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THE CONCENTRATION-DEPENDENCE OF CRF-LIKE DIURETIC PEPTIDE: MECHANISMS OF ACTION

THOMAS M. CLARK[1], TIMOTHY K. HAYES[2], G. M. HOLMAN[3] AND KLAUS W. BEYENBACH[4,*]

[1]*Department of Zoology, Washington State University, Pullman, WA 99164, USA,* [2]*Bayer Corporation, 1017 Main Campus Drive, Suite 3800, Raleigh, NC 27606, USA,* [3]*Food Animal Protection Research Laboratory, USDA, College Station, TX 77845, USA* and [4]*Section Physiology, VRT 8014, Cornell University, Ithaca, NY 14853, USA*

*Author for correspondence (e-mail: KWB1@Cornell.edu)

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Summary

The mechanism of action of synthetic CCRF-DP, the corticotropin-releasing factor (CRF)-related diuretic peptide of the salt marsh mosquito *Culex salinarius*, was investigated in isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. A low concentration of CCRF-DP (10^{-9} mol l^{-1}) caused a small but insignificant increase in transepithelial secretion of NaCl and fluid, but significantly reduced transepithelial voltage and resistance without a change in short-circuit current, pointing to the stimulation of passive Cl^- transport through the paracellular pathway as the principal mechanism of a mild diuresis. Significant changes in voltage and resistance but not in short-circuit current were duplicated by the ionophore A23187 (0.4 μmol l^{-1}), suggesting Ca^{2+} as a second messenger at 10^{-9} mol l^{-1} CCRF-DP. A high concentration of CCRF-DP (10^{-7} mol l^{-1}) significantly increased transepithelial secretion of NaCl and fluid and significantly increased short-circuit current, pointing to the stimulation of active Na^+ transport through the transcellular pathway as the mechanism of a strong diuresis. This effect was mimicked by dibutyryl-cAMP, suggesting cAMP as a second messenger at 10^{-7} mol l^{-1} CCRF-DP. Dibutyryl-cGMP had no effects. These results suggest dose-dependent, receptor-mediated effects of CCRF-DP that target discrete transport pathways *via* discrete second messengers: low concentrations of CCRF-DP cause a mild diuresis, apparently *via* Ca^{2+}-mediated effects on paracellular Cl^- transport, and high concentrations cause a strong diuresis *via* cAMP-mediated effects on active transcellular Na^+ transport in addition to the effects on the paracellular pathway.

Key words: epithelial transport, CRF-like diuretic peptide, secretory diuresis, cyclic AMP, Ca^{2+}, short-circuit current, corticotropin-releasing factor.

Introduction

In a recent study of Malpighian tubules of the yellow fever mosquito *Aedes aegypti*, we reported that the insect diuretic peptide CCRF-DP affects both paracellular and transcellular transport pathways in a dose-dependent manner (Clark *et al.* 1998). At concentrations lower than 10^{-8} mol l^{-1} the peptide exerts an effect mainly on paracellular transport, and at concentrations higher than 10^{-8} mol l^{-1} the peptide stimulates transcellular transport in addition to paracellular transport. These conclusions were reached in electrophysiological studies that were interpreted on the basis of an electrical circuit model. In the present study, we sought to confirm these electrophysiological conclusions by making physico-chemical measurements of transepithelial electrolyte and fluid secretion. In addition, we probed the second messenger pathways of CCRF-DP that might mediate selective effects on paracellular and transcellular transport.

While these physico-chemical studies support the electrophysiological conclusion that CCRF-DP has dose-dependent effects on paracellular and transcellular transport, the present study indicates that separate second messenger pathways may mediate these dose-dependent effects.

Materials and methods

Mosquitoes and Malpighian tubules

The *Aedes aegypti* colony was maintained as described by Pannabecker *et al.* (1993). On the day of the experiment, a female mosquito (3–7 days post-eclosion) was cold-anesthetized and then decapitated. Malpighian tubules were removed from the abdominal cavity under Ringer's solution. Tubule segments near the blind end of the tubule (between 0.5 and 1 mm long) that are known to secrete salt and water were used (Williams and Beyenbach, 1983, 1984; Beyenbach and Petzel, 1987; Beyenbach, 1995).

Composition of Ringer's solution

Aedes Ringer's solution contained the following: 150 mmol l^{-1} NaCl, 25 mmol l^{-1} Hepes, 3.4 mmol l^{-1} KCl,

7.5 mmol l^{-1} NaOH, 1.8 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $MgSO_4$, 1.7 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} glucose. The pH was adjusted to 7.1; osmotic pressure measured approximately 310 mosmol kg^{-1}. Dibutyryl-cAMP, dibutyryl-cGMP and the Ca^{2+} ionophore A23187 were purchased from Sigma.

Culex *CRF-like diuretic peptide (CCRF-DP)*

The peptide was isolated from whole-body extracts of wild mosquitoes caught in the marshlands of Anahuac National Wildlife Refuge in Southeast Texas (Hayes *et al.* 1994). More than 90 % of the captured population belonged to the species *Culex salinarius*, the saltwater mosquito. The isolation, purification and synthesis of *Culex* CRF-like diuretic peptide (CCRF-DP) were accomplished in the laboratories of G. M. Holman (Texas A&M University). Initially, the peptide was tracked by its ability to increase cAMP concentrations in Malpighian tubules of *Manduca sexta* (F. L. Clottens and others, in preparation). The amino acid sequence of CCRF-DP was then determined to be TKPSLSIVNPL-DVLRQRIILEMARRQMRENTRQVERNKAILREIamide (F. L. Clottens and others, in preparation). This sequence resembles that of the growing family of CRF-like (corticotropin-releasing factor) diuretic peptides identified in insects (Schooley, 1993; Coast, 1996).

CCRF-DP was shipped to us (at the laboratory of Beyenbach) in dry form in two vials containing respectively 1 and 100 pmol of CCRF-DP and 1000 μg of β-lactoglobulin (to minimize CCRF-DP 'sticking' to vial walls). To each vial, 200 μl of Ringer's solution was added to produce stock solutions. On the day of the experiment, the appropriate CCRF-DP stock solution (5 μl) was added to the peritubular Ringer bath (20 μl) to yield the desired test concentration of CCRF-DP, 10^{-9} or 10^{-7} mol l^{-1}.

Measurement of transepithelial fluid secretion

Rates of transepithelial fluid secretion were measured by the method of Ramsay (1954) modified by us as described in Petzel *et al.* (1987). In brief, the blind end of the tubule was suspended in a 20 μl droplet of Ringer's solution under light paraffin oil, and the open end of the tubule was held in light paraffin oil. Thus, fluid secreted into the tubule lumen was free to exit the tubule into paraffin oil, where it was allowed to accumulate for 30 min periods. After the first 30 min period (control), secreted fluid was removed and allowed to sink to the bottom of the paraffin oil dish. The volume of this perfectly spherical droplet was determined by measuring its diameter. Volume divided by time yielded the fluid secretion rate $\dot{V}_s$. Thereafter, CCRF-DP was added to the peritubular Ringer of the tubule to observe the effects on fluid secretion in the next 30 min. Thus, each tubule served as its own control, making the data suitable for analysis using the Student's *t*-test for paired samples. Control and experimental samples of secreted fluid were saved for analysis of elemental composition by X-ray analysis (electron probe).

The product of the fluid secretion rate $\dot{V}_s$ and the concentration of solute X in the secreted fluid $[X]_s$, yields the rate of transepithelial solute secretion $\dot{Q}_X$:

$$\dot{Q}_x = \dot{V}_s[X]_s . \quad (1)$$

Electron probe analysis of secreted fluid

Fluid secreted by Malpighian tubules was analyzed for solute composition using the electron probe methods described by Roinel (1975) as modified in our laboratory (Williams *et al.* 1983). Briefly, a volumetric pipette of approximately 30 pl (prepared with a microforge) was used to deposit equal volumes of (1) secreted fluid, (2) peritubular Ringer's solution, and (3) standard solutions of known concentrations of Na^+, K^+ and Cl^- on the polished 1 cm^2 surface of a beryllium block (under light paraffin oil to prevent evaporation). The oil was subsequently removed with chloroform, which also removed water from the samples leaving solutes behind on the polished beryllium surface as specks of small crystals. These specks were then analyzed in a JEOL 733 Superprobe scanning electron microscope using wavelength dispersive spectroscopy (WDS) as described previously (William and Beyenbach, 1983).

Electrophysiological investigations

In electrophysiological studies, the blind (closed) end of the tubule was opened with small, sharp dissection needles so that the tubule lumen could be perfused *in vitro* (Beyenbach and Dantzler, 1990). Briefly, the tubule was suspended between two holding pipettes in a Ringer bath, and the tubule lumen was cannulated with a double-barrelled pipette of 10 μm outer diameter (Theta-borosilicate glass, 1402401, Hilgenberg, Malsfeld, Germany). One barrel of this pipette was used to perfuse the tubule lumen with Ringer's solution at a rate of approximately 5 nl min^{-1}. At this perfusion rate, the entire volume of luminal fluid is replaced every second. The perfusion barrel was also used to measure transepithelial voltage (V_t) with respect to ground in the peritubular Ringer bath (Beyenbach and Dantzler, 1990). The other barrel was used to inject 100 nA into the tubule lumen for measurements of transepithelial resistance (R_t) by cable analysis (Helman, 1972). The peritubular bath (500 μl) was perfused with Ringer's solution at a rate of 4 ml min^{-1}. In view of the high perfusion rates of both tubule lumen and peritubular bath, transepithelial voltage and resistance were measured in the virtual absence of transepithelial ion gradients, i.e. in symmetrical solutions, which is required when the short-circuit current is used as a measure of active transport (Clark *et al.* 1998). Transepithelial voltage was measured continuously and recorded on a Gould Brush chart recorder. Transepithelial resistance was measured periodically, when of interest, and stored in digital form together with voltage.

Malpighian tubules that under control conditions had maintained a stable V_t of at least 20 mV (lumen-positive) for 5 min were used for further study. At that time, bath flow was

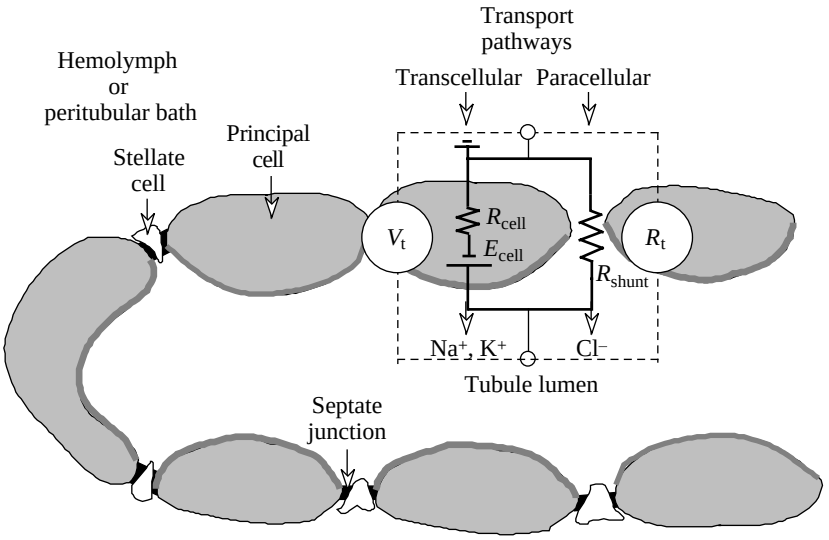


Fig. 1. Electrical equivalent circuit of transepithelial NaCl and KCl secretion in Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. Na^+ and K^+ are secreted into the tubule lumen *via* active transport mechanisms residing in the principal cells, and the accompanying counterion Cl^- is secreted into the lumen *via* passive transport (electrodiffusion) through the paracellular pathway. V_t, transepithelial voltage; R_t, transepithelial resistance; E_{cell}, electromotive force for cation secretion; R_{cell}, transcellular resistance of the active transport pathway; R_{shunt}, resistance of the paracellular pathway.

stopped (with negligible effects on voltage and resistance) because the available quantities of synthetic CCRF-DP did not allow waste of the peptide by testing in a flowing peritubular bath. The agent of interest, CCRF-DP, cAMP, cGMP or A23187, was then added to the peritubular Ringer. The effects of second messengers were also tested under stop-flow conditions.

Equivalent electrical circuit of transepithelial secretion of Na^+, K^+ and Cl^- in Malpighian tubules of Aedes aegypti

Fig. 1 illustrates the simplest electrical circuit model of transepithelial NaCl and KCl secretion across Malpighian tubules of *Aedes aegypti*. The electrical circuit consists of transcellular and shunt transport pathways in parallel. The transcellular transport pathway mediates active transport, and the shunt mediates passive transport. The active transport pathway is modeled with an electromotive force of the active transport system, E_{cell}, in series with the resistance of the active transport pathway, R_{cell}. The shunt pathway consists of the single resistance R_{shunt}. Previous studies have shown conclusively that Na^+ and K^+ are secreted into the tubule lumen by active transport through principal cells and that Cl^- is secreted into the tubule lumen by passive transport through the shunt, which for the most part is a paracellular pathway (Williams and Beyenbach, 1984; Beyenbach and Petzel, 1987; Pannabecker *et al.* 1993; Beyenbach, 1995). Since the transcellular and shunt pathways span the epithelium in parallel (Fig. 1), they are electrically coupled such that the transcellular current, I_e, is equal to the shunt current, i.e. the transepithelial cationic current is equal to the transepithelial anionic current. Indeed, Na^+ and K^+ are secreted into the tubule lumen as the salts of Cl^-, as confirmed in the present study (see Table 1; Fig. 2) and in previous studies (Williams and Beyenbach, 1983; Petzel *et al.* 1987; Plawner *et al.* 1991).

Conventional analysis of the model circuit of Fig. 1 shows that the transepithelial voltage V_t is:

$$V_t = I_e R_{cell} + E_{cell} = I_e R_{shunt} = \frac{E_{cell} R_{shunt}}{R_{cell} + R_{shunt}} , \quad (2)$$

the transepithelial resistance R_t is:

$$R_t = \frac{R_{cell} R_{shunt}}{R_{cell} + R_{shunt}} , \quad (3)$$

and the short-circuit current I_{sc} is:

$$I_{sc} = \frac{V_t}{R_t} = \frac{E_{cell}}{R_{cell}} , \quad (4)$$

where E_{cell} is the electromotive force for cation secretion. I_{sc} defines the current flowing through the active transport pathway alone, namely the maximum current when the shunt resistance, R_{shunt}, is zero (Fig. 1). When R_{shunt} is zero, transepithelial voltage is zero, defining the short-circuit condition (equation 2).

Typically, short-circuit current is measured directly in Ussing-chamber experiments where V_t is voltage-clamped to 0 mV. However, Malpighian tubules are difficult to voltage clamp since this requires passing a wire down the tubule lumen (Spring, 1972). Fortunately, the electrical model of Fig. 1 and equations 2–4 describing that model allow an estimate of I_{sc} as the ratio of the transepithelial voltage (equation 2) and transepithelial resistance (equation 3). Since this estimate of I_{sc} is calculated, equation 4 yields a virtual short-circuit current rather than a real short-circuit current.

Statistical evaluation of the data

Each tubule was used as its own control. Accordingly, the data were analyzed for the differences between paired samples, control *versus* experimental, in each tubule (paired Student's *t*-test). When of interest, analysis of variance (ANOVA)

followed by Student–Newman–Keuls multiple-comparisons test was used (Sokal and Rohlf, 1969).

Results

Effect of lactoglobulin on rates of transepithelial electrolyte and fluid secretion

Malpighian tubules of *Aedes aegypti* spontaneously secrete fluid *in vitro* (Williams and Beyenbach, 1983, 1984). To test the effect of CCRF-DP on the rate of transepithelial fluid secretion, three sets of experiments were performed. In the first set, we tested the effects of β-lactoglobulin on transepithelial fluid secretion since this globulin was used to minimize binding of CCRF-DP to shipment vials (Eppendorf tubes).

The addition of β-lactoglobulin to the peritubular medium at a concentration of $1\,mg\,ml^{-1}$ (the concentration in the presence of CCRF-DP) had no significant effects on transepithelial secretion. The change in the rate of fluid secretion, from 0.64±0.12 to 0.66±0.10 $nl\,min^{-1}$, was insignificant (N=10 tubules). Similarly, the analysis of secreted fluid revealed negligible changes in the concentrations of secreted electrolytes. $[Na^+]_s$ increased insignificantly from 159.3±11.8 to 163.2±11.7 $mmol\,l^{-1}$, $[K^+]_s$ decreased insignificantly from 25.9±3.5 to 23.1±3.7 $mmol\,l^{-1}$ and $[Cl^-]_s$ increased insignificantly from 177.7±13.9 to 188.4±12.1 $mmol\,l^{-1}$. Since none of these changes was significant, it follows that β-lactoglobulin has no effect on transepithelial electrolyte and fluid secretion.

Effect of CCRF-DP on rates of transepithelial electrolyte and fluid secretion

The addition of Ringer containing CCRF-DP (and β-lactoglobulin, $1\,mg\,ml^{-1}$) to yield a peritubular CCRF-DP concentration of $10^{-9}\,mol\,l^{-1}$ caused the rate of transepithelial fluid secretion to increase from 0.87 $nl\,min^{-1}$ (control) to 1.05 $nl\,min^{-1}$ in 18 tubules (Table 1). This increase did not reach statistical significance, nor did the changes in the concentration of secreted Na^+, K^+ and Cl^- (Table 1). Although rates of transepithelial Na^+ and Cl^- movement increased, these increases were not significant (Fig. 2A). Rates of K^+ secretion were hardly affected at all (Fig. 2A). Thus, although a $10^{-9}\,mol\,l^{-1}$ concentration of CCRF-DP tended to increase the rates of transepithelial NaCl and fluid secretion, the changes were not statistically significant (Fig. 2A).

The addition of CCRF-DP to yield a concentration of $10^{-7}\,mol\,l^{-1}$ significantly (P<0.05) increased the rate of transepithelial fluid secretion from 0.70 to 0.93 $nl\,min^{-1}$ in 10 tubules with insignificant changes in the concentrations of secreted Na^+, K^+ and Cl^- (Table 1). However, the product of fluid secretion and electrolyte concentration (equation 1) revealed significant increases (P<0.01) in the rates of transepithelial Na^+ and Cl^- secretion, but not of K^+ secretion (Fig. 2B). Thus, at a concentration of $10^{-7}\,mol\,l^{-1}$, CCRF-DP significantly increased transepithelial secretion rates of NaCl and fluid (Table 1; Fig. 2B).

Effects of $10^{-9}\,mol\,l^{-1}$ CCRF-DP on tubule electrophysiology

While $10^{-9}\,mol\,l^{-1}$ CCRF-DP increased the rates of transepithelial secretion of NaCl and fluid, albeit by insignificant amounts (Fig. 2A), this concentration of CCRF-DP had significant effects on tubule electrophysiology: transepithelial voltage, V_t, repeatedly depolarized in a transient fashion, i.e. V_t oscillated, depolarizing and then repolarizing between 5 and 20 times per minute. Invariably, as voltage

Table 1. *The effects of voltage-depolarizing and -hyperpolarizing concentrations of CCRF-DP on transepithelial fluid secretion, the concentrations of Na^+, K^+ and Cl^- in secreted fluid, and transepithelial electrochemical potentials of Na^+, K^+ and Cl^-*

	Voltage-depolarizing concentration			Voltage-hyperpolarizing concentration		
	Control	$10^{-9}\,mol\,l^{-1}$ CCRF-DP	*P*	Control	$10^{-7}\,mol\,l^{-1}$ CCRF-DP	*P*
Rate of fluid secretion ($nl\,min^{-1}$)	0.87±0.10 (18)	1.05±0.15 (18)	NS	0.70±0.11 (10)	0.93±0.14 (10)	0.05
$[Na^+]$ ($mmol\,l^{-1}$)	167.6±4.5 (18)	165.9±5.9 (18)	NS	150.7±12.4 (10)	159.7±11.1 (10)	NS
$[K^+]$ ($mmol\,l^{-1}$)	38.4±4.9 (18)	33.2±4.4 (18)	NS	22.5±2.2 (10)	20.2±2.7 (10)	NS
$[Cl^-]$ ($mmol\,l^{-1}$)	187.3±7.4 (18)	182.9±7.6 (18)	NS	164.0±16.2 (18)	164.4±15.9 (18)	NS
V_t (mV)	40.9±3.3 (6)	23.4±5.1 (6)	0.02	34.7±3.8 (6)	60.5±7.1 (6)	0.01
R_t (kΩ cm)	6.3±1.0 (6)	3.1±0.6 (6)	0.05	4.2±0.9 (6)	2.7±0.6 (6)	0.05
I_{sc} ($\mu A\,cm^{-1}$)	7.6±1.4 (6)	8.0±2.1 (6)	NS	9.2±1.2 (6)	24.8±4.6 (6)	0.01
μ_{ec} Na^+ (mV)	42.2	22.8	*	33.3	60.6	*
μ_{ec} K^+ (mV)	103.0	80.1	*	83.1	65.8	*
μ_{ec} Cl^- (mV)	–36.3	–17.9	*	–33.6	–59.3	*

Peritubular Ringer concentrations of Na^+, K^+ and Cl^- were, respectively, 159.3, 3.4 and 156.8 $mmol\,l^{-1}$.

NS, not significant; * not evaluated, because electrolyte concentrations and voltage were not measured simultaneously in the same tubule; positive and negative electrochemical potentials indicate, respectively, uphill and downhill transepithelial electrolyte transport into the tubule lumen.

μ_{ec}, transepithelial electrochemical potential; P, statistical significance compared with the control value (*t*-test).

Values are means ± S.E.M. (N).

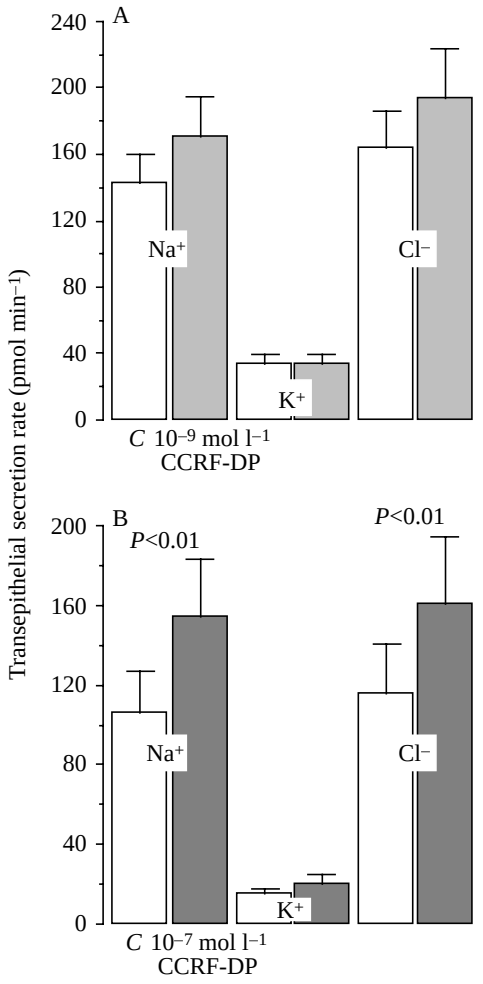


Fig. 2. Effect of synthetic CCRF-D on transepithelial electrolyte secretion in isolated Malpighian tubules of *Aedes aegypti*. (A) Effects of 10^{-9} mol l^{-1} CCRF-DP. Each of 18 tubules was first studied under control conditions (*C*) for 30 min and then for another 30 min after CCRF-DP had been added to the peritubular Ringer. Although rates of transepithelial NaCl and fluid secretion increased in the presence of CCRF-DP, the changes were not statistically significant. (B) Effects of 10^{-7} mol l^{-1} CCRF-DP (10 tubules). CCRF-DP significantly increased rates of transepithelial NaCl secretion, but not of K^+ secretion. Values are means + S.E.M.

decreased towards 0 mV, transepithelial resistance, R_t, decreased as well; as voltage returned to control values (in the presence of CCRF-DP), transepithelial resistance also returned to control values. In six tubules where the effects of 10^{-9} mol l^{-1} CCRF-DP were studied, V_t decreased significantly from 40.9 to 23.4 mV (lumen-positive) in parallel with the significant decrease in R_t from 6.3 to 3.1 kΩ cm (Table 1). Furthermore, upon wash-out of CCRF-DP, V_t and R_t returned to control values. Thus, the reversibility of the effects of CCRF-DP and the voltage and resistance oscillations observed in the presence of 10^{-9} mol l^{-1} CCRF-DP both encompassed parallel changes in transepithelial voltage and resistance. As a result, the short-circuit current, I_{sc}, remained unchanged during the excursions of voltage and resistance, indicating that the active transport pathway did not mediate the effects of 10^{-9} mol l^{-1} CCRF-DP on voltage and resistance. By exclusion, then, 10^{-9} mol l^{-1} CCRF-DP affects the shunt pathway (Fig. 1).

Calculations of transepithelial electrochemical potentials indicate that, under control and experimental conditions, Na^+ and K^+ are secreted into the tubule lumen against their electrochemical potentials (requiring active transport mechanisms) and that Cl^- is secreted into the tubule lumen down its electrochemical potential (requiring no input of external energy).

In separate experiments, we tested the effects of β-lactoglobulin on transepithelial voltage and found no significant effects (data not shown).

Effects of 10^{-7} mol l^{-1} CCRF-DP on tubule electrophysiology

At a concentration of 10^{-7} mol l^{-1}, CCRF-DP stimulated transepithelial secretion of NaCl and fluid to significant levels (Table 1; Fig. 2B). This concentration of CCRF-DP also had significant effects on transepithelial voltage (Table 1). However, the voltage response to high concentrations of CCRF-DP (10^{-7} mol l^{-1}) was biphasic, in contrast to the oscillations in the presence of low concentrations (10^{-9} mol l^{-1}). Upon addition of 10^{-7} mol l^{-1} CCRF-DP to the peritubular medium, V_t first underwent a single deep depolarization, then repolarized and continued to hyperpolarize to a peak value before settling at a value that, in general, was greater than that of the control.

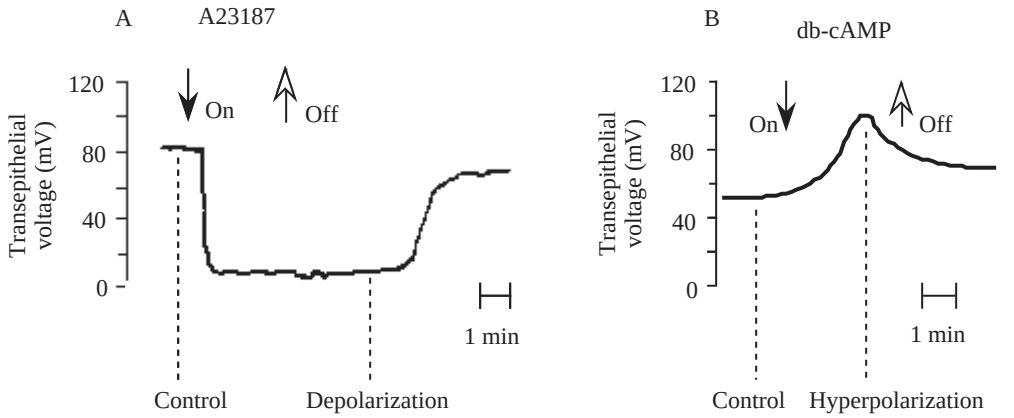
The initial voltage depolarization in the presence of 10^{-7} mol l^{-1} CCRF-DP mimicked the response to 10^{-9} mol l^{-1} CCRF-DP in that V_t and R_t decreased together with no change in I_{sc}. In particular, V_t decreased significantly from 34.7±3.8 to 20.9±5.7 mV in parallel with the significant decrease in R_t from 4.2±0.9 to 2.7±0.4 kΩ cm (six tubules). Thus, voltage and resistance decreased with no significant change in short-circuit current: 9.2±1.2 μA cm^{-1} under control condition and 9.9±3.6 μA cm^{-1} during the voltage depolarization, as in the presence of 10^{-9} mol l^{-1} CCRF-DP, pointing again to the shunt pathway that mediates parallel changes in voltage and resistance.

During the subsequent significant hyperpolarization of V_t to 60.5 mV, transepithelial resistance remained depressed at 2.7 kΩ cm (Table 1). Consequently, short-circuit current increased significantly to 24.8 μA cm^{-1} (Table 1), indicating the stimulation of the active transport pathway. Indeed, direct measurements of transepithelial electrolyte and fluid secretion indicate the stimulation of transepithelial Na^+ secretion (Fig. 2B). It follows that secretion of Cl^- also increases since, in the steady state, transepithelial cationic current is equal to anionic current (Figs 1, 2B; Table 1).

Upon wash-out of 10^{-7} mol l^{-1} CCRF-DP, the voltage, resistance and short-circuit current returned to control values.

Effects of Ca^{2+} ionophore on tubule electrophysiology

The addition of the Ca^{2+} ionophore A23187 to the peritubular Ringer significantly depolarized the transepithelial



Voltage (mV)	31.8±4.6	5.0±1.2**	33.1±5.3	57.7±4.6**
Resistance (kΩ cm)	11.0±3.5	2.4±0.6*	4.9±0.6	3.5±0.4†
Short-circuit current (μA cm^{-1})	4.0±1.4	2.1±0.1	6.9±1.1	17.4±2.6**
	N=6 tubules		*N*=5 tubules	

Fig. 3. Effects of the Ca^{2+} ionophore A23187 (0.4 μmol l^{-1}) and the nucleotide db-cAMP (10 μmol l^{-1}) on the electrophysiology of isolated Malpighian tubules of *Aedes aegypti*. (A) The Ca^{2+} ionophore caused significant reductions in transepithelial voltage and resistance without a significant change in short-circuit current. (B) The nucleotide dibutyryl-cAMP caused a significant increase in transepithelial voltage in parallel with a significant reduction in transepithelial resistance, leading to a large significant increase in short-circuit current. The voltage traces illustrate a representative experiment; the tabulated values are mean ± S.E.M. *P<0.05; †P<0.02; **P<0.005.

voltage and decreased the transepithelial resistance (Fig. 3A). Upon the addition of 0.4 μmol l^{-1} A23187 to the peritubular Ringer's solution, V_t dropped significantly, on average from 31.8 to 5.0 mV, in parallel with a significant drop in R_t from 11.0 to 2.4 kΩ cm (Fig. 3). Although the short-circuit current decreased from 4.0 to 2.1 μA cm^{-1}, this decrease was not statistically significant in view of the large standard error in control short-circuit current. Thus, the effects of A23187 mimicked those of 10^{-9} mol l^{-1} CCRF-DP in that both decreased V_t and R_t with no change in I_{sc} (Fig. 3A).

Like the effects of low concentrations of CCRF-DP, the effects of A23187 were fully reversible upon wash-out of the ionophore, provided that A23187 was applied for short periods (Fig. 3A). The long-term exposure to A23187 needed to assess the effects on transepithelial fluid and electrolyte secretion in the Ramsay assay had detrimental effects on the tubules. The tubules ceased secreting fluid, presumably because of Ca^{2+} overload in the presence of A23187 when extracellular [Ca^{2+}] remained at 1.7 mmol l^{-1}.

Effects of db-cAMP on tubule electrophysiology

Cyclic AMP is widely believed to mediate the effects of insect CRF-like diuretic peptides (Audsley *et al.* 1995; Schooley, 1993; Coast, 1995, 1996). In fact, synthetic CCRF-DP was made after natural CCRF-DP had been isolated by virtue of its ability to raise cAMP concentrations in isolated Malpighian tubules of *Manduca sexta* (F. L. Clottens and others, in preparation). For this reason, the effects of cAMP on tubule electrophysiology were of interest. In spite of the use of the membrane-permeable form of the nucleotide, db-cAMP had no effects on transepithelial voltage at concentrations of 10^{-8}, 10^{-7} and 10^{-6} mol l^{-1} (data not shown). However, at a concentration of 10^{-5} mol l^{-1}, db-cAMP caused the transepithelial voltage to hyperpolarize (Fig. 3B). In particular, 10^{-5} mol l^{-1} db-cAMP significantly hyperpolarized V_t and reduced R_t (Fig. 3B), confirming previous observations with db-cAMP concentrations 10 and 100 times greater (Sawyer and Beyenbach, 1985; Williams and Beyenbach, 1984). Transepithelial voltage increased transiently from 33.1 to 57.7 mV (Fig. 3B). At the same time, transepithelial resistance dropped from 4.9 to 3.5 kΩ cm (Fig. 3B). Because of the rise in V_t and the fall in R_t, the short-circuit current increased significantly nearly threefold from 6.9 to 17.4 μA cm^{-1} (Fig. 3B). Thus, the effects of db-cAMP mimicked those of high concentrations of CCRF-DP in that both stimulate active transport of Na^+ (Table 1). The effects of db-cAMP were reversible upon wash-out, as were those of high concentrations of CCRF-DP (Fig. 3B).

Voltage depolarizations were never observed in the presence of db-cAMP. Instead, the voltage hyperpolarizations induced

by db-cAMP were consistently transient, reaching a peak and then declining to above-control values in the presence of db-cAMP, effects similar to those observed in response to 10^{-7} mol l^{-1} CCRF-DP (Table 1).

Since db-cAMP failed to mimic all the electrophysiological effects of CCRF-DP, we tested another second messenger system known to be active in Malpighian tubules: cGMP (Dow *et al.* 1994; Davies *et al.* 1995).

Lack of effect of db-cGMP on tubule electrophysiology

The addition of db-cGMP (1 mmol l^{-1}) to the peritubular bath had no effect on transepithelial voltage and resistance. In five tubules where the effects of db-cGMP were studied, transepithelial voltage was 45.1±9.3 mV and transepithelial resistance was 6.5±0.7 kΩ cm under control conditions; in the presence of db-cGMP transepithelial voltage was 42.4±8.3 mV and transepithelial resistance was 6.7±0.6 kΩ cm. The short-circuit current remained unchanged at 6.5±0.9 μA cm^{-2} under control conditions and 6.2±1.0 μA cm^{-2} in the presence of db-cGMP. Thus, db-cGMP had no effect on tubule electrophysiology even at millimolar concentrations.

Since changes in transepithelial electrolyte and fluid secretion elicit immediate changes in transepithelial voltage and resistance in highly electrogenic epithelia such as Malpighian tubules of *Aedes aegypti*, the lack of an effect of cGMP on V_t and R_t signals the lack of an effect on electrolyte and fluid secretion. For this reason, the effect of cGMP on transepithelial electrolyte and fluid secretion was not studied.

Discussion

The isolated tubule under control conditions

Isolated Malpighian tubules of *Aedes aegypti* secrete a fluid *in vitro* that is essentially iso-osmotic to the peritubular Ringer's solution of approximately 310 mosmol kg^{-1} (Williams and Beyenach, 1983; Petzel *et al.* 1987; Beyenbach, 1995). Almost 80 % of the osmotic pressure of secreted fluid stems from NaCl and KCl; other salts account for the remaining percentage (Table 1).

The mean concentration of Na^+ in secreted fluid was 161.0±4.8 mmol l^{-1} under control conditions (N=38 tubules), close to the Na^+ concentration of 159.3 mmol l^{-1} in the peritubular Ringer. Although luminal [Na^+] is nearly equimolar to peritubular [Na^+], transepithelial secretion of Na^+ requires active transport mechanism(s) because of the large lumen-positive transepithelial voltages (Table 1). The electrochemical potentials against which Na^+ is moved into the tubule lumen range from 23 to 61 mV in the present study (Table 1) and exceed 100 mV in the presence of cAMP (Williams and Beyenbach, 1984). Transport of Na^+ into the lumen against these electrochemical gradients requires the transport pathway for Na^+ to go through cells in general, and principal cells in particular, where the energy for transport is generated (Williams and Beyenbach, 1983, 1984; Sawyer and Beyenbach, 1985).

The mean concentration of K^+ in secreted fluid was 30.9±2.7 mmol l^{-1} under control conditions (N=38 tubules), significantly above the K^+ concentration of 3.4 mmol l^{-1} in the peritubular Ringer. Given the lumen-positive transepithelial voltages, K^+ is secreted into the lumen against electrochemical potentials ranging from 66 to 103 mV in the present study (Table 1) and in excess of 140 mV in the presence of cAMP (Williams and Beyenbach, 1984). An electrochemical K^+ potential of 140 mV is equivalent to a 237-fold concentration difference or an equivalent luminal K^+ concentration of 806 mmol l^{-1} (at V_t=0 mV) when peritubular K^+ concentration is 3.4 mmol l^{-1}. Again, transepithelial transport of K^+ must take a transcellular route (through principal cells) to tap the energy needed for uphill transport into the tubule lumen.

The mean concentration of Cl^- in secreted fluid was 178.6±6.4 mmol l^{-1} under control conditions (N=38 tubules), significantly above the Cl^- concentration of 156.8 mmol l^{-1} in the peritubular Ringer, indicating transepithelial Cl^- secretion against chemical potentials of 3–4 mV (Nernst). Since the electrical potential in the tubule lumen is on average 38.0±2.0 mV lumen-positive (N=20 tubules), the net electrochemical potential of Cl^- (–34 mV) favors the passive, downhill movement of Cl^- into the lumen (Table 1). Indeed, Cl^- transport into the tubule lumen was always downhill, whether under control conditions or in the presence of low or high concentrations of CCRF-DP (Table 1). Thus, thermodynamic criteria indicate passive (downhill) transport of Cl^- into the tubule lumen. Since passive transport into the tubule lumen need not involve passage through cells, Cl^- may bypass cells. An extracellular pathway is ideally suited for such a bypass transport route, as indicated in the equivalent electrical circuit of Fig. 1. Accordingly, the active transport pathway for Na^+ and K^+ is placed through the principal cells, and the passive transport pathway for Cl^- is placed between the principal cells. Whereas O'Donnell *et al.* (1996) believed that Cl^- takes a transcellular route through stellate cells in Malpighian tubules of *Drosophila melanogaster*, our evidence in Malpighian tubules of *Aedes aegypti* indicates that Cl^- takes mostly, though not exclusively, a paracellular route; namely, a transepithelial route through septate junctions (Pannabecker *et al.* 1993; Clark *et al.* 1998).

CRF-like diuretic peptides in insects

Of the many agents that have diuretic effects in insects, the CRF-related peptides, so-called because of structural similarities with vertebrate urotensin I, sauvagine and corticotropin-releasing factor (CRF), have received the most attention. The laboratory of Schooley was the first to isolate and sequence an insect CRF-related peptide from the tobacco hornworm *Manduca sexta* (Kataoka *et al.* 1989). Since then, a second CRF-like diuretic peptide has been found in *M. sexta* (Blackburn *et al.* 1991), and other CRF-like diuretic peptides have been identified in the cricket (Kay *et al.* 1991*a*), the locust (Kay *et al.* 1991*b*; Lehmberg *et al.* 1991), the cockroach (Kay *et al.* 1992), the housefly and stable fly (Clottens *et al.* 1994), the mealworm (Furuya *et al.* 1995) and, most recently, the salt marsh mosquito *Culex salinarius* (F. L. Clottens and others, in preparation).

The synthetic CRF-like peptide from *Culex* (CCRF-DP) was used in the present study to elucidate its mechanism of diuresis in isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. While dose-dependent effects are the norm in physiology and pharmacology, the dose-dependent stimulation of active cationic and passive anionic transport pathways by a single extracellular peptide, as shown in the present study, is new in epithelial transport physiology.

Effects of low doses of CCRF-DP on, presumably, the paracellular transport pathway

A low concentration of CCRF-DP ($1\,nmol\,l^{-1}$) caused a small, but insignificant, increase in transepithelial NaCl and fluid secretion, suggesting stimulation of a mild diuresis that was not statistically significant in the Ramsay assay (Table 1; Fig. 2). However, concomitant effects on transepithelial voltage and resistance were significant (Table 1), which is not surprising given the greater resolution of electrophysiological measurements of transport compared with measurements of concentrations and volume in the Ramsay assay. Thus, low concentrations of CCRF-DP bring about a mild diuresis that is best observed in the electrophysiological correlates of transport.

Significantly, low concentrations of CCRF-DP caused voltage and resistance to change in parallel: voltage and resistance fell during voltage depolarizations and rose together during voltage repolarizations, suggesting that voltage tracks resistance (Table 1). According to the equivalent electrical circuit (Fig. 1), voltage tracks resistance in the same direction (decrease or increase) upon changes in shunt resistance, pointing to the effects of low concentrations of CCRF-DP on, presumably, the paracellular transport pathway. In addition, measurements of the short-circuit current rule out effects on the transcellular transport pathway because the short-circuit current remains constant during voltage depolarizations and repolarizations (Table 1). Thus, both circuit analysis and estimates of the short-circuit current (equation 4) show that low concentrations of CCRF-DP affect primarily, if not solely, the shunt pathway (Fig. 1). Further analysis of the electrical circuit in Fig. 1 predicts that a reduction in shunt resistance is expected to increase rates of transepithelial electrolyte secretion under the open-circuit conditions that prevail in the Ramsay assay, bringing about the mild diuresis that is, indeed, observed (Fig. 2).

The effects of low concentrations of CCRF-DP on the shunt pathway are strikingly similar to the effects of leucokinin in spite of enormous structural differences between the leucokinins (which have eight amino acids) and the CRF-related peptides (which have 30–50 amino acids) (Holman *et al.* 1987; Hayes *et al.* 1989; Pannabecker *et al.* 1993; Schooley, 1993). Like low concentrations of CCRF-DP, leucokinin causes parallel reductions in transepithelial resistance and voltage which, in the case of leucokinin, are due to the increase in paracellular Cl^- conductance (Pannabecker *et al.* 1993; Wang *et al.* 1996). As paracellular Cl^- conductance increases, both transepithelial resistance and voltage decrease concomitantly. At the same time, rates of transepithelial electrolyte and fluid secretion increase as Cl^-, serving as counterion to both Na^+ and K^+, encounters less resistance in its passage through the paracellular pathway (Pannabecker *et al.* 1993). Similarly, an increase in shunt Cl^- permeability could underlie the mild diuresis induced by low concentrations of CCRF-DP (Fig. 2; Table 1).

The effects of Ca^{2+} on tubule electrophysiology are consistent with this hypothesis for the following reason. O'Donnell *et al.* (1996) showed conclusively in *Drosophila melanogaster* Malpighian tubules that leucokinin increases anion transport in general and Cl^- transport in particular. More importantly, they established that Ca^{2+} serves as the second messenger of leucokinin (O'Donnell *et al.* 1996). Experimental maneuvers that increase intracellular free [Ca^{2+}] duplicate the effects of leucokinin and those that immobilize intracellular Ca^{2+} suppress the action of leucokinin (O'Donnell *et al.* 1996). Employing a similar strategy in Malpighian tubules of *Aedes aegypti*, we find that the Ca^{2+} ionophore A23187, which is expected to increase intracellular free [Ca^{2+}], mimics the effects of low concentrations of CCRF-DP (and leucokinin) on the shunt pathway by reducing transepithelial voltage and resistance without a change in short-circuit current (Fig. 3A). In view of the strikingly similar electrophysiological responses to low concentrations of CCRF-DP and Ca^{2+} ionophore, Ca^{2+} appears to serve as a second messenger for low concentrations of CCRF-DP in *Aedes aegypti* Malpighian tubules, mediating an increase in shunt Cl^- conductance.

Effects of high doses of CCRF-DP on, presumably, paracellular and transcellular transport pathways

In contrast to the transient voltage reductions observed in the presence of low concentrations of CCRF-DP, a high dose of CCRF-DP prompted a biphasic voltage response. The immediate response was a significant decrease in the transepithelial voltage together with a decrease in resistance without a change in short-circuit current, duplicating the effects of a low dose of CCRF-DP on the shunt pathway. Thereafter, voltage hyperpolarized at reduced resistance, leading to a 150 % increase in short-circuit current that signified a significant effect on the active transport pathway (Table 1). Significantly, the increase in the short-circuit current paralleled the increase in the rate of transepithelial NaCl secretion and not KCl secretion, revealing stimulation of active Na^+ transport and not of K^+ transport (Fig. 2; Table 1).

Compared with the effects of a low concentration of CCRF-DP ($1\,nmol\,l^{-1}$), the diuresis triggered by a high concentration ($100\,nmol\,l^{-1}$) is strong (Fig. 2; Table 1). Moreover, the selective increase in transepithelial secretion of NaCl and not of KCl by high concentrations of CCRF-DP is reminiscent of the stimulation of NaCl secretion by cAMP observed in previous studies (Williams and Beyenbach, 1983; Sawyer and Beyenbach, 1985). Moreover, cAMP also increases transepithelial voltage and decreases resistance with a significant increase in short-circuit current, duplicating the effects of a high dose of CCRF-DP (Fig. 3B). Thus, cAMP appears to serve as a second messenger at high concentrations

of CCRF-DP in *Aedes aegypti* Malpighian tubules. Mediation *via* cAMP supports the widely held view that CRF-related diuretic peptides, to which CCRF-DP belongs, act to raise [cAMP] in insect Malpighian tubules (Clark and Spring, 1992; Schooley, 1993; Audsley *et al.* 1995; Coast, 1995, 1996; O'Donnell *et al.* 1996). In *Aedes aegypti* Malpighian tubules, cAMP is known to stimulate the active transport pathway by increasing the Na^+ conductance of the basolateral membrane of principal cells (Sawyer and Beyenbach, 1985). The selective increase in basolateral Na^+ conductance is responsible for the stimulation of transepithelial NaCl secretion and not KCl secretion, as apparently is also the case in the presence of high concentrations of CCRF-DP (Table 1; Fig. 2).

The biphasic voltage response to a high concentration of CCRF-DP reflects the sequential stimulation of first the shunt pathway (presumably *via* Ca^{2+}) and then the transcellular transport pathway (presumably *via* cAMP). We are cautious about the second messengers involved because we have yet to show that a low concentration of CCRF increases intracellular [Ca^{2+}], on the one hand, and that a high concentration of CCRF-DP increases intracellular [Ca^{2+}] and [cAMP], on the other, although this hypothesis is intellectually satisfying given the clear demonstration of the role of Ca^{2+} in mediating anion transport and the role of cAMP in mediating cation transport in Malpighian tubules of *Drosophila melanogaster* (O'Donnell *et al.* 1996). Whereas Ca^{2+} regulates cation transport and cAMP regulates anion transport in *Drosophila melanogaster* Malpighian tubules, the present study in *Aedes aegypti* Malpighian tubules suggests that a single peptide, CCRF-DP, is able to activate the two second messengers in a dose-dependent fashion.

Relevance to the literature on insect diuretic peptides

Biphasic voltage responses such as those observed in the presence of high concentrations of CCRF-DP are not new in studies of Malpighian tubules. Most relevant are our own observations of the mosquito natriuretic peptide, MNP, which triggers a biphasic voltage response like that produced by high concentrations of CCRF-DP (Petzel *et al.* 1985). Furthermore, MNP selectively triggers a stimulation of transepithelial NaCl secretion *via* an elevation of intracellular cAMP concentration (Petzel *et al.* 1985) similar to the effects of high concentrations of CCRF-DP observed in the present study (Fig. 2; Table 1). However, functional resemblance does not indicate structural resemblance, and CCRF-DP has more than twice the molecular mass of MNP (Petzel *et al.* 1986; Holman *et al.* 1987). Nonetheless, the two peptides may share similar functional groups. The high doses of CCRF-DP that are needed (10^{-7} mol l^{-1}) to elicit effects in the present study may reflect (1) the study of a peptide that has been isolated from one species (*Culex* sp.) and is studied in another species (*Aedes aegypti*) and (2) that our studies were carried out *in vitro* rather than *in vivo*.

Veenstra (1988) observed in *Aedes aegypti* Malpighian tubules that 5-hydroxytryptamine (5-HT) caused sequential depolarization and hyperpolarization of the transepithelial voltage similar to the effects of MNP (Petzel *et al.* 1985) and high concentrations of CCRF-DP (Table 1). Veenstra (1988) hypothesized that the biphasic voltage response caused by 5-HT was due to activation of different receptors and second messengers, a situation well documented in responses to biogenic amines (Prince and Berridge, 1973; Berridge, 1980; Peroutka 1988). Similarly, CCRF-DP may be interacting with two distinct receptor types. Low concentrations of CCRF may bind to a high-affinity receptor to trigger an increase in cytoplasmic free [Ca^{2+}], which raise the Cl^- conductance of the paracellular pathway, thereby causing a mild diuresis (Fig. 2; Table 1). High concentrations of CCRF may bind in addition to a low-affinity receptor to trigger the stimulation of adenylate cyclase, thereby raising cytoplasmic [cAMP] and leading to the stimulation of active Na^+ transport and causing a strong diuresis of a NaCl-rich fluid (Fig. 2; Table 1). However, the phenomenon of multiple receptor types for a single agonist is not common amongst peptide hormones. Accordingly, CCRF-DP may interact with a single receptor at the basolateral membrane of epithelial cells that activates two different signal pathways, just as insulin binds to a single receptor and thereby activates multiple signaling pathways (Saltiel, 1996).

In a previous study (Clark *et al.* 1998), high doses of CCRF-DP that stimulated both transcellular and paracellular transport were observed to increase short-circuit current to a greater extent than the sum of their single effects. This synergism stems in part from the properties of electrical circuits and from possible biochemical amplification schemes that lead to synergistic physiology (Clark *et al.* 1998).

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References

AUDSLEY, N., KAY, I., HAYES, T.K. AND COAST, G. M. (1995). Cross-reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol.* A **110**, 87–93.

BERRIDGE, M. J. (1980). The role of cyclic nucleotides and calcium in the regulation of chloride transport. *Ann. N.Y. Acad. Sci.* **341**, 157–171.

BEYENBACH, K. W. (1995). Mechanism and regulation of electrolyte transport in Malpighian tubules. *J. Insect Physiol.* **41**, 197–207.

BEYENBACH, K. W. AND DANTZLER, W. H. (1990). Comparative kidney tubule sources, isolation, perfusion and function. *Meth. Enzymol.* **191**, 167–226.

BEYENBACH, K. W. AND PETZEL, D. H. (1987). Diuresis in mosquitoes: role of a natriuretic factor. *News physiol. Sci.* **2**, 171–175.

BLACKBURN, M. B., KINGAN, T. G., BODNAR, W., SHABANOWITZ, J., HUNT, D. F., KEMPE, T., WAGNER, R. M., RAINA, A. K., SCHNEE, M. E. AND MA, M. C. (1991). Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. biophys. Res. Commun.* **181**, 927–932.

CLARK, T. M., HAYES, T. K. AND BEYENBACH, K. W. (1998). Dose-dependent effects of CRF-like diuretic peptide on transcellular and paracellular pathways. *Am. J. Physiol.* (in press).

CLARK, T. M. AND SPRING, J. H. (1992). Excretion in the house cricket. Role of second messengers in Malpighian tubule function. *Can. J. Zool.* **70**, 1347–1353.

CLOTTENS, F. L., HOLMAN, G. M., COAST, G. M., TOTTY, N. F., HAYES, T. K., KAY, I., MALLET, A. I., WRIGHT, M. S., CHUNG, J.-S., TRUONG, O. AND BULL, D. L. (1994). Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides* **15**, 971–979.

COAST, G. M. (1995). Synergism between diuretic peptides controlling ion and fluid transport in insect Malpighian tubules. *Regul. Peptides* **57**, 283–296.

COAST, G. M. (1996). Neuropeptides implicated in the control of diuresis in insects. *Peptides* **17**, 327–336.

DAVIES, S. A., HUESMAN, G. R., MADDRELL, S. H. P., O'DONNELL, M. J., DOW, J. A. T. AND TUBLITZ, N. J. (1995). CAP2b, a cardioaccelerator peptide, is present in *Drosophila* and stimulates fluid secretion by Malpighian tubules *via* cyclic GMP. *Am. J. Physiol.* **269**, R1321–R1326.

DOW, J. A. T., MADDRELL, S. H. P., DAVIES, S. A., SKAER, N. J. V. AND KAISER, K. (1994). A novel role for the nitric oxide/cyclic GMP signaling pathway: the control of fluid secretion in *Drosophila*. *Am. J. Physiol.* **266**, R1716–R1719.

FURUYA, K., SCHREGG, K. M., WANG, H., KING, D. S. AND SHOOLEY, D. A. (1995). Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor*. *Proc. natn. Acad. Sci. U.S.A.* **92**, 12323–12327.

HAYES, T. K., HOLMAN, G. M., PANNABECKER, T. L., WRIGHT, M. S., STREY, A. S., NACHMAN, R. J., HOEL, D. F., OLSON, J. K. AND BEYENBACH, K. W. (1994). Culekinin depolarizing peptide: a mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Peptides* **52**, 235–248.

HAYES, T. K., PANNABECKER, T. L., HINCKLEY, D. J., HOLMAN, G. M., NACHMAN, R. J., PETZEL, D. H. AND 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. AND NACHMAN, R. J. (1987). Isolation, primary structure and synthesis of leucokinins VII and VIII: the final members of a new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae*. *Comp. Physiol. Biochem.* **88**, 31–34.

KATAOKA, H., TROETSCHLER, R. G., LI, J. P., KRAMER, S. J., CARNEY, R. L. AND SCHOOLEY, D. A. (1989). Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2976–2980.

KAY, I., COAST, G. M., CUSINATO, O., WHEELER, C. H., TOTTY, N. F. AND GOLDSWORTHY, G. J. (1991*a*). Isolation and characterization of a diuretic peptide from *Acheta domesticus* – Evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe Seyler* **372**, 505–512.

KAY, I., PATEL, M., COAST, G. M., TOTTY, N. F., MALLET, A. I. AND GOLDSWORTHY, G. J. (1992). Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L. *Regul. Peptides* **42**, 111–122.

KAY, I., WHEELER, C. H., COAST, G. M., TOTTY, N. F., CUSINATO, O., PATEL, M. AND GOLDSWORTHY, G. J. (1991*b*). Characterization of a diuretic peptide from *Locusta migratoria*. *Biol. Chem. Hoppe Seyler* **372**, 929–934.

LEHMBERG, E., OTA, R. B., FURUYA, K., KING, D. S., APPLEBAUM, S. W., FRENZ, H. J. AND SCHOOLEY, D. A. (1991). Identification of a diuretic hormone of *Locusta migratoria*. *Biochim. biophys. Res. Commun.* **179**, 1036–1041.

O'DONNELL, M. J., DOW, J. A. T., HUESMANN, G. R., TUBLITZ, N. J. AND MADDRELL, S. H. P. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. exp. Biol.* **199**, 1163–1175.

PANNABECKER, T. L., HAYES, T. K. AND BEYENBACH, K. W. (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63–76.

PEROUTKA, S. J. (1988). 5-Hydroxytryptamine receptor subtypes. *A. Rev. Neurosci.* **11**, 45–60.

PETZEL, D. H., BERG, M. M. AND BEYENBACH, K. W. (1987). Hormone-controlled, cyclic AMP-mediated fluid secretion in yellow fever mosquito. *Am. J. Physiol.* **253**, R701–R711.

PETZEL, D. H., HAGEDORN, H. H. AND BEYENBACH, K. W. (1985). Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol.* **249**, R379–R386.

PETZEL, D. H., HAGEDORN, H. H. AND BEYENBACH, K. W. (1986). Peptide nature of two mosquito natriuretic factors. *Am. J. Physiol.* **250**, R328–R332.

PLAWNER, L., PANNABECKER, T. L., LAUFER, S., BAUSTIAN, M. D. AND BEYENBACH, K. W. (1991). Control of diuresis in the yellow fever mosquito *Aedes aegypti*: Evidence for similar mechanisms in the male and female. *J. Insect Physiol.* **37**, 119–128.

PRINCE, W. T. AND BERRIDGE, M. J. (1973). The role of calcium in the action of 5-hydroxytryptamine and cyclic AMP on salivary glands. *J. exp. Biol.* **58**, 367–384.

RAMSAY, J. A. (1954). Active transport of water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. exp. Biol.* **31**, 104–113.

ROINEL, N. (1975). Electron microprobe quantitative analysis of lyophilized 10^{-10}l volume samples. *J. Microscop., Paris* **22**, 261–268.

SALTIEL, A. R. (1996). Diverse signaling pathways in the cellular actions of insulin. *Am. J. Physiol.* **270**, E375–E385.

SAWYER, D. B. AND BEYENBACH, K. W. (1985). Dibutyryl-cAMP increases basolateral sodium conductance of mosquito Malpighian tubules. *Am. J. Physiol.* **248**, R339–R345.

SCHOOLEY, D. A. (1993). Insect diuretic hormones with homology to sauvagine–CRF–urotensin I. In *Molecular Comparative Physiology*, vol. 12, *Structure and Function of Primary Messengers in Invertebrates: Insect Diuretic and Antidiuretic Peptides* (ed. K. W. Beyenbach), pp. 22–37. Basel: S. Karger AG.

SOKAL, R. R. AND ROHLF, F. J. (1969). *Biometry*. First edition. New York: W. H. Freeman and Co.

SPRING, K. (1972). Insertion of an axial electrode into renal proximal tubule. *Yale J. Biol. Med.* **45**, 426–431.

VEENSTRA, J. A. (1988). Effects of 5-hydroxytryptamine on Malpighian tubules of *Aedes aegypti. J. Insect Physiol.***34**, 299–304.

WANG, S., RUBENFELD, A. B., HAYES, T. K. AND BEYENBACH, K. W. (1996). Leucokinin increases paracellular permeability in insect Malpighian tubules. *J. exp. Biol.* **199**, 2537–2542.

WILLIAMS, J. C., JR AND BEYENBACH, K. W. (1983). Differential effects of secretagogues on Na^+ and K^+ secretion in Malpighian tubules of *Aedes aegypti* L. *J. comp. Physiol.* B **149**, 511–517.

WILLIAMS, J. C., JR AND BEYENBACH, K. W. (1984). Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. *J. comp. Physiol.* B **154**, 301–309.