The Journal of Experimental Biology 199, 2537–2542 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0599

LEUCOKININ INCREASES PARACELLULAR PERMEABILITY IN INSECT MALPIGHIAN TUBULES

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Accepted 17 July 1996

Summary

There are two major transport pathways across epithelia: a transcellular pathway through cells and a paracellular pathway between cells. Previous electrophysiological studies in mosquito Malpighian tubules suggested that the neuropeptide leucokinin-VIII (LK-VIII) increases the chloride permeability of the paracellular pathway. To test the effect of LK-VIII on the paracellular pathway further, we measured transepithelial permeabilities of inulin and sucrose in isolated Malpighian tubules from the mosquito *Aedes aegypti*. Cell membranes are impermeable to inulin and sucrose, leaving the paracellular pathway as the only route for their transepithelial permeation. LK-VIII (10^{-6} mol l^{-1}) significantly increased transepithelial permeability to both inulin (by 73.8 %) and sucrose (by 32.4 %) in parallel with a significant increase in rates of transepithelial fluid secretion (by 75–90 %). Cyclic adenosine monophosphate (cyclic AMP, 10^{-4} mol l^{-1}), which is known to stimulate transcellular transport, also increased rates of transepithelial fluid secretion (by 57–59 %), but it did so without increasing the permeability to sucrose and inulin. Thus, LK-VIII increases the permeability of the paracellular pathway whereas cyclic AMP does not.

Key words: insect neuropeptide, epithelial transport, inulin, sucrose, paracellular pathway, cyclic adenosine monophosphate, mosquito, *Aedes aegypti*.

Introduction

The Malpighian tubule of the yellow fever mosquito consists of two types of epithelial cells, principal cells and stellate cells, which define the transcellular transport pathway (Beyenbach, 1995). The space between these epithelial cells, including tight junctions (septate junctions in insects), defines the paracellular transport pathway (Bradley and Snyder, 1989). Frömter and Diamond (1972) were the first to recognize the important role of the paracellular pathway in determining the general function of an epithelium. A high permeability of this pathway makes the epithelium suitable for high rates of transepithelial transport, as in 'leaky epithelia', whereas a low permeability of this pathway makes the epithelium more suitable for storage functions, as in 'tight epithelia'. Since epithelia have control over their relative leakiness or tightness, there is considerable interest in the physiological regulation of the paracellular pathway (Anderson and van Itallie, 1995).

The leucokinins are a class of insect neuropeptides that were first isolated from the cockroach *Leucophaea maderae* by Holman *et al.* (1987). They increase fluid secretion by Malpighian tubules (Hayes *et al.* 1989), and they stimulate contractions of the insect hindgut (Holman *et al.* 1991). Thus, leucokinins functionally couple Malpighian tubules and hindgut for the rapid excretion of unwanted solutes and fluid.

At the level of Malpighian tubules, leucokinin-VIII increases transepithelial fluid secretion by increasing a Cl^- permeability that cannot be localized in principal cells (Pannabecker *et al.* 1993). Moreover, leucokinin-VIII changes the Malpighian tubule from a moderately tight epithelium to a leaky epithelium, pointing to an increase in paracellular permeability (Pannabecker *et al.* 1993).

The purpose of this study, then, was to determine whether leucokinin-VIII increases paracellular permeability. Since cell membranes are impermeable to inulin and sucrose, they provide convenient markers of transport between epithelial cells through the paracellular pathway. Thus, if leucokinin-VIII increases paracellular permeability, it will increase the transepithelial permeability to inulin or sucrose.

Materials and methods

General experimental design

Malpighian tubules were isolated from females of the yellow fever mosquito (*Aedes aegypti*) and prepared for *in*

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vitro measurements of transepithelial fluid secretion by the method of Ramsay (1954) modified by us as described previously (Pannabecker *et al.* 1993). In each tubule experiment, rates of transepithelial fluid secretion and isotopic inulin (or sucrose) flux were first measured for 30 min in the absence of leucokinin and then for 30 min in the presence of leucokinin. Thus, each tubule served as its own control, allowing statistical analysis using the Student's *t*-test for paired samples (control *versus* experiment). Data are presented as means ± S.E.M.; statistical significance is considered for values of $P<0.05$.

Mosquitoes

Adult female mosquitoes, 3–7 days post-emergence, were used in this study. Mosquitoes were reared as described by Pannabecker *et al.* (1993) and maintained in a controlled environment at 26 °C and a light:dark cycle of 12 h:12 h. The mosquitoes were fed 3 % sucrose *ad libitum*.

Ringer's solutions

Mosquito Ringer solution was prepared fresh on the day of the experiment and contained (in mmol l^{-1}): NaCl, 150; KCl, 3.4; $NaHCO_3$, 1.8; $CaCl_2$, 1.7; $MgSO_4$, 1; Hepes, 25; glucose, 5; and NaOH, 7.5. The pH was adjusted to 7.1. The osmolarity of this Ringer solution was approximately 320 mosmol l^{-1}. This Ringer solution was used to isolate Malpighian tubules and to bathe them during the experiments. For measurements of transepithelial inulin and sucrose permeability, the peritubular Ringer bath (50 µl volume) was supplemented with [^{14}C]inulin or [^{14}C]sucrose (Sigma) to yield a specific activity of 740 kBq ml^{-1}. Synthetic leucokinin-VIII was made in the laboratory of T. K. Hayes (Texas A&M University) and used at a concentration of 10^{-6} mol l^{-1} (Hayes *et al.* 1989). Dibutyryl cyclic AMP (db-cAMP) was purchased from Sigma and used at a concentration of 10^{-4} mol l^{-1}.

Measurement of experimental variables

Rates of transepithelial fluid secretion were measured as described previously (Petzel *et al.* 1987). After a 30 min control (or experimental) period, the secreted fluid that had accumulated at the open end of the tubule (suspended in oil) was collected with a pipette and expelled into oil. The diameter of this droplet was measured for the determination of volume. The droplet was subsequently transferred to a scintillation vial containing 200 µl of milli-Q water to which 3 ml of scintillation fluid (ACS, Amersham) was added. A peritubular bath sample was prepared for ^{14}C counting in the same way. A Beckman LS6800 liquid scintillation counter was used in the channel ratio mode to correct for quenching.

The diffusion of non-electrolytes such as sucrose (or inulin) across isolated Malpighian tubules is described by the Fick equation (equation 1):

$$J_{solute} = P_{solute}\Delta[solute], \quad (1)$$

where J_{solute} is the rate of transepithelial sucrose or inulin secretion, i.e. the product of the rate of transepithelial fluid secretion and the concentration of sucrose or inulin in the secreted fluid, P_{solute} is the transepithelial sucrose permeability, and Δ[solute] is the difference between the solute concentrations in the peritubular medium and secreted fluid. Solute concentration was calculated from the known specific activities of ^{14}C-carboxylated inulin (111 MBq g^{-1}) and sucrose (12.65 GBq $mmol^{-1}$). Since the units of flux and concentration difference are mol min^{-1} and mol l^{-1}, respectively, it follows that solute permeability has the dimensions of volume/time in the present study. The conventional dimensions of permeability are length/time, which requires an exact knowledge of the surface area of the Malpighian tubule available for the transport of inulin or sucrose. Since this surface area is not known, we measure permeability as volume/time. However, tubule surface area remains the same throughout each experiment since each tubule is used as its own control.

Results

Variation of control data

Rates of transepithelial fluid secretion in isolated Malpighian tubules of *Aedes aegypti* are known to be quite variable (Beyenbach and Oviedo, 1993). The present study conducted over the course of 2 years using different populations of *Aedes aegypti* again illustrated the appreciable variability of measured baseline parameters (Figs 1, 2). To allow for this variability, each tubule was used as its own control.

The sham experiment

Since isolated Malpighian tubules are disturbed when secreted fluid is collected and when leucokinin-VIII or db-cAMP is added to the peritubular bath, it was important to show that these manipulations did not lead to artifactual changes in fluid secretion rate and epithelial permeability. Accordingly, seven Malpighian tubules were studied in a sham experiment that included all experimental steps except the inclusion of leucokinin-VIII or cyclic AMP when the peritubular Ringer was changed for the experimental period. During the control period, the mean rate of fluid secretion was 0.80±0.10 nl min^{-1} and the mean sucrose permeability was 249±20 pl min^{-1} (mean ± S.E.M., N=7 tubules). During the sham period, the mean rate of fluid secretion was 0.86±0.14 nl min^{-1} and the mean sucrose permeability was 274±43 pl min^{-1}. Neither change in fluid secretion rate nor sucrose permeability reached statistical significance.

Effects of leucokinin-VIII on fluid secretion and sucrose permeability

Leucokinin-VIII significantly increased transepithelial fluid secretion from 0.63±0.04 nl min^{-1} (control) to 1.20± 0.05 nl min^{-1} (Fig. 1A). In parallel, the permeability of the tubule to sucrose increased significantly from 101.8±15.0 to 134.8±19.4 pl min^{-1} (Fig. 1A).

Effects of db-cAMP on fluid secretion and sucrose permeability

The nucleotide db-cAMP (10^{-4} mol l^{-1}) increased

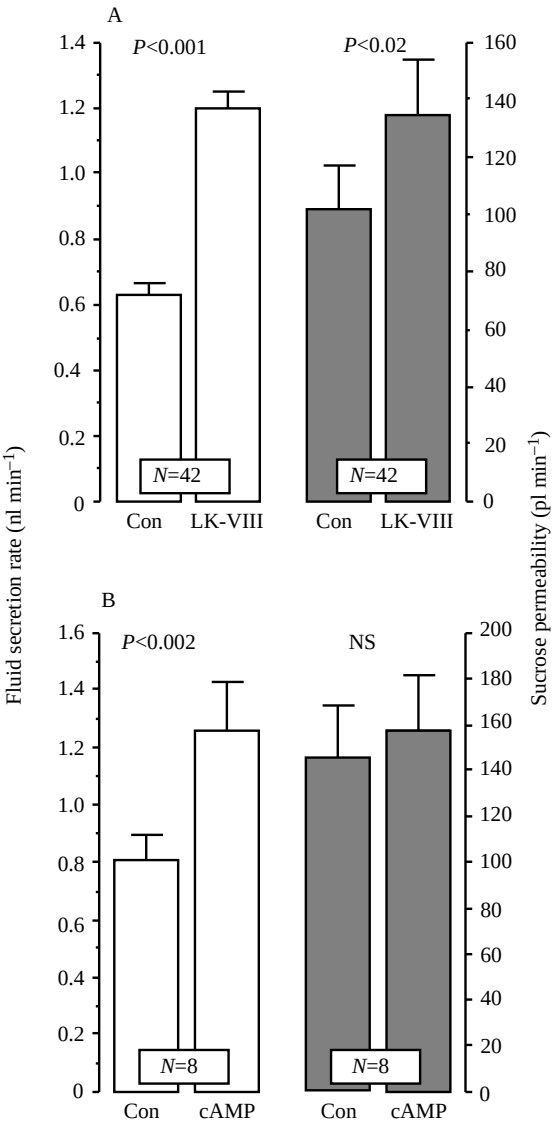

Fig. 1. Effects of leucokinin-VIII (LK-VIII) (A) and dibutyryl cyclic AMP (cAMP) (B) on rates of transepithelial fluid secretion (left ordinate) and sucrose permeability (right ordinate) in isolated Malpighian tubules of *Aedes aegypti*. Data are means + S.E.M. Con, control tubules; NS, not significant.

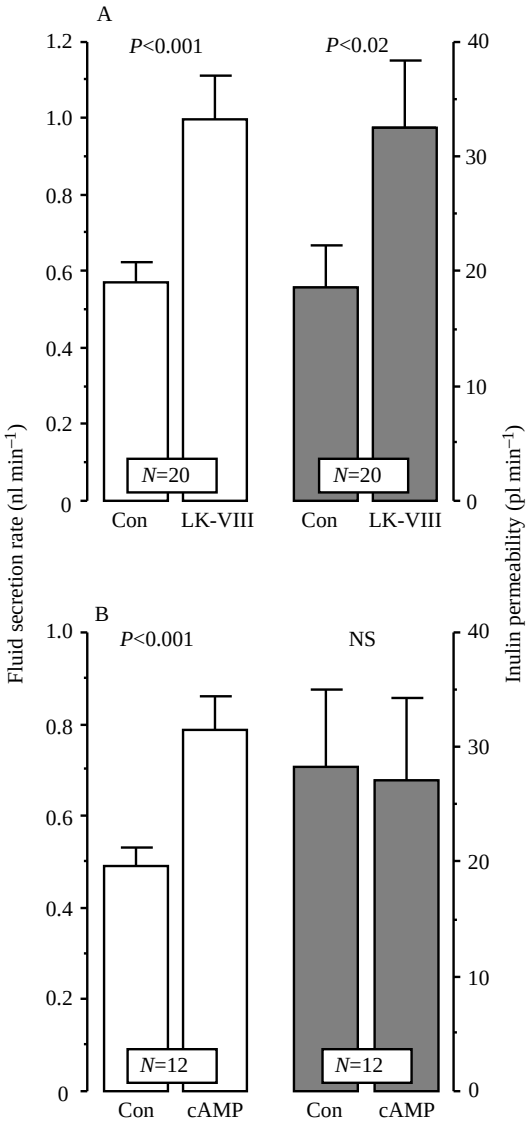

Fig. 2. Effects of leucokinin-VIII (LK-VIII) (A) and dibutyryl cyclic AMP (cAMP) (B) on rates of transepithelial fluid secretion (left ordinate) and inulin permeability (right ordinate) in isolated Malpighian tubules of *Aedes aegypti*. Data are means + S.E.M. Con, control tubules; NS, not significant.

transepithelial fluid secretion significantly from 0.80± 0.09 nl min^{-1} (control) to 1.26±0.17 nl min^{-1} (Fig. 1B). In contrast, the increase in sucrose permeability from 144.9±23.0 pl min^{-1} to 157.4±23.5 pl min^{-1} was not significant (Fig. 1B).

Effects of leucokinin-VIII on fluid secretion and inulin permeability

Leucokinin-VIII increased transepithelial fluid secretion significantly from 0.57±0.05 nl min^{-1} (control) to 1.00±0.11 nl min^{-1} (Fig. 2A). In parallel, the permeability of the tubule to inulin increased significantly from 18.7±3.6 to 32.5±5.7 pl min^{-1} (Fig. 2A).

Effects of db-cAMP on fluid secretion and inulin permeability

The nucleotide db-cAMP (10^{-4} mol l^{-1}) increased transepithelial fluid secretion significantly from 0.49±0.04 nl min^{-1} (control) to 0.78±0.08 nl min^{-1} (Fig. 2B). In contrast, the decrease in inulin permeability from 28.1±6.8 to 27.0±7.2 pl min^{-1} was not significant (Fig. 2B).

Discussion

Unusual effects of leucokinin-VIII

In a previous study that probed the mechanism of action of leucokinin-VIII in Malpighian tubules, we made the unusual

observation that this insect octapeptide transformed a moderately tight epithelium to a leaky epithelium in a matter of seconds (Pannabecker *et al.* 1993). No sooner had leucokinin-VIII entered the perfusion bath than multiple electrophysiological changes followed in parallel: (1) the transepithelial voltage dropped from 59 to 6 mV, (2) the transepithelial resistance fell from 58 to 10 Ω cm^2, and (3) the basolateral and apical membrane voltages of principal cells hyperpolarized and depolarized, respectively, to meet at voltages between 90 and 100 mV. These electrophysiological changes are expected from an epithelium that short-circuits itself, and such self-short-circuiting had not been observed before, let alone induced by an extracellular neuropeptide such as leucokinin-VIII. Moreover, these effects of leucokinin-VIII were dependent on the presence of Cl^- in the extracellular solutions. The symmetrical transepithelial Cl^- diffusion potentials and the increased magnitude of these potentials for bath-to-lumen- and lumen-to-bath-directed Cl^- gradients after treatment with leucokinin-VIII suggested that the principal effect of leucokinin-VIII was to increase the Cl^- permeability of the paracellular pathway. This hypothesis seemed to be supported by the non-selective increase in transepithelial secretion rates of both NaCl and KCl as the mechanism for increasing transepithelial fluid secretion (Pannabecker *et al.* 1993), but definitive proof of the increase in paracellular permeability was lacking.

The significant barrier to paracellular transport in epithelia is the tight junction, in insects referred to as septate junctions (Frömter and Diamond, 1972; Bradley and Snyder, 1989). Unfortunately, 'tight' junction is a misnomer that suggests an absolute barrier (Cereijido, 1992). Since Frömter's and Diamond's discovery of the important role of the paracellular pathway in the physiology of epithelia, numerous studies have shown that junctional barriers are not absolute and that solute and water permeabilities can vary over several orders of magnitude in different epithelia (Frömter and Diamond, 1972; Powell, 1981; Anderson and van Itallie, 1995). That an insect neuropeptide such as leucokinin-VIII had the power to alter junctional permeability in a single epithelium had not been observed before (Pannabecker *et al.* 1993). Accordingly, the uniqueness of this observation required that we obtain independent confirmation of rapid, reversible physiological changes in junctional permeability.

Junctional permeability

Tight junctions display ionic permselectivities (Tabei and Imai, 1986; Bakker and Groot, 1989; Weinstein *et al.* 1989). In Malpighian tubules of *Aedes aegypti*, the junctions are permselective to Cl^- (Pannabecker *et al.* 1993). This junctional Cl^- permeability offers a paracellular pathway for Cl^-, allowing Cl^- to serve as a counterion for the cations Na^+ and K^+ that are secreted across the epithelium by energy-dependent, and therefore transcellular, pathways (Beyenbach, 1995). Thus, transcellular secretion of Na^+ and K^+ is electrically coupled to paracellular secretion of Cl^-, and rates of NaCl and KCl secretion increase when paracellular Cl^- permeability increases in the presence of leucokinin-VIII (Pannabecker *et al.* 1993; Beyenbach, 1995). In addition to offering an electrodiffusive pathway for ions, junctions also offer a transepithelial pathway for molecules as large as sucrose and inulin (O'Donnell and Maddrell, 1983; Skaer *et al.* 1987; Whittembury *et al.* 1986; Hernandez *et al.* 1995). Hence, if transepithelial permeability to sucrose or inulin increases in the presence of leucokinin-VIII, a mechanism involving junctions would be indicated.

As shown in the present study, Malpighian tubules are permeable to sucrose and inulin (Figs 1, 2). However, permeabilities are low. First, concentrations of sucrose in fluid secreted into the tubule lumen were one-sixth of the concentrations in the peritubular Ringer medium where sucrose was added. Concentrations of inulin in secreted fluid were even lower, only 1/22 the concentration in the peritubular Ringer. Second, under control conditions, the permeability of Malpighian tubules to sucrose was approximately eight times greater than the permeability to inulin. These differences probably reflect the different cross-sectional diameters of these molecules: 1.5 nm for inulin and 0.9 nm for sucrose (Ma *et al.* 1995; Bhakdi *et al.* 1986). Although leucokinin-VIII significantly increased permeability to inulin and sucrose, these permeabilities are still orders of magnitude lower than Cl^- permeabilities.

Mechanisms of paracellular transport

In a recent study of paracellular permeation in Malpighian tubules of *Rhodnius prolixus*, Hernandez *et al.* (1995) have examined the transepithelial flux of a number of extracellular fluid markers in relation to transepithelial water flow. Good correlation between transepithelial solute and water flow was observed in the case of small solutes such as sucrose, indicating entrainment of solute by water, i.e. solvent drag. No correlations were observed between the transepithelial flux of inulin and water flow, indicating the movement of inulin across the epithelium by diffusion (Whittembury *et al.* 1986). Analyses of other solute and water fluxes suggested that solvent drag of small molecules takes place through a slit 1.1 nm wide, presumably located in the paracellular pathway of *Rhodnius* Malpighian tubules. Our studies in *Aedes* Malpighian tubules confirm these observations in *Rhodnius*. We consistently observed good correlations ($0.58<r<0.87$) between the net secretory flux of sucrose and rates of transepithelial fluid secretion, but we did not observe any correlations between inulin and fluid secretion. Thus, the paracellular pathways in Malpighian tubules of *Aedes* and *Rhodnius* appear to be functionally similar in providing transepithelial extracellular routes for diffusion of large molecules and for solvent drag of small molecules.

Effects on the paracellular pathway in general, and on tight junctions in particular, have always been difficult to prove since one cannot always rule out effects on the transcellular pathway. In this regard the known effects of db-cAMP on transepithelial electrolyte and fluid secretion in *Aedes* Malpighian tubules served as a welcome negative control. The

nucleotide is known to stimulate transepithelial secretion of NaCl (and water) because of its selective effect on the transcellular pathway, where it increases the Na^+ conductance of the basolateral membrane of principal cells (Sawyer and Beyenbach, 1985). Thus, db-cAMP primarily affects transcellular transport pathways. In the present study, db-cAMP significantly increased transepithelial fluid secretion but it had no effect on the permeabilities of inulin and sucrose. In contrast, leucokinin-VIII increased transepithelial permeability to inulin and sucrose while increasing transepithelial fluid secretion (Figs 1, 2). These results confirm independent mechanisms for stimulating transepithelial fluid secretion in *Aedes* Malpighian tubules: (1) stimulation of Na^+-dependent, transcellular transport by cyclic AMP (Beyenbach and Petzel, 1987), and (2) stimulation of Cl^--dependent, paracellular transport by leucokinin-VIII (Pannabecker *et al.* 1993). In the present study, both cyclic AMP and leucokinin-VIII increased fluid secretion as well as transepithelial net secretion of inulin and sucrose. But only leucokinin-VIII increased the transepithelial permeability to inulin and sucrose. Since inulin and sucrose are confined to the extracellular space, this increase must take place in the paracellular pathway. Thus, the conclusion from this study is that leucokinin-VIII increases junctional permeability in *Aedes* Malpighian tubules, and the marked drop in transepithelial resistance and the self-short-circuiting of the Malpighian tubule that we previously observed in the presence of leucokinin-VIII stem largely from the increase in paracellular permeability (Pannabecker *et al.* 1993).

As to the mechanisms that couple leucokinin-VIII to the increase in junctional permeability in Malpighian tubules, studies of vertebrate tight junctions provide the first ideas of how septate junctions might be regulated in insects. In brief, vertebrate tight junctions are associated with integral and peripheral membrane proteins that interact with the cell cytoskeleton, in particular a perijunctional ring of actin and myosin. Contractions of this perijunctional ring are thought to affect tight junction structure and permeability (Madara, 1989). The dynamic regulation of this perijunctional ring *via* intracellular signalling pathways is presently considered to be the clearest mechanism for the regulation of tight junctional permeability (Anderson and van Itallie, 1995).

The authors would like to thank the National Science Foundation for financial support (DCB 8403305, IBN 9220464). Parts of this work were submitted by A.R. and S.W. as undergraduate theses in fulfillment for graduating from Cornell with Honors in Biology.

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