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The mechanism of action of the antidiuretic peptide Tenmo ADFa in Malpighian tubules of *Aedes aegypti*

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Summary

The mechanism of action of *Tenebrio molitor* antidiuretic factor 'a' (Tenmo ADFa) was explored in isolated Malpighian tubules of *Aedes aegypti*. In the Ramsay assay of fluid secretion, Tenmo ADFa (10^{-9} mol l^{-1}) significantly inhibited the rate of fluid secretion from 0.94 nl min^{-1} to 0.44 nl min^{-1} without significant effects on the concentrations of Na^+, K^+ and Cl^- in secreted fluid. In isolated perfused tubules, Tenmo ADFa had no effect on the transepithelial voltage (V_t) and resistance (R_t). In principal cells of the tubule, Tenmo ADFa had no effect on the basolateral membrane voltage (V_{bl}) and the input resistance of principal cells (R_{pc}). Tenmo ADFa significantly increased the intracellular concentration of cyclic guanosine monophosphate (cGMP) from 2.9 μmol l^{-1} (control) to 7.4 μmol l^{-1}. A peritubular [cGMP] of 20 μmol l^{-1} duplicated the antidiuretic effects of Tenmo ADFa without inducing electrophysiological effects. In contrast, 500 μmol l^{-1} cGMP significantly depolarized V_{bl}, hyperpolarized V_t, and reduced R_t and R_{pc}, without increasing antidiuretic potency beyond that of 20 μmol l^{-1} cGMP. A plot of peritubular cGMP concentration *vs* V_{bl} revealed a steep dose–response between 300 μmol l^{-1} and 700 μmol l^{-1} with an EC_{50} of 468 μmol l^{-1}. These observations suggest a receptor- and cGMP-mediated mechanism of action of Tenmo ADFa. Tenmo ADFa and physiological concentrations of cGMP (<20 μmol l^{-1}) reduce the rate of isosmotic fluid secretion by quenching electroneutral transport systems. The inhibition reveals that as much as 50% of the normal secretory solute and water flux can stem from electrically silent mechanisms in this highly electrogenic epithelium.

Key words: antidiuresis, isosmotic fluid secretion, cGMP, inhibition of electroneutral transport, Na/H exchange, Na/K/2Cl cotransport, *Tenebrio molitor*, antidiuretic factor 'a' (Tenmo ADFa), Malpighian tubule, *Aedes aegypti*.

Introduction

Malpighian tubules execute the renal mechanisms of extracellular fluid homeostasis in insects. The tubules respond directly to compositional changes in the hemolymph and indirectly to diuretic and anti-diuretic factors (Beyenbach, 2003). Diuretic factors accelerate the renal excretion of salt and water, lightening the young mosquito in advance of first flight (Chen et al., 1994) or relieving the adult mosquito of the unwanted weight of a blood meal (Williams and Beyenbach, 1983). In blood-feeding insects, the distension of the abdominal wall triggers the release of diuretic hormone(s) into the hemolymph (Adams, 1999; Stobbart, 1977; Wheelock et al., 1988). When these primary messengers arrive at Malpighian tubules, they initiate a potent stimulation of fluid secretion (Williams and Beyenbach, 1983, 1984). The physiological cascade triggered by abdominal distension is so fast in the *Anopheles* mosquito that the diuresis begins even before the mosquito has finished her meal.

The diuresis wanes with time due to the excretion and/or inactivation of diuretic hormones (Laenen et al., 2001; Williams et al., 1983). The utility of antidiuretic factors terminating the diuresis has also been proposed. Quinlan et al. (1997) reported an increase in cyclic guanosine monophosphate (cGMP) in *Rhodnius* Malpighian tubules as the diuresis decreases, and suggested that this may be important in bringing the diuresis to a conclusion.

Spring et al. (1988) were first to demonstrate the existence of an antidiuretic mechanism in Malpighian tubules of an insect. Subjecting house crickets to dehydration, Spring and coworkers detected antidiuretic activity in the hemolymph that inhibited fluid secretion in Malpighian tubules. The antidiuretic agent in the hemolymph of house crickets has not yet been identified.

The first peptide found to have antidiuretic potency, among other activities, is Manse CAP_{2b}, the cardioacceleratory peptide of *Manduca sexta* (Huesmann et al., 1995). It is an octapeptide with diverse physiological and species-specific

activities. It increases the heart rate in *Manduca* and *Drosophila* (Huesmann et al., 1995), and it has antidiuretic effects in *Rhodnius prolixus* (Quinlan et al., 1997). It is also a weak antidiuretic in *Tenebrio molitor*, with an EC_{50} of 85 nmol l^{-1}. In contrast, it has diuretic activity in *Drosophila* Malpighian tubules, where it elevates nitric oxide (NO) and then cGMP to cause diuresis (Davies et al., 1995). cGMP has also been shown to cause diuresis in *Manduca sexta* although no peptide has yet been discovered that elevates cGMP in this species (Skaer et al., 2002).

Tenmo ADFa is the antidiuretic factor of the beetle *Tenebrio molitor*. The peptide inhibits fluid secretion in *Tenebrio* Malpighian tubules with an EC_{50} of 10 fmol l^{-1}, employing cGMP as second messenger (Eigenheer et al., 2003; Wiehart et al., 2002). FopADF is the antidiuretic factor of the forest ant *Formica polyctena* (Laenen et al., 2001). It decreases fluid secretion in Malpighian tubules to values 29% of control by blocking the entry of K^+ from the hemolymph into the cell and by inhibiting the proton pump at the apical membrane of epithelial cells (Laenen et al., 2001). The primary structure of FopADF has not yet been sequenced.

The present study sought to elucidate the mechanism of action of Tenmo ADFa in Malpighian tubules of *Aedes aegypti*. We found that Tenmo ADFa significantly inhibits the transepithelial secretion of both NaCl and KCl. At the same time the antidiuretic has no effect on transepithelial voltage and resistance, nor on basolateral membrane voltage and input resistance in principal cells. Tenmo ADFa significantly increases intracellular cGMP concentration, and low concentrations of peritubular cGMP (20 μmol l^{-1}) mimic the effects of Tenmo ADFa. Higher cGMP concentrations (500 μmol l^{-1}) induce electrophysiological effects but without increasing antidiuretic effects. Thus, Tenmo ADFa and physiological concentrations of cGMP reduce isosmotic fluid secretion by inhibiting electroneutral and non-conductive transport pathways.

Materials and methods

Mosquitoes

Mosquitoes *Aedes aegypti* L. were reared and maintained according to methods described by Pannabecker et al. (1993). Adult mosquitoes were fed a diet of 3% sucrose solution. Only Malpighian tubules from 3- to 10-day-old female mosquitoes were used. Moreover, only the blind (distal) end of Malpighian tubules was studied. The proximal end of the tubule was removed when tubules were perfused *in vitro*. In the Ramsay fluid secretion assay the proximal end extended into the oil, serving as conduit for fluid secreted by the distal blind end.

Ringer's solution, Tenmo ADFa, cGMP and Ba^{2+}

Ringer solution contained in mmol l^{-1}: 150 NaCl, 3.4 KCl, 1.7 $CaCl_2$, 1.8 $NaHCO_3$, 1.0 $MgSO_4$, 25 Hepes, and 5 glucose. Tenmo ADFa is a 14-amino-acid peptide that was originally isolated from head extracts of *Tenebrio molitor* (Eigenheer et al., 2002). Tenmo ADFa with the sequence Val-Val-Asn-Thr-Pro-Gly-His-Ala-Val-Ser-Tyr-His-Val-Tyr was synthesized by K. Schegg and the gift of D. Schooley. It is unlikely that Tenmo ADFa, the antidiuretic peptide of *Aecheta*, is produced in mosquitoes, although related peptides are probably produced. So far, Malpighian tubules of insects have been remarkably responsive to heterologous peptides such as CRF-like peptides and leucokinin (Audsley et al., 1995). The dose–response of the effects of Tenmo ADFa on fluid secretion of isolated Malpighian tubules in *Tenebrio molitor* puts the EC_{50} at 10^{-14} mol l^{-1} (Eigenheer et al., 2002). Accordingly, we prepared a stock solution of 10^{-8} mol l^{-1} in Ringer solution and kept it frozen in 1 ml portions until use. In all studies, Tenmo ADFa was used at a peritubular concentration of 10^{-9} mol l^{-1}. The effect of other concentrations of ADFa were not explored since the focus of this study was on physiological mechanisms of action.

Cyclic GMP was obtained from Sigma-Aldrich (St Louis, MO, USA) and used at peritubular concentrations between 10 μmol l^{-1} and 1000 μmol l^{-1}. The effect of Ba^{2+} on fluid and electrolyte secretion was tested at a concentration of 5 mmol l^{-1} $BaCl_2$, which is a saturating concentration for electrophysiological effects (Masia et al., 2000). In these experiments, $MgCl_2$ rather than $MgSO_4$ was used in the Ringer solution. In addition, the control Ringer solution was fortified with 15 mmol l^{-1} mannitol for osmotic balance.

Ramsay fluid secretion assay

One advantage of Malpighian tubules of *Aedes aegypti* is their spontaneous secretion of fluid *in vitro*. The tubules do not require pre-stimulation with a secretagogue before experiments on the rates and mechanisms of fluid secretion can commence. Rates of transepithelial fluid secretion were measured in isolated Malpighian tubules using the method of Ramsay (1953) as described previously (Hegarty et al., 1991). In brief, the isolated Malpighian tubule was transferred to 40 μl Ringer solution under oil. The open end of the tubule was pulled out of the Ringer droplet into the oil and draped to a needle post that had been coated with 0.125 mg ml^{-1} poly-lysine for stickiness. Needle posts with a length of approximately 2 mm and an outer diameter of 0.2mm were the broken tips of jeweler's brooches (purchased from local cooperative jewelers), which were also used to trim the ends of Malpighian tubules. The brooches are made of hardened steel that resists bending when the tips are filed to sharpness. Two such sharpened jewelers brooches are routinely used to fray open the end of Malpighian tubules for *in vitro* microperfusion and for fluid secretion assays by the method of Ramsay. The use of blunt instruments tends to crush the tubule and seal the lumen.

The volume of secreted fluid was calculated by measuring the diameter of the spherical aqueous droplet emerging from the open end of the tubule. Volume measurements were made every 5 min for at least 30 min each for the control and experimental periods. After the initial 30 min control period, the cumulative secreted volume was collected for analysis by

electron probe (Williams and Beyenbach, 1983). Thereafter, 4 μl of Ringer solution was removed and replaced with Tenmo ADFa or cGMP to yield the desired concentration. Secreted volume was then measured again as in the control period and the cumulative volume was collected for electron probe analysis after at least 30 min.

The method of Ramsay yields measures of net transepithelial volume transport that includes secretory and absorptive fluxes. Accordingly, a decrease in secretory flow rate can reflect the inhibition of a secretory flux and/or the stimulation of an absorptive flux. Since the blind (distal) end of Malpighian tubules initiates the formation of urine by tubular secretion, we interpret changes in secretion rates to reflect primarily changes in secretory transport activity.

Electron probe analysis of secretions

The concentrations of Na^+, K^+ and Cl^- in secreted fluid were measured by electron probe analysis as previously described (Williams and Beyenbach, 1983). In brief, picoliter volumes of (1) fluid secreted by tubules in the Ramsay assay, (2) standard solutions of Na^+, K^+ and Cl^- and (3) the peritubular Ringer solution were transferred in quintuplets onto the polished surface of a beryllium block. Unknown, standard and Ringer samples were then dehydrated and irradiated with a beam current of 50 nA in a JEOL 8900 (Tokyo, Japan) electron microprobe. The X-ray emissions of the Na^+, K^+ and Cl^- present in the standard solutions were used to construct standard curves, against which the unknown Na^+, K^+ and Cl^- concentrations in secreted fluid were read. The X-ray emissions from Ringer solutions served as internal controls. For each measurement we used the mean of five samples.

In vitro *microperfusion of Malpighian tubules*

The method used for measuring transepithelial and membrane voltage and resistance in isolated perfused Malpighian tubules is described in Williams and Beyenbach (1984) and Yu and Beyenbach (2001). Briefly, a 100 μm segment of Malpighian tubule was trimmed open at both ends and suspended between two holding pipettes in a Ringer bath. The tubule lumen was cannulated with a double-barreled perfusion pipette. One barrel of the pipette was used to perfuse the tubule lumen with Ringer solution and to measure the transepithelial voltage V_t with respect to ground in the peritubular Ringer bath. The other barrel was used to inject current (I=50 nA) into the tubule lumen for the measurement of the transepithelial resistance (R_t) by cable analysis (Helman, 1972; Yu and Beyenbach, 2001). The typical experiment began with collecting control data in the presence of normal Ringer solution. Thereafter the peritubular Ringer solution was switched to include Tenmo ADFa or cGMP and experimental data were collected.

Two-electrode voltage clamp of principal cells

The method of voltage-clamping single principal cells in intact Malpighian tubules is described in Masia et al. (2000). An isolated Malpighian tubule was placed on the bottom of a perfusion bath, and a principal cell was impaled with two conventional microelectrodes filled with 3 mol l^{-1} KCl (20–30 MΩ). When the basolateral membrane voltage (V_{bl}) measured by both electrodes were within 5 mV of each other, the experiment was continued. V_{bl} and the input resistance (R_{pc}) of principal cells were measured under control conditions and then in the presence of peritubular Tenmo ADFa or cGMP. R_{pc} was determined in current–voltage plots obtained from five voltage clamp steps in increments of 10 mV on the positive and negative side of the spontaneous V_{bl}.

Ba^{2+} is known to block K^+ channels in Malpighian tubules of insects (Leyssens et al., 1993; Masia et al., 2000; Nicolson and Isaacson, 1987; Weltens et al., 1992). We used Ba^{2+} in the peritubular Ringer bath to block a major conductive pathway of the basolateral membrane of principal cells in order to uncover small changes in V_{bl} and R_{pc} that might be induced by Tenmo ADFa and cGMP.

Intracellular cGMP concentration

The effect of Tenmo ADFa on intracellular cGMP concentration was measured by enzyme immunoassay of cGMP using the cyclic GMP EIA kit from Cayman Chemicals (Ann Arbor, MI, USA). All five tubules were removed from a single cold-anesthetized female mosquito and placed in 25 μl of Ringer solution including 100 μmol l^{-1} zaprinast, a phosphodiesterase inhibitor. To one set of five tubules 25 μl of Ringer solution was added to serve as control. To another set of five tubules 25 μl of Ringer and 2×10^{-9} mol l^{-1} Tenmo ADFa was added. The two sets of tubules were incubated at room temperature for 15 min. Thereafter, 200 μl of ice-cold ethanol was added, and the tubules were sonicated on ice-water for 15 min. After centrifugation at 16 000 ***g*** and 4°C for 10 min, 200 μl of the supernatant was removed and evaporated to dryness in a vacuum centrifuge. The residue was subsequently reconstituted in 200 μl of buffer solution (EIA kit) and duplicate or triplicate 50 μl samples transferred into the wells of the kit plate. Cyclic GMP, AChE (acetylcholinesterase) tracer and cyclic GMP EIA antiserum were added. After incubation for 18 h, the plates were developed in Ellman's reagent for 90 min. The absorbance was read at 412 nm (Broderick et al., 2003; Eigenheer et al., 2002; Quinlan et al., 1997).

The cytoplasmic cGMP concentration of Malpighian tubules was measured against cGMP standards ranging from 0.24 to 30.0 pmol l^{-1}. A sigmoidal regression (SigmaPlot V 8.0) provided the best fit, against which the absorbance of unknown cGMP concentrations was read.

Since cGMP was extracted from whole Malpighian tubules, the intracellular cGMP concentration was estimated from the measured amount of cGMP extracted from five Malpighian tubules and the cellular volume of the tubules. The latter was estimated by subtracting the volume of the tubule lumen from the total tubule volume of the typical female Malpighian tubule with the following dimensions: length 3.5 mm, o.d. 100 μm, i.d. 10 μm.

Statistical data

Control rates of fluid secretion can vary a great deal in isolated Malpighian tubules, as can values of transepithelial voltage, resistance and other electrophysiological variables. In the case of transepithelial fluid secretion, the difference between low and high rates of secretion is more a difference of magnitude rather than of kind. For example, male Malpighian tubules secrete fluid at much lower rates (0.093 nl min^{-1}) than female Malpighian tubules (0.64 nl min^{-1}), but the kind of fluid secreted by both is the same, consisting of NaCl, KCl and water (Plawner et al., 1991). In female Malpighian tubules, control rates of fluid secretion can be as low as 0.1 nl min^{-1} and as high as 1.0 nl min^{-1}, but NaCl and KCl are consistently the major osmolytes of secreted fluid. To deal with highly variable secretion data, we perform paired experiments. Each tubule is used as its own control. First, the control variable of the tubule under study is measured, then the experimental agent is introduced, and the experimental variable is measured. The statistical analysis tests the significance of the difference between control and experimental values according to the paired Student's *t*-test. We prefer this treatment of variable data over the selection of Malpighian tubules according to certain physiological criteria.

The measurement of intracellular cGMP concentrations precluded paired measurements. Accordingly, the significant difference of population means was tested.

Results

Effects of Tenmo ADFa on transepithelial electrolyte and fluid secretion

Under control conditions, nine isolated Malpighian tubules secreted fluid at a rate of from 0.94±0.08 nl min^{-1} (mean ± S.E.M.) by secreting the Cl^- salts of Na^+ and K^+ (Fig. 1A). The Na^+ concentration in secreted fluid was 94.6±13.2 mmol l^{-1}, K^+ concentration 55.7±7.0 mmol l^{-1} and Cl^- concentration 146.0±14.2 mmol l^{-1} in the same nine Malpighian tubules (Fig. 1B). The product of the fluid secretion rate and the ion concentration in secreted fluid yields the transepithelial ion secretion rate, which was 81.9±5.1 pmol min^{-1} for Na^+, 52.9±7.2 pmol min^{-1} for K^+ and 131.3±8.6 pmol min^{-1} for Cl^- (Fig. 1C). The sum of the concentrations of Na^+, K^+ and Cl^- in secreted fluid was 296.3±29.1 mmol l^{-1}, close to 296 mOsm kg^{-1} H_2O, the osmotic pressure of secreted fluid (Williams et al., 1983).

The addition of Tenmo ADFa (10^{-9} mol l^{-1}) to the peritubular Ringer solution significantly ($P<0.01$) decreased the rate of transepithelial fluid secretion from 0.94 nl min^{-1} to 0.44±0.08 nl min^{-1} (Fig. 1A). However, Tenmo ADFa had no effect on the concentrations of Na^+, K^+, and Cl^- in secreted fluid. In the presence of 10^{-9} mol l^{-1} Tenmo ADFa, the Na^+ concentration in secreted fluid was 98.6±16.0 mmol l^{-1}, K^+ concentration 54.9±8.2 mmol l^{-1}, and Cl^- concentration 144.7±20.2 mmol l^{-1} (Fig. 1B). In the absence of significant effects on the concentrations of secreted ions, the rates of transepithelial ion secretion fell proportionally with the decrease in fluid secretion. The rate of transepithelial Na^+ significantly dropped from 81.9 pmol min^{-1} to 46.9±12.2 pmol min^{-1}. Transepithelial K^+ secretion significantly fell from 52.9 pmol min^{-1} to 23.4±4.6 pmol min^{-1}, and transepithelial Cl^- secretion significantly decreased from 131.3 pmol min^{-1} to

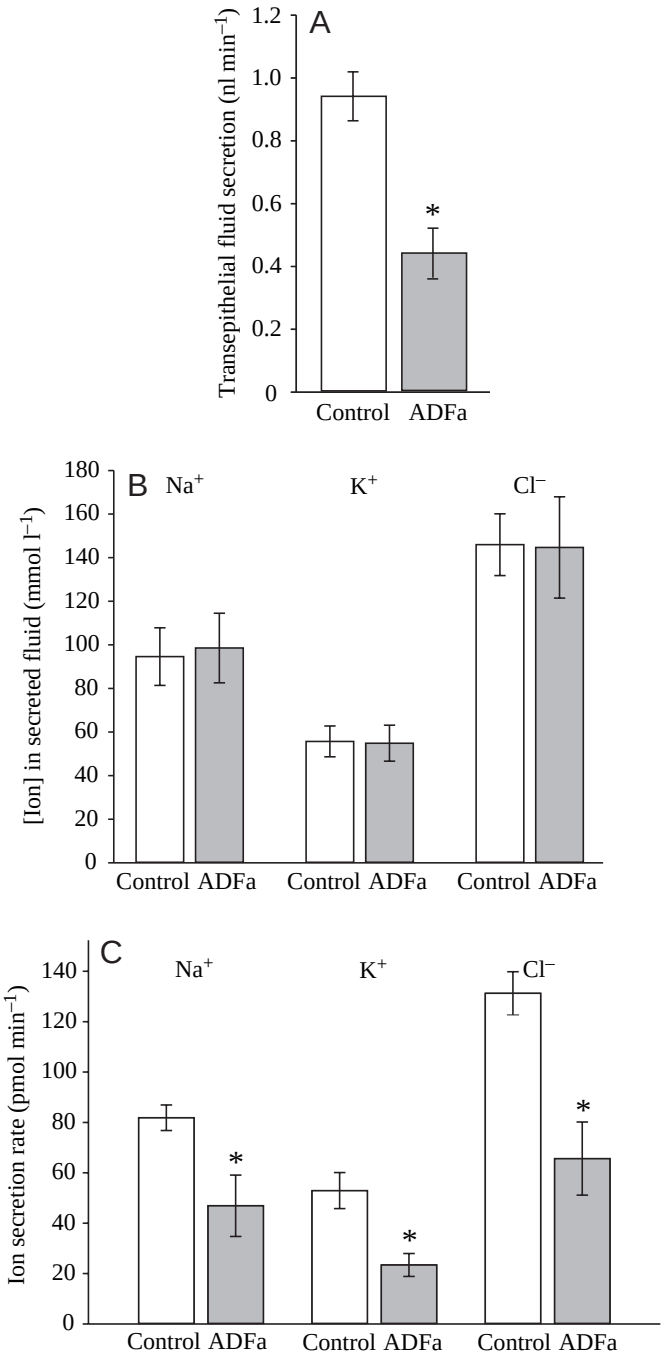

Fig. 1. Effects of *Tenebrio molitor* antidiuretic factor 'a' (Tenmo ADFa, 10^{-9} mol l^{-1}) on transepithelial fluid secretion (A), concentrations of Na^+, K^+, and Cl^- in secreted fluid (B), and transepithelial rates of Na^+, K^+ and Cl^- secretion (C) in isolated Malpighian tubules of *A. aegypti*. Values are means ± S.E.M. (N=9); *$P<0.05$, paired Student's *t*-test.

65.6±14.5 pmol min^{-1}. In the presence of Tenmo ADFa, the sum of the concentrations of Na^+, K^+, and Cl^- in secreted fluid was 298.1±42.0 mmol l^{-1} and not significantly different from control. Significant reduction in the rates of transepithelial ion and water secretion without significant effects on the concentrations of secreted Na^+, K^+ and Cl^- (and hence osmotic pressure) reveals Tenmo ADFa as an inhibitor of isosmotic fluid secretion.

Effects of Tenmo ADFa on electrophysiological variables of Malpighian tubules

Under perfusion of the tubule lumen with the same Ringer solution present in the peritubular bath, the transepithelial voltage V_t was 32.7±9.4 mV (lumen-positive) in seven Malpighian tubules and remained near that value at 31.3±9.5 mV in the presence of 10^{-9} mol l^{-1} Tenmo ADFa (Fig. 2A). The transepithelial resistance R_t was 14.3±1.6 kΩcm in the same seven Malpighian tubules under control conditions and did not change significantly (14.6±1.7 kΩcm) in the presence of Tenmo ADFa (Fig. 2B). Thus, Tenmo ADFa reduces the rate of isosmotic fluid secretion without affecting transepithelial electrogenic and conductive transport pathways.

In studies of principal cells of the tubule by the method of two-electrode voltage clamp, Tenmo ADFa (10^{-9} mol l^{-1}) had no effect on the basolateral membrane voltage V_{bl} and no effect on the input resistance of principal cells R_{pc} (Fig. 2C,D). The small depolarization of V_{bl} from –78.9±3.9 mV (control) to –77.9±4.7 mV in the presence of Tenmo ADFa was not significant (Fig. 2C). Likewise, the small increase in R_{pc} from 450.2±32.2 kΩ (control) to 470.0±20.2 kΩ was not significant (Fig. 2D). Higher Tenmo ADFa concentrations (10^{-8}, 10^{-7} and 10^{-6} mol l^{-1}) did not elicit significant effects on V_{bl} or R_{pc} (data not shown).

Effects of cGMP on transepithelial electrolyte and fluid secretion

Since cGMP is thought to be the second messenger of Tenmo ADFa, the effects of this nucleotide were of interest. In 24 Malpighian tubules, the addition of 500 μmol l^{-1} cGMP to the peritubular Ringer bath significantly (P<0.01) reduced the rate of transepithelial fluid secretion from 0.39±0.03 nl min^{-1} to 0.19±0.02 nl min^{-1} (Fig. 3A). In a subset of 12 Malpighian tubules in which secreted ion concentrations were measured, the rate of fluid secretion again fell significantly (P<0.05) from 0.37±0.04 nl min^{-1} to 0.22±0.03 nl min^{-1}. In the same 12 tubules the Na^+ concentration in secreted fluid increased from 112.6±10.5 mmol l^{-1} to 126.7±12.9 mmol l^{-1}, the K^+ concentration decreased from 53.0±8.7 mmol l^{-1} to 41.6±6.0 mmol l^{-1}, and the Cl^- concentration increased from 140.3±6.9 mmol l^{-1} to 151.1±17.9 mmol l^{-1} (Fig. 3B). Only the decrease in the K^+ concentration reached statistical significance (P<0.05). As expected from the reciprocal change in Na^+ and K^+ concentration, the sum of the concentrations of Na^+, K^+ and Cl^- in secreted fluid did not change significantly: 306.0±13.4 mmol l^{-1} under control conditions and 319.4±32.4 mmol l^{-1} in the presence of 500 μmol l^{-1} cGMP.

Like Tenmo ADFa, cGMP significantly inhibited transepithelial ion secretion (Fig. 3C). Transepithelial Na^+ secretion significantly decreased from 39.0±3.3 pmol min^{-1} to 26.1±2.6 pmol min^{-1}; K^+ secretion significantly fell from 21.7±5.0 pmol min^{-1} to 8.9±1.8 pmol min^{-1} and Cl^- secretion significantly fell from 52.1±6.9 pmol min^{-1} to 30.7±3.3 pmol min^{-1} (Fig. 3C).

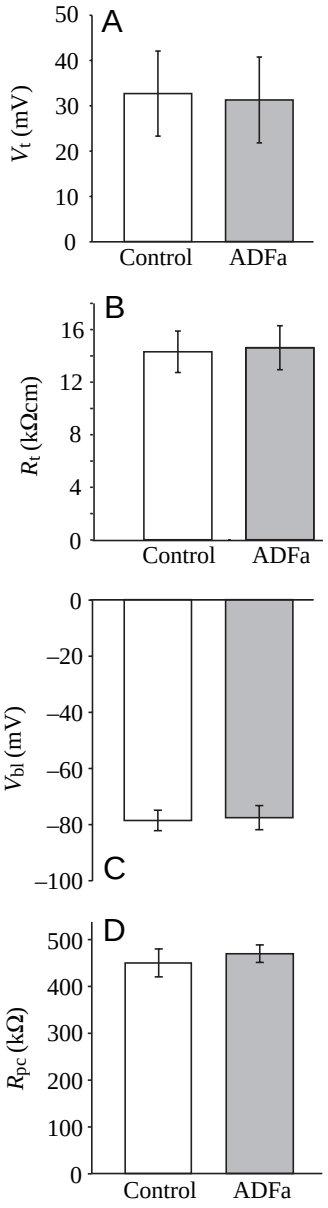

Fig. 2. Effects of Tenmo ADFa (10^{-9} mol l^{-1}) on the transepithelial voltage V_t (A) and resistance R_t (B) in isolated perfused Malpighian tubules and on the basolateral membrane voltage V_{bl} (C) and input resistance R_{pc} (D) of principal cells of Malpighian tubules of *A. aegypti*. Values are means ± S.E.M. (N=7).

Effects of cGMP on electrophysiological variables of Malpighian tubules

To evaluate the effects of cGMP on electrogenic transport mechanisms, V_t and resistance were measured in isolated perfused Malpighian tubules. In this series of experiments V_t was 20.4±2.8 mV (lumen-positive) in 20 Malpighian tubules and R_t was 8.2±1.2 kΩcm (Fig. 4A,B). The addition of 500 μmol l^{-1} cGMP to the peritubular bath significantly (P<0.05) hyperpolarized V_t from 20.4 mV to 41.8±6.1 mV and significantly (P<0.05) reduced R_t from 8.2 kΩcm to 6.2±0.7 kΩcm.

At a concentration of 500 μmol l^{-1}, cGMP also had significant effects on V_{bl} and R_{pc} (Fig. 4C,D). Upon addition of cGMP to the peritubular bath of the tubule, V_{bl} significantly

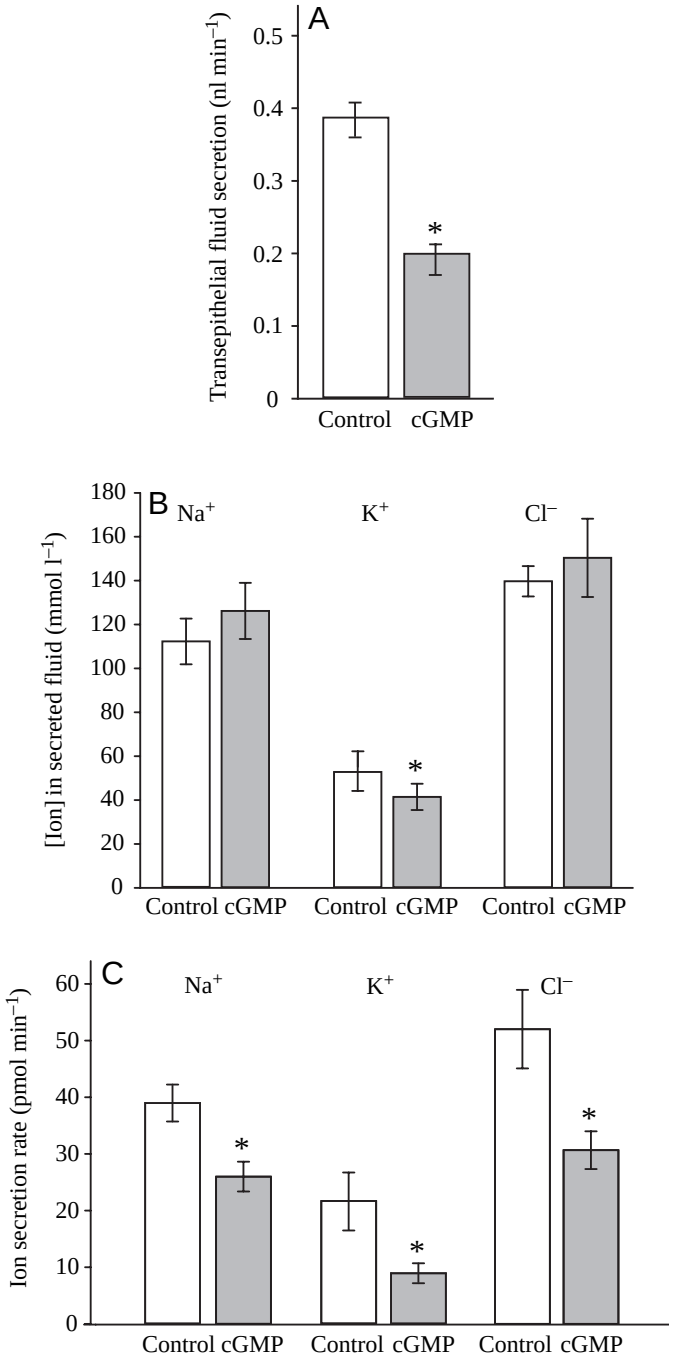

Fig. 3. Effects of 500 μmol l^{-1} cyclic guanosine monophosphate (cGMP) on transepithelial fluid secretion (A), on the concentrations of Na^+, K^+ and Cl^- in secreted fluid (B), and on transepithelial rates of Na^+, K^+ and Cl^- secretion in isolated Malpighian tubules of *A. aegypti* (C). Values are means ± S.E.M.; N=24 tubules (A), N=12 tubules (B,C); *P<0.05, paired Student's t-test.

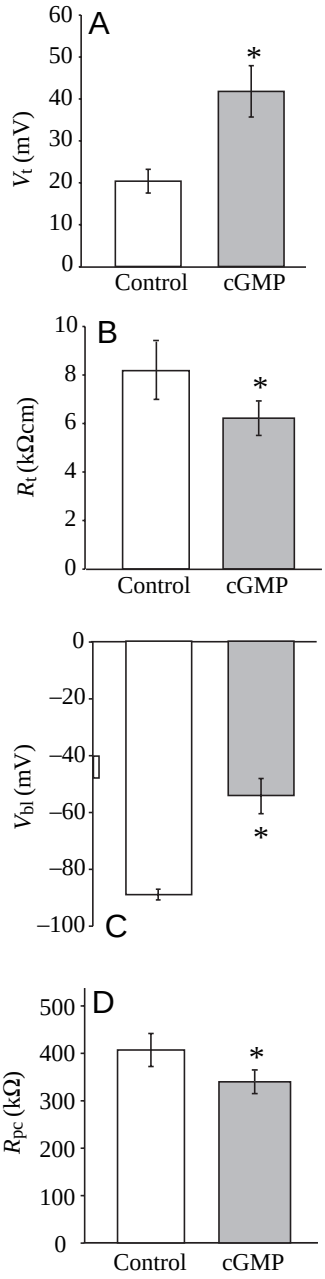

Fig. 4. Effects of cGMP (500 μmol l^{-1}) on the transepithelial voltage V_t (A) and resistance R_t (B) in isolated perfused Malpighian tubules and on the basolateral membrane voltage V_{bl} (C) and input resistance R_{pc} (D) of principal cells of Malpighian tubules of *A. aegypti*. Values are means ± S.E.M.; N=20 (A–C), N=21 (D) tubules or cells; *P<0.05, paired Student's t-test.

(P<0.01) depolarized from –89.0±1.9 mV to –54.3±6.6 mV (N=20 principal cells). In parallel, R_{pc} significantly (P<0.05) decreased from 406.0±34.7 kΩ to 339.2±24.8 kΩ (N=21 principal cells). Since V_t hyperpolarized by 21.4 mV and V_{bl} depolarized by 34.7 mV in the presence of 500 μmol l^{-1} cGMP, it follows that the apical membrane also depolarized by 13.3 mV. Thus, high concentrations of cGMP affected both apical and basolateral membranes of principal cells.

Dose response curve to cGMP

Similar effects of Tenmo ADFa and cGMP on transepithelial electrolyte and fluid secretion are consistent with the second messenger role of cGMP (Figs 1, 3). However, Tenmo ADFa did not affect electrophysiological variables (Fig. 2) whereas 500 μmol l^{-1} cGMP did (Fig. 4). To investigate the difference, we consulted a dose–response curve of the effects of cGMP on V_{bl} and R_{pc}.

As shown in Fig. 5, cGMP had no effect on voltage and resistance at peritubular concentrations of less than 300 μmol l^{-1}. At concentrations higher than 300 μmol l^{-1}, cGMP significantly depolarized V_{bl} in parallel with significant reductions in R_{pc}. Typically, V_{bl} responded to cGMP with an initial steep depolarization that subsequently recovered to a new steady state value (Fig. 5A, insert). A dose–response curve drawn to peak voltage depolarizations yielded an estimate of 380 μmol l^{-1} as the half maximum (EC_{50}) cGMP concentration (Fig. 5A). The EC_{50} was 468 μmol l^{-1} for the dose–response drawn to new steady state values in the presence of cGMP. The EC_{50} for the effect on R_{pc} was 351 μmol l^{-1}, similar to the EC_{50} of peak depolarization (Fig. 5B).

Measurement of intracellular cGMP concentration

Since cGMP is thought to be the second messenger of Tenmo ADFa (Eigenheer et al., 2002), measurements of intracellular cGMP concentrations in the absence and presence of ADFa were of interest. Female Malpighian tubules of *Aedes aegypti* have a cytoplasmic volume of approximately 27.2 nl (Wu and Beyenbach, 2003), enabling us to estimate the intracellular cGMP concentration from the cGMP content of homogenates of Malpighian tubules. Under control conditions, the mean intracellular cGMP concentration was 2.90±0.77 μmol l^{-1} (19 measurements of five Malpighian tubules each). In the presence of 10^{-9} mol l^{-1} Tenmo ADFa, the intracellular cGMP concentration significantly (P<0.05) increased to 7.43±1.78 μmol l^{-1} (21 determinations of five Malpighian tubules each).

The intracellular cGMP concentration of 7.4 μmol l^{-1} measured in the presence of antidiuretic concentrations of Tenmo ADFa (Fig. 1) is much lower than 300 μmol l^{-1}, the lowest peritubular cGMP concentration that affects V_{bl} and R_{pc} (Fig. 5). Suspecting pharmacological effects of high concentrations of cGMP on tubule electrophysiology, it was of interest to investigate whether a low concentration of cGMP could inhibit fluid secretion without inducing effects on voltage and resistance. A peritubular cGMP concentration of 20 μmol l^{-1} was chosen for these experiments.

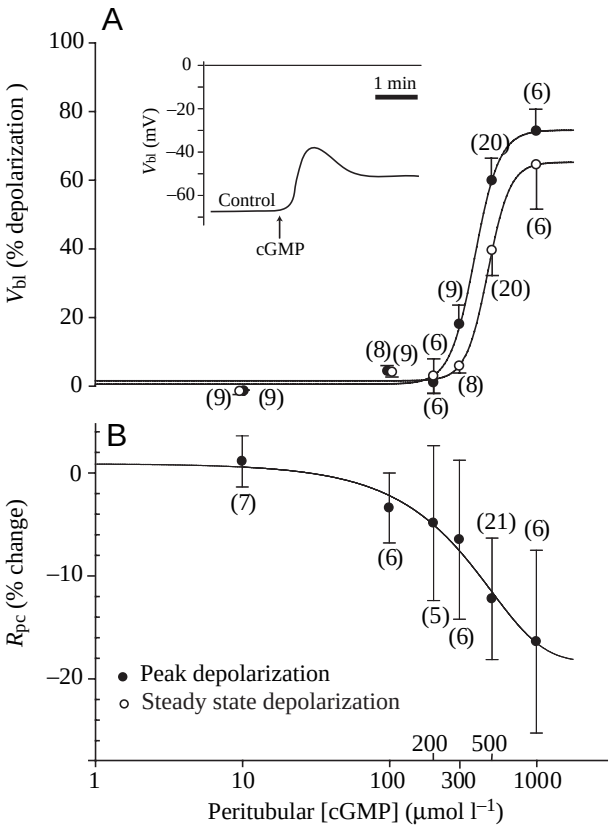

Fig. 5. Dose–response of the effects of cGMP on the basolateral membrane voltage V_{bl} (A), and the input resistance R_{pc} (B) of principal cells in isolated Malpighian tubules of *A. aegypti*. The insert in A illustrates the usual biphasic response of V_{bl} to 500 μmol l^{-1} cGMP. Values are means ± S.E.M.; the number of principal cells is indicated for each data point; *P<0.05, paired Student's t-test. Open circles, steady state depolarization; closed circles, peak depolarisation.

Effects of a low peritubular concentration of cGMP

The addition of 20 μmol l^{-1} cGMP to the peritubular Ringer bath significantly decreased the rate of transepithelial fluid secretion from 0.23±0.01 nl min^{-1} to 0.12±0.01 nl min^{-1} (N=9). The 48% decrease is similar to 51% measured in the presence of 500 μmol l^{-1} cGMP (Fig. 3A) and 53% in the presence of 10^{-9} mol l^{-1} Tenmo ADFa (Fig. 1A). Thus, a low peritubular cGMP concentration of only 20 μmol l^{-1}, duplicated the antidiuretic effect of Tenmo ADFa and elicited antidiuretic effects similar to those of 500 μmol l^{-1} cGMP. Like Tenmo ADFa, 20 μmol l^{-1} cGMP had no significant effects on V_t and R_t. The control V_t was 27.4±5.8 mV in eight Malpighian tubules. The small increase to 28.1±5.4 mV in the presence of 20 μmol l^{-1} cGMP was not significant, nor was the small increase in R_t from 13.9±4.0 kΩcm (control) to 14.2±4.2 kΩcm.

A peritubular cGMP concentration of 10 μmol l^{-1} had no effect on V_{bl} or R_{pc}. In these experiments, V_{bl} was –90.4±2.5 mV under control conditions and –91.8±2.3 mV in the presence of 10 μmol l^{-1} cGMP (N=9 cells). In the same

cells R_{pc} was 426.7±35.1 kΩ and 431.1±35.4 kΩ under control and experimental conditions, respectively.

Ba^{2+} block of K^+ channels does not uncover effects of ADFa and low concentrations of cGMP

Concerned that the high K^+ conductance of the basolateral membrane of principal cells (Beyenbach and Masia, 2002) might shunt the voltage effects of Tenmo ADFa and low concentrations of cGMP, we repeated experiments in the presence of barium, a known blocker of K^+ channels in principal cells of *Aedes* Malpighian tubules (Masia et al., 2000).

Under control conditions, V_{bl} was –85.1±3.9 mV and R_{pc} was 476.9±23.2 kΩ in six principal cells. The addition of 5 mmol l^{-1} Ba^{2+} to the peritubular bath immediately and significantly (P<0.05) hyperpolarized the membrane voltage from –85.1 mV to –103.0±5.1 mV, and significantly (P<0.05) increased the input resistance from 476.9 kΩ to 740.7±94.4 kΩ (N=6 principal cells). In the presence of Ba^{2+}, Tenmo ADFa (10^{-9} mol l^{-1}) had no effect on V_{bl} (–103.5±6.1 mV) nor on R_{pc} (755.5±84.1 kΩ, N=6 principal cells).

Similar observations were made with cGMP at a peritubular concentration of 20 μmol l^{-1}. In this series of experiments V_{bl} was –80.6±4.8 mV under control conditions, which again significantly (P<0.05) hyperpolarized to –86.9±5.5 mV in the presence of 5 mmol l^{-1} Ba^{2+} (N=8 principal cells). The control R_{pc} was 452.6±41.9 kΩ, which significantly (P<0.05) increased to 585.3±55.4 kΩ in the presence of Ba^{2+} in same eight principal cells. In the presence of Ba^{2+}, the addition of 20 μmol l^{-1} cGMP to the peritubular bath had no significant effects on V_{bl} (–86.9±5.7 mV) and R_{pc} (595.9±81.2 kΩ). Thus, significant electrophysiological effects of Tenmo ADFa and physiological concentrations of cGMP were not observed in Ba^{2+}-treated tubules that should have revealed small changes on voltage and resistance.

Discussion

Our experiments give way to five conclusions: (1) Tenmo ADFa, the antidiuretic peptide of *Tenebrio molitor* has antidiuretic effects in blind-ended (distal) segments of Malpighian tubules of the yellow fever mosquito *Aedes aegypti*; (2) Tenmo ADFa increases intracellular cGMP concentration twofold, and low concentrations of cGMP (20 μmol l^{-1}) duplicate the antidiuretic effects of Tenmo ADFa, consistent with a second messenger role; (3) Tenmo ADFa and low doses of cGMP inhibit isosmotic fluid secretion, reducing the rates of transepithelial NaCl and KCl secretion but not their concentrations in secreted fluid; (4) the inhibition of isosmotic fluid secretion targets electroneutral and non-conductive transport systems; and (5) high concentrations of cGMP (500 μmol l^{-1}) increase an unknown conductance of the basolateral membrane and depolarize the basolateral membrane voltage, supplemental to and independent of the electroneutral mechanism of action of low concentrations of cGMP. High concentrations of cGMP (500 μmol l^{-1}) do not elicit antidiuretic effects beyond those of low concentrations of cGMP (20 μmol l^{-1}).

Diuretic and antidiuretic factors of Tenebrio

The mealworm *Tenebrio molitor* has so far yielded two diuretic and two antidiuretic hormones. The diuretic hormones belong to the family of sauvagine/corticotropin-releasing factor/urotensin I-related insect diuretic peptides (Furuya et al., 1995, 1998). The two hormones increase the production of cAMP, the nucleotide that is widely believed to mediate diuresis in insects (Beyenbach, 2003; Cady and Hagedorn, 1999; Hazelton et al., 2002; O'Donnell et al., 1996; Xu and Marshall, 2000). Cyclic AMP increases fluid secretion in Malpighian tubules of *Aedes aegypti* by increasing a Na^+-conductance and bumetanide-sensitive transport system with the effect of stimulating transepithelial NaCl secretion, and not KCl secretion (Hegarty et al., 1991; Sawyer and Beyenbach, 1985).

The two antidiuretic hormones of *Tenebrio molitor*, Tenmo ADFa and ADFb, have 14 and 13 amino acids, respectively (Eigenheer et al., 2002, 2003). Although they bear no structural resemblance to each other, they inhibit fluid secretion in *Tenebrio* Malpighian tubules, and both are thought to use cGMP as second messenger. Tenmo ADFa is about 24 000 times more potent than Tenmo ADFb. In the present study we used synthetic Tenmo ADFa on Malpighian tubules of female *Aedes aegypti*. The inhibition of fluid secretion, concomitant with the increase in intracellular cGMP concentration, indicates that the mosquito possesses an ADFa-like receptor-mediated mechanism of antidiuresis.

Diverse effects of intracellular cGMP

The nucleotide cGMP has species-specific effects. It stimulates fluid secretion in Malpighian tubules of *Drosophila* (Coast et al., 2001), *Locusta* (Morgan and Mordue, 1985), *Teleogryllus* (Xu and Marshall, 2000), and *Manduca* (Skaer et al., 2002), but it inhibits fluid secretion in Malpighian tubules of *Rhodnius* (Quinlan et al., 1997), *Tenebrio* (Eigenheer et al., 2002) and *Aedes* (Fig. 3). Since it increases fluid secretion in *Drosophila* and reduces it in Malpighian tubules of *Aedes*, cGMP has opposite effects in species of the same order, Diptera, but similar effects across orders, namely *Aedes* (Diptera) and *Rhodnius* (Hemiptera).

Next to species differences, cGMP exhibits heterogeneities along the length of Malpighian tubules. The nucleotide stimulates fluid secretion in the main segment but not in the distal segment of Malpighian tubules of the black field cricket *Teleogryllus* (Xu and Marshall, 2000). Similar heterogeneities have been reported in *Rhodnius* Malpighian tubules (O'Donnell and Quinlan, 1998).

In the present study we observe dose-dependent effects of cGMP in Malpighian tubules of *Aedes aegypti*. The nucleotide inhibited fluid secretion at all concentrations tested. At concentrations less than 200 μmol l^{-1} cGMP had no electrophysiological effects (Fig. 5). In contrast, cGMP concentrations above 300 μmol l^{-1} had significant effects on

voltage and resistance. Since high concentrations of cGMP did not inhibit fluid secretion any more than low cGMP concentrations, the electrophysiological effects of high cGMP concentrations are apparently independent of the effects on epithelial transport (Fig. 4). The ionic conductance activated by high concentrations of cGMP is unknown.

Dose-dependent effects, functional heterogeneities along the length of Malpighian tubules, and opposite effects in tubules of different species, reflect the confusion that can arise in the search for unifying themes. At the level of epithelial cells, cGMP, cAMP, calcium, inositol trisphosphate, diacyl glycerol, ATP and prostaglandins are but some intracellular regulators that not only influence transport but also interact with each other. Interactive effects of cGMP and cAMP can be antagonistic, as in *Tenebrio* Malpighian tubules, where the stimulatory effects of cAMP can be neutralized by cGMP (Quinlan et al., 1997; Wiehart et al., 2002). Quinlan and O'Donnell (1998) suggest that in *Rhodnius* Malpighian tubules, cGMP activates the phosphodiesterase of cAMP, thereby reducing intracellular cAMP concentrations and hence rates of transepithelial electrolyte and fluid secretion (Quinlan and O'Donnell, 1998).

Inhibition of isosmotic fluid secretion

The inhibition of transepithelial fluid secretion by Temno ADFa without changes in the concentrations of secreted Na^+, K^+ and Cl^- signifies the inhibition of isosmotic fluid secretion. How isosmotic fluid secretion is reduced at mechanistic and molecular levels is an intriguing question. Since water follows solute by osmosis, the reduction in transepithelial fluid secretion must reflect the inhibition of solute secretion. Indeed, Tenmo ADFa reduces transepithelial secretion rates of Na^+, K^+ and Cl^- (Fig. 1) without affecting their concentrations in secreted fluid and without affecting electrophysiological variables of the tubule and its principal cells. Thus, Tenmo ADFa inhibits the kinetics of transepithelial transport and not the transepithelial electrochemical potentials of transported ions. Three hypothetical mechanisms come to mind.

The first hypothesis proposes metabolic control of transepithelial transport. By reducing the energy supply of active transport systems, the rate of isosmotic fluid secretion is reduced without affecting transepithelial concentration differences of transported ions and without affecting the transepithelial and cell membrane voltages and resistances.

The second hypothesis considers a reduction in transepithelial water permeability. Leaky epithelia characteristically transport solute and water in isosmotic proportions due to high transepithelial permeabilities to both. Accordingly, a decrease in water permeability is expected to decrease transepithelial volume flow without affecting solute concentrations.

The third hypothesis suggests an effect on transepithelial Cl^- transport. Of the three ions secreted into the tubule lumen, the transepithelial secretion of Cl^- correlates more strongly with the secretion of water than the secretion of Na^+ and K^+ (Beyenbach et al., 1993). Since Cl^- is the counterion of transepithelial Na^+ and K^+ secretion, inhibition of Cl^- transport is expected to reduce Na^+ and K^+ transport and hence transepithelial solute and water transport. However, this mechanism would be expected to significantly increase the transepithelial resistance, which was not observed in the present study.

The three hypotheses illustrate how little we know about Malpighian tubules. To our knowledge there are no modern investigations of the energetics of transepithelial transport in Malpighian tubules that might give further insights into the metabolic inhibition proposed above. The evidence for the presence of aquaporin water pathways is just now emerging (Echevarria et al., 2001; Dow and Davies, 2003), but transepithelial water permeabilities in the presence and absence of diuretic or antidiuretic agents have not yet been measured in Malpighian tubules. As to possible effects of ADFa on transepithelial Cl^- transport, there is no consensus on the mechanism and the route of transepithelial Cl^- transport in Malpighian tubules (O'Donnell et al., 1998; Yu and Beyenbach, 2002).

Inhibition of electroneutral transport systems

Tenmo ADFa and low, physiological concentrations of cGMP reduced transepithelial NaCl, KCl and water secretion by approximately the same extent (~50%), without effects on voltage and resistance measured across the tubule or in principal cells. Thus, Tenmo ADFa and low concentrations of cGMP inhibit electroneutral transport mechanisms. The large contribution of electroneutral transport to transepithelial secretion surprised us because Malpighian tubules of *Aedes aegypti* usually display parallel effects on epithelial transport and electrophysiology. Stimulators of secretion, mosquito natriuretic peptide, cAMP and leucokinin all affect voltage and resistance (Beyenbach, 2003; Pannabecker et al., 1993; Petzel et al., 1987; Sawyer and Beyenbach, 1985), and inhibitors of secretion, Ba^{2+} and bafilomycin, also affect voltage and resistance (Beyenbach et al., 2000; Masia et al., 2000). In contrast, Tenmo ADFa and its second messenger elicit no electrophysiological effects in spite of the significant reduction in secretion. Accordingly, effects on transport must not always be mirrored in the electrophysiology of the tubule and its epithelial cells.

We have recently started to investigate the relative contributions of electrogenic and electroneutral transport systems to transepithelial secretion in *Aedes* Malpighian tubules (Scott et al., 2004). Transport mechanisms across the basolateral membrane of principal cells received our first attention. The basolateral membrane is dominated by a K^+-conductance that accounts for 64% of the total membrane conductance (Beyenbach and Masia, 2002). Blocking this conductance with Ba^{2+} inhibits transepithelial fluid secretion by 80%. The remaining K^+ secretion (20%) is blocked by bumetanide, the blocker of Na/K/2Cl cotransport in vertebrate tissues (Scott et al., 2004). Bumetanide alone blocks transepithelial K^+ secretion by 70%, as much as channel block by Ba^{2+} (Hegarty et al., 1991; Scott et al., 2004). Since Ba^{2+}

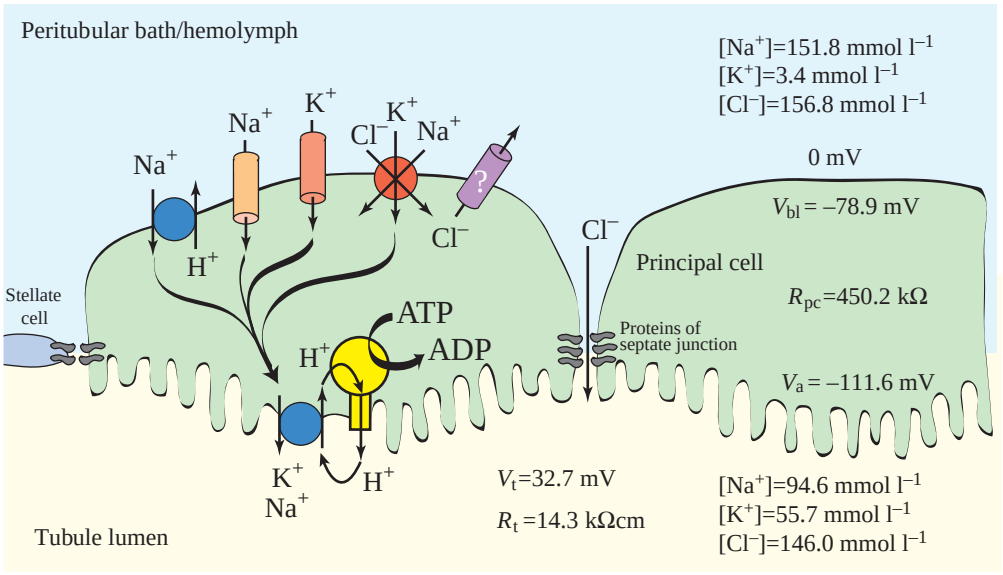

Fig. 6. Model of transepithelial electrolyte and fluid secretion in isolated Malpighian tubules of *A. aegypti*. The tubule consists of principal and stellate cells in a ratio of 5:1 (Beyenbach, 2003). Transepithelial secretion of Na^+ and K^+ is transcellular, and secretion of Cl^- is paracellular and/or through stellate cells under control conditions. The transepithelial Cl^- flux is largely paracellular through septate junctions under diuretic conditions stimulated by leucokinin (Yu and Beyenbach, 2004). The V-type H^+-ATPase located at the apical membrane powers transcellular and paracellular transport *via* electrical coupling (Beyenbach, 2003). The Na/K ATPase may be present in stellate cells, but its contribution to transepithelial electrolyte and fluid secretion appears to be minor or negligible in view of a high V-type H^+-ATPase activity and a low Na/K ATPase measured in *Aedes* Malpighian tubules (Weng et al., 2003). Control data for the Tenmo ADFa experiment of Fig. 1 are shown. The Cl^- channel in the basolateral membrane of principal cells is hypothetical to allow the exit of Cl^- such that the epithelial cell remains in steady state. V_{bl}, basolateral membrane voltage; V_a, apical membrane voltage; V_t, transepithelial voltage; R_{pc}, input resistance, principal cell; R_t, transepithelial resistance.

inhibits transepithelial K^+ secretion by 80% and bumetanide by 70%, it appears that electroconductive and electroneutral transport mechanisms are functionally coupled, where the block of K^+-channels reduces K^+ transport *via* Na/K/2Cl transport and *vice versa* (Fig. 6). In the thick ascending limb of the loop of Henle, this kind of functional coupling serves to recycle K^+ across the apical membrane. Apical membrane K^+-channels return intracellular K^+ to the tubule lumen, thereby ensuring a sufficient K^+ supply for Na/K/2Cl cotransport across that membrane (Vuillemin et al., 1992), because glomerular filtration may not always present sufficient quantities of K^+ to the luminal face of the Na/K/2Cl cotransporter.

A second electroneutral transport system that might be inhibited by ADFa is Na/H exchange (Giannakou and Dow, 2001; Petzel, 2000). In Malpighian tubules of *Aedes aegypti*, 1 mmol l^{-1} amiloride inhibits transepithelial fluid secretion by 60% without significant effects on electroconductive transport pathways (Hegarty et al., 1992). The inhibition of Na/H exchange across the basolateral membrane inhibits not only transepithelial NaCl secretion but also KCl secretion, suggesting again the coupling of electroconductive and electroneutral transport.

Since Tenmo ADFa and low concentrations of cGMP inhibit electroneutral transport in *Aedes* Malpighian tubules, Na/K/2Cl and Na/H transporters are possible targets of their antidiuretic action (Fig. 6). Cyclic GMP is reported to inhibit Na/K/2Cl transport in rabbit cardiomyocytes (Clemo and Baumgarten, 1995; Lew et al., 1997) and HeLa cells (Kort and Koch, 1990). The nucleotide is known to inhibit Na/H exchange in renal proximal tubules (Roczniak and Burns, 1996), small intestine (Fawcus et al., 1997), rat mesangial cells (Schulte et al., 1999) and in epithelial cell lines such as Caco-2 (Gill et al., 2002) and human intestinal C2/bbe (McSwine et al., 1998). Accordingly, a fourth hypothesis regarding the mechanism of action of ADF and cGMP proposes the inhibition of Na/H exchange transport and Na/K/2Cl cotransport.

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