.W., Frömter, E. 1985. Electrophysiological evi-

dence for Cl secretion in shark renal proximal tubules. *Am. J. Physiol.* **248:**F282–295

Beyenbach, K.W., Petzel, D.H. 1987. Diuresis in mosquitoes: role of a natriuretic factor. *News Physiol. Sci.* **2:**1171–1175

Beyenbach, K.W., Koeppen, B.M., Dantzler, W.H., Helman, S.I. 1980. Na concentration and the electrical properties of the snake distal tubules. *Am. J. Physiol.* **239:**F412–F419

Burg, M., Grantham, J., Abramow, M., Orloff, J. 1966. Preparation and study of fragments of single rabbit nephrons. *Am. J. Physiol.* **210:**1293–1298

Cohen, S.A., Tarvin, T.L., Bidlingmeyer, B.A. 1984. Analysis of amino acids using precolumn derivatization with phenylisothiocyanate. *Amer. Lab.* **16:**48–53

Erspamer, V. 1981. The tachykinin peptide family. *Trend. Neurosci.* **4:**267–269

Hayes, T.K., Pannabecker, T.L., Hinckley, D.J., Holman, G.M., Nachman, R.J., Petzel, D.H., Beyenbach, K.W. 1989. Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44:**1259–1266

Helman, S.I. 1972. Determination of electrical resistance of the isolated cortical collecting tubule and its possible anatomical location. *Yale J. Biol. Med.* **45:**339–345

Holman, G.M., Cook, B.J., Nachman, R.J. 1986*a*. Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea madera:* Members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C:**205–211

Holman, G.M., Cook, B.J., Nachman, R.J. 1986*b*. Primary structure and synthesis of two additional neuropeptides from *Leucophaea maderae:* Members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C:**271–276

Holman, G.M., Cook, B.J., Nachman, R.J. 1987*a*. Isolation, primary structure, and synthesis of leucokinins V and VI: Myotropic peptides of *Leucophaea maderae. Comp. Biochem. Physiol.* **88C:**27–30

Holman, G.M., Cook, B.J., Nachman, R.J. 1987*b*. Isolation, primary structure, and synthesis of leucokinins VII and VIII: The final members of this new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae. Comp. Biochem. Physiol.* **88C:**31–34

Holman, G.M., Nachman, R.J., Schoofs, L., Hayes, T.K., Wright, M.S., DeLoof, A. 1991. The *Leucophaea maderae* hindgut preparation: A rapid and sensitive bioassay tool for the isolation of insect myotropins of other insect species. *Insect Biochem.* **21:**107–112

Hudson, D., Kain, K., Ng, D. 1986. High yielding fully automatic synthesis of oecropin A amide and analogues. *In:* Peptide Chemistry. Y. Kiso, editor. pp 413–418. Osaka Protein Research Foundation, Osaka, Japan

Isaacson, L.C., Nicolson, S.W., Fisher, D.W. 1989. Electrophysiological and cable parameters of perfused Malpighian tubules. *Am. J. Physiol.* **257:**R1190–R1198

Lord, B.A.P., DiBona, D.R. 1976. Role of the septate junction in the regulation of paracellular transepithelial flow. *J. Cell. Biol.* **71:**967–972

Maddrell, S.H.P. 1978. Transport across insect excretory epithelia. *In:* Membrane Transport in Biology, G. Giebisch, D.C. Tosteson, H.H. Ussing, editors. pp 239–272. Springer Verlag, New York

Pannabecker, T.L., Aneshansley, D.J., Beyenbach, K.W. 1992. Unique electrophysiological effects of dinitrophenol in Malpighian tubules. *Am. J. Physiol.* **263:**R609–614

Petzel, D., Hagedorn, H.H., Beyenbach, K.W. 1985. Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol.* **249:**R379–R386

Petzel, D., Hagedorn, H.H., Beyenbach, K.W. 1986. Peptide nature of mosquito natriuretic factor. *Am. J. Physiol.* **250:**R328–R332

Petzel, D.H., Berg, M.M., Beyenbach, K.W. 1987. Hormone-controlled, cAMP-mediated fluid secretion in yellow fever mosquito. *Am. J. Physiol.* **253:**R701–R711

Phillips, J. 1981. Comparative physiology of insect renal function. *Am. J. Physiol.* **241:**R241–R257

Phillips, J. 1983. Endocrine control of salt and water balance: secretion. *In:* Endocrinology of Insects, R.G.H. Downer, H. Laufer, editors. pp 411–425. Alan Liss, New York

Pilcher, D. 1970. The influence of the diuretic hormone on the process of urine secretion by the Malpighian tubules of *Carausius morosus. J. Exp. Biol.* **53:**465–484

Pollard, H.B., Creutz, C.E., Pazoles, C.J., Hansen, J. 1977. Caclium-binding properties of monovalent anions commonly used to substitute for chloride in physiological salt solutions. *Anal. Biochem.* **83:**311–314

Ramsay, J.A. 1954. Active transport of water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. Exp. Biol.* **31:**1004–113

Ussing, H.H., Windhager, E.E. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61:**484–504

Wieczorek, H., Weerth, S., Schindlbeck, M., Klein. U. 1989. A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264:**11143–11148

Williams, J.C., Jr., Beyenbach, K.W. 1983. Differential effects of secretagogues on Na and K secretion in Malpighian tubules of *Aedes aegypti. J. Comp. Physiol.* **149:**511–517

Williams, J.C., Jr., Beyenbach, K.W. 1984. Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. *J. Comp. Physiol.* **154:**301–309

Yonath, J., Civan, M.M. 1971. Determination of the driving force for the Na pump in toad bladder by means of vasopressin. *J. Membrane Biol.* **5:**366–385

Received 19 May 1992; revised 9 September 1992