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Effects of leucokinin-VIII on *Aedes* Malpighian tubule segments lacking stellate cells

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Summary

The diuretic peptide leucokinin is known to increase fluid secretion in Malpighian tubules of the yellow fever mosquito *Aedes aegypti* by increasing a transepithelial Cl^- conductance. The present study sought to examine whether stellate cells provided this transepithelial conductance in *Aedes* Malpighian tubules as they do in *Drosophila* Malpighian tubules. *Aedes* Malpighian tubule segments with and without stellate cells were perfused *in vitro* for measurements of the transepithelial voltage (V_t), resistance (R_t) and Cl^- diffusion potentials (DP_{Cl}). In 11 tubule segments containing both principal cells and stellate cells, 1 μmol l^{-1} leucokinin-VIII added to the peritubular bath immediately and significantly decreased V_t from 39.3±14.3 mV to 2.3±0.7 mV, decreased R_t from 12.4±2.6 kΩcm to 2.4±0.3 kΩcm, and increased DP_{Cl} from 8.2±1.2 mV to 42.1±5.4 mV. These effects of leucokinin-VIII were qualitatively and quantitatively similar in six tubule segments containing no stellate cells; V_t decreased from 37.8±7.0 mV to 3.4±0.6 mV, R_t decreased from 8.8±2.1 kΩcm to 1.7±0.2 kΩcm, and DP_{Cl} increased from 5.8±2.6 mV to 50.0±2.1 mV. Thus, stellate cells are not required for signaling or mediating the effects of leucokinin in Malpighian tubules of *Aedes aegypti*. The results further support previous observations that principal cells signal the effects of leucokinin to increase the Cl^- conductance of the paracellular pathway through septate (or tight) junctions.

Key words: leucokinin, paracellular Cl^- conductance, tight junction, septate junction, Malpighian tubule, yellow fever mosquito, *Aedes aegypti*.

Introduction

The leucokinins are considered cephalomyotropic peptides, to indicate their isolation from the head of the cockroach *Leucophaea maderae* and their ability to increase muscular contractions in the cockroach hindgut (Holman et al., 1987). Curious that agents which stimulate hindgut contractions might also enhance excretory activity in Malpighian tubules upstream, we discovered that leucokinin has diuretic effects in Malpighian tubules of the yellow fever mosquito *Aedes aegypti* (Hayes et al., 1989). Since then, the stimulation of fluid secretion by leucokinin and other kinins has been found in Malpighian tubules of the house cricket (Coast et al., 1990), locust (Thompson et al., 1995), tobacco hornworm (Blackburn et al., 1995), fruit fly (O'Donnell et al., 1996) and housefly (Iaboni et al., 1998). In Malpighian tubules of the yellow fever mosquito, locust and cricket, leucokinin is thought to increase transepithelial secretion of Cl^-, thereby increasing the transepithelial secretion of Na^+, K^+ and water (Coast, 2001; Pannabecker et al., 1993). In Malpighian tubules of the yellow fever mosquito, leucokinin-VIII increases a transepithelial Cl^- conductance (Pannabecker et al., 1993), thereby increasing intraepithelial transport currents with the effect of increasing transepithelial secretion of NaCl, KCl and water (Beyenbach, 2001).

There is good agreement that the transepithelial Cl^- conductance activated by leucokinin does not pass through principal cells of Malpighian tubules (O'Donnell et al., 1996; Pannabecker et al., 1993). Stellate cells are thought to offer the transepithelial Cl^- conductance in *Drosophila melanogaster* Malpighian tubules, responding to drosokinin, the leucokinin-like diuretic in this species (O'Donnell et al., 1998). Currents measured near stellate cells, not principal cells, were found to be sensitive to Cl^- channel blockers, consistent with the activation of a transcellular Cl^- conductance through stellate cells (O'Donnell et al., 1998). Moreover, the drosokinin receptor and Ca^{2+} signaling pathway have been localized in stellate cells (Pollock et al., 2003; Radford et al., 2002). In contrast, studies of *Aedes aegypti* Malpighian tubules in our laboratory have found that leucokinin activates a Ca^{2+} signaling pathway in principal cells (Yu and Beyenbach, 2002), which in turn increases the transepithelial Cl^- conductance of the paracellular pathway (Pannabecker et al., 1993). Thus, the site of the transepithelial Cl^- conductance activated by kinins seems to be species-specific: the paracellular pathway in *Aedes* Malpighian tubules and a transcellular pathway through stellate cells in *Drosophila* Malpighian tubules.

In Malpighian tubules of four mosquito species, including

Aedes aegypti, stellate cells comprise 14–21% of the cell population (Satmary and Bradley, 1984). Thus, 1 in 5 epithelial cells along the length of the tubule is a stellate cell. The paucity of stellate cells makes it possible to select and dissect for study tubule segments that do not contain stellate cells. Leucokinin-VIII activated a transepithelial Cl^- conductance in these tubule segments free of stellate cells, indicating that stellate cells are neither needed for signaling nor for mediating the effects of leucokinin on transepithelial Cl^- conductance. These observations strengthen the case for a diuretic mechanism mediated by principal cells and executed *via* alterations in the paracellular pathway through septate junctions.

Materials and methods

Mosquitoes, Malpighian tubules and Ringer solution

The colony of mosquitoes *Aedes aegypti* L. was maintained as described previously (Pannabecker et al., 1993). On the day of an experiment, a female mosquito (3–7 days post-eclosion) was cold-anesthetized and decapitated. The Malpighian tubules, approximately 3 mm in length, were removed from the abdominal cavity under Ringer solution. After removing the terminal blind end (400–600 μm) of the tubule, 150–300 μm segments of the secretory portion of the tubule were isolated for study *in vitro*. To ensure that only principal cells and no stellate cells were present in the tubule segments, we inspected the tubules under a stereomicroscope (StereoZoom 4, Bausch & Lomb, Rochester, NY, USA) prior to dissection and perfusion. In addition, we kept the distance between the two glass pipettes holding the tubule segment in the Ringer bath between 100 μm and 200 μm, i.e. a length that is less than two principal cells and that contains no stellate cells (Fig. 1B). When stellate cells were present, the tubule segment was aspirated into the holding pipette where stellate cells could be isolated from the peritubular Ringer bath using a Sylgard® 184 seal (Fig. 1). Prior to *in vitro* microperfusion, tubule segments were carefully examined for the presence/absence of stellate cells. Stellate cells are easy to identify. They are slender and transparent, with rounded endings at their extensions, in sharp contrast to bulky opaque principal cells (Figs 2C, 3A), as in *Drosophila* Malpighian tubules (Rosay et al., 1997).

Ringer solution contained, in mmol l^{-1}: 150.0 NaCl, 7.5 NaOH, 3.4 KCl, 1.8 $NaHCO_3$, 1.7 $CaCl_2$, 1.0 $MgSO_4$, 5.0 glucose and 25.0 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes). The pH was adjusted to 7.1 with 1 mol l^{-1} NaOH. Synthetic leucokinin-VIII was a gift from Ron Nachman (USDA, College Station, TX, USA), and was used at a concentration of 1 μmol l^{-1}, which is required to exert maximal effects on tubule electrophysiology and fluid secretion (Hayes et al., 1989; Veenstra et al., 1997). Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and Fisher Scientific (Suwanee, GA, USA).

In vitro *microperfusion of Malpighian tubules*

Fig. 1A illustrates the method for measuring transepithelial voltage and resistance in isolated perfused Malpighian tubules (Burg et al., 1966; Helman, 1972). The tubule lumen was cannulated with a double-barreled perfusion pipette of outer diameter approximately 10 μm (Theta-Borosilicate glass, #1402401; Hilgenberg, D-34323 Malsfeld, Germany). One barrel of this pipette was used to perfuse the tubule lumen with Ringer solution and to measure 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 measurements of the transepithelial resistance (R_t) by cable analysis (Helman, 1972). The peritubular bath (500 μl) was perfused with Ringer solution at a rate of 6 ml min^{-1}. V_t was recorded continuously, and R_t was measured periodically when of interest. The electric isolation of the tubule lumen was ensured with pipette dimensions that fitted the outer and inner diameters of the tubule. In addition, a viscous resin of high dielectric constant, Sylgard® 184 (Dow Corning Inc., Auburn, MI, USA), was used to secure electrical insulation in the collecting pipette (Fig. 1A,B). All voltage measurements were done with custom-made high impedance amplifiers (Burr-Brown, $10^{11}\Omega$). A permanent recording of voltage during the experiment was produced using a strip chart recorder (Model BD 64, Kipp and Zonen, Bohemia, NY, USA).

Fig. 1A illustrates a tubule segment that includes a stellate cell, and Fig. 1B the perfusion of a tubule segment devoid of stellate cells. Fig. 1C shows an equivalent electrical circuit of transepithelial electrolyte secretion in *Aedes* Malpighian tubules. The transcellular active transport pathways for Na^+ and K^+ through principal cells at basolateral and apical membranes are lumped together in a single resistor R_c, the transcellular resistance. Active transcellular transport is driven by an electromotive force (E_c) generated at the apical membrane by the V-type H^+-ATPase. Parallel to active transcellular cation transport is passive transport of Cl^- through the epithelial shunt (R_{sh}) located outside principal cells. The active and passive transport pathways form an intraepithelial electric circuit (Fig. 1C). Since the intraepithelial loop current (I) is the same in active and passive transport pathways, it follows that the rate of transcellular cation (Na^+ and K^+) secretion equals the rate of Cl^- secretion through the shunt, thereby conserving the electroneutrality of the fluid on both sides of the epithelium.

Transepithelial Cl^- diffusion potential

The amplitude of transepithelial Cl^- diffusion potentials was measured as the change in transepithelial voltage upon a tenfold isosmotic replacement of peritubular Cl^- with isethionate. The measurement assumes a low permeability of the tubule to isethionate (Yu and Beyenbach, 2001). The Cl^- concentration in the tubule lumen was maintained at 156.8 mmol l^{-1} by perfusing the tubule lumen with normal Ringer at rates less than 5 nl min^{-1}. The tenfold reduction in the peritubular Cl^- concentration drove the diffusion of Cl^- from the tubule lumen to the peritubular bath, generating lumen-positive transepithelial diffusion potentials with magnitudes proportional to transepithelial Cl^- conductance.

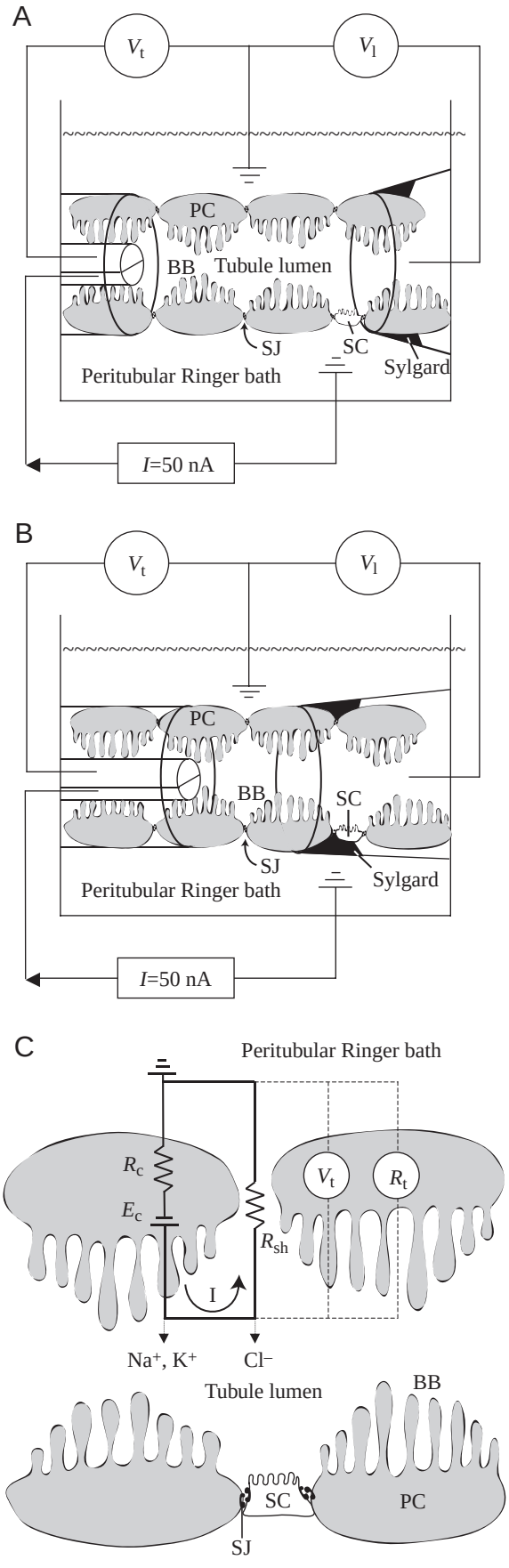

Fig. 1. *In vitro* microperfusion of Malpighian tubules of the yellow fever mosquito, *Aedes aegypti*. (A) Perfusion of a tubule segment containing a stellate cell. (B) Perfusion of a tubule segment without stellate cells. The lumen of Malpighian tubules was cannulated with a double-barreled glass pipette for perfusion of the tubule lumen and for measurements of the transepithelial voltage (V_t) and resistance (R_t). V_l is the voltage measured at the distal end of the perfused segment. A current (I=50 nA) was injected into the tubule lumen when measurements of R_t were desired. BB, brush border; PC, principal cell; SC, stellate cell; SJ, septate junction. (C) Simple electrical equivalent circuit of transepithelial electrolyte transport across Malpighian tubules. E_c, electromotive force of the active transport pathway; I, intraepithelial current; R_c, transcellular resistance; R_{sh}, shunt resistance. R_t is the total resistance of R_c and R_{sh} in parallel.

Light and electron microscopy

For light microscopy, *Aedes* Malpighian tubules were transferred to 80 µl Ringer solution on a poly-L-lysine coated microscope slide and covered with a 22 mm × 30 mm coverslip. The edges of the coverslip were sealed with Permount® to prevent evaporation of the Ringer solution. Digital images were taken with an upright microscope (Leica DMLB, Wetslar, Germany) equipped with a digital camera (MagnaFire S99802, Optronics, Goleta, CA, USA), hardware (IEEE-1394 PCI host controller) and software (MagnaFire 2.1A). For electron microscopy, the Malpighian tubules were prepared as described previously (O'Connor and Beyenbach, 2001). Sections were cut to a thickness of 70 nm. Electron micrographs of the tubules were produced with a Philips Tecnai 12 Biotwin transmission electron microscope (FEI, Eindhoven, Netherlands).

Statistical evaluation of data

Each tubule served as its own control. Accordingly, the data were analyzed for the differences between paired samples, control *versus* experimental (leucokinin-VIII), using the paired Student's *t*-test.

Results

Principal cells and stellate cells in the Aedes Malpighian tubules

Fig. 2A illustrates the excretory system in the yellow fever mosquito *Aedes aegypti*, including the five Malpighian tubules, which empty their secretions into the gut at the junction of the midgut and the hindgut. As in other mosquitoes, principal cells dominate the makeup of the Malpighian tubule, accounting for 79–86% of the cell population (Satmary and Bradley, 1984). The remaining cells are stellate cells. Malpighian tubules of females are much larger than those of males (Plawner et al., 1991). Moreover, principal cells in Malpighian tubules from females contain large quantities of intracellular concretions, which obstruct a view of the tubule lumen (Bradley et al., 1982). Intracellular concretions reflect light, making their

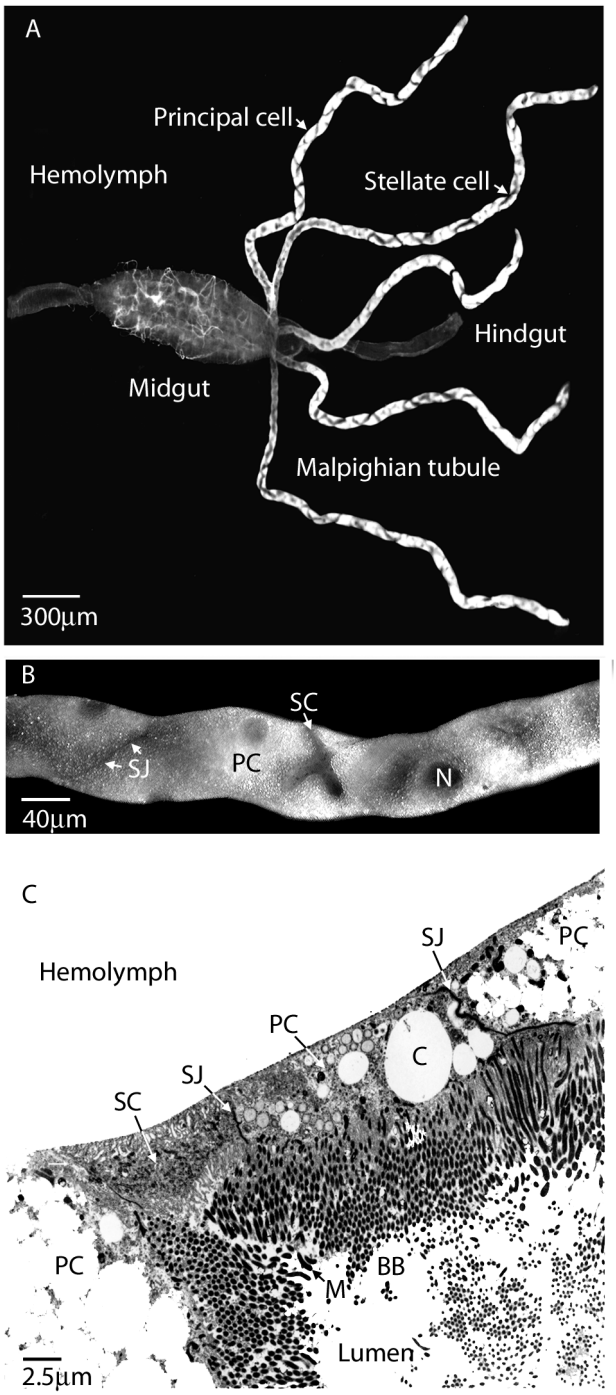

Fig. 2. Malpighian tubules of the female yellow fever mosquito, *Aedes aegypti*, examined by light (A,B) and electron (C) microscopy. BB, brush border; C, intracellular concretion; M, mitochondrion; N, nucleus; PC, principal cell; SC, stellate cell; SJ, septate junction. Scale bars, 300 μm (A), 40 μm (B), 2.5 μm (C).

principal cells appear white when the tubule is viewed against a dark background (Fig. 2A,B). In principal cells of *Drosophila* Malpighian tubules, there are two types of concretions (Wessing et al., 1992): type I, which contain Ca^{2+} and Mg^{2+}, and type II, which contain K^+.

Principal cells are large and spindle-shaped, 80–120 μm long (Fig. 2B; Masia et al., 2000). Their thickness is greatest (~30 μm) near the cell center, where the large nucleus is located. As illustrated in Fig. 2C, cell thickness decreases towards the lateral edges of principal cells where they contact other principal or stellate cells at septate junctions. The lateral interstitial space between epithelial cells is much diminished in view of the fusiform shape of principal cells. Accordingly, transepithelial diffusion potentials reflecting paracellular permeability reflect primarily the permeability of septate junctions.

In contrast to the large, fusiform, and opaque principal cells, stellate cells are small, thin and transparent (Fig. 2B,C). In addition, stellate cells have the characteristic shape of a star with rounded points (Fig. 2B; Wessing et al., 1992). Stellate cells are 50–150 μm long and less than 5 μm thick (Fig. 2B,C). Their thinness and lack of intracellular concretions make them transparent, yielding a view of the tubule lumen (Fig. 2B). Due to their small size and number, stellate cells appear sporadically along the length of the tubule. Therefore, tubule segments 200–250 μm long frequently lack stellate cells (Fig. 2A,B).

Fig. 2C illustrates principal and stellate cells and their septate junctions. Principal cells have a prominent brush border where each long microvillus is home to a mitochondrion. The brush border of stellate cells is short and devoid of mitochondria. In addition, stellate cells have many deep infoldings of the basal membrane facing the hemolymph (Bradley et al., 1982).

The effects of leucokinin-VIII on Malpighian tubule segments with and without stellate cells

Fig. 3 summarizes the effects of leucokinin-VIII in segments of *Aedes* Malpighian tubules with (A–D) and without (E–H) stellate cells. A stellate cell is clearly seen in Fig. 3A. In contrast, Fig. 3E illustrates a tubule segment consisting only of principal cells and septate junctions. Principal cells thinning out towards their lateral edges also make them transparent near the septate junctions. However, the presence of intracellular concretions in these lateral transparent zones clearly identifies principal cells rather than transparent extensions of stellate cells.

Eleven Malpighian tubule segments containing both principal and stellate cells were studied in the absence (control) and presence of leucokinin-VIII (Fig. 3A–D). The perfused tubule segments were 290.9±16.3 μm long. In the absence of leucokinin-VIII and under symmetrical perfusion with Ringer solution in the tubule lumen and peritubular bath, the transepithelial voltage was 39.3±7.3 mV and the transepithelial resistance was 12.4±2.6 kΩcm (Fig. 3B,C). The tubule segments displayed small transepithelial Cl^- diffusion potentials, 8.2±1.2 mV, in response to a tenfold replacement of peritubular Cl^- with isethionate (Fig. 3D). Upon the addition of 1 μmol l^{-1} leucokinin-VIII to the peritubular bath, the transepithelial voltage dropped significantly from 39.3 mV to 2.3±0.7 mV, and the transepithelial resistance dropped

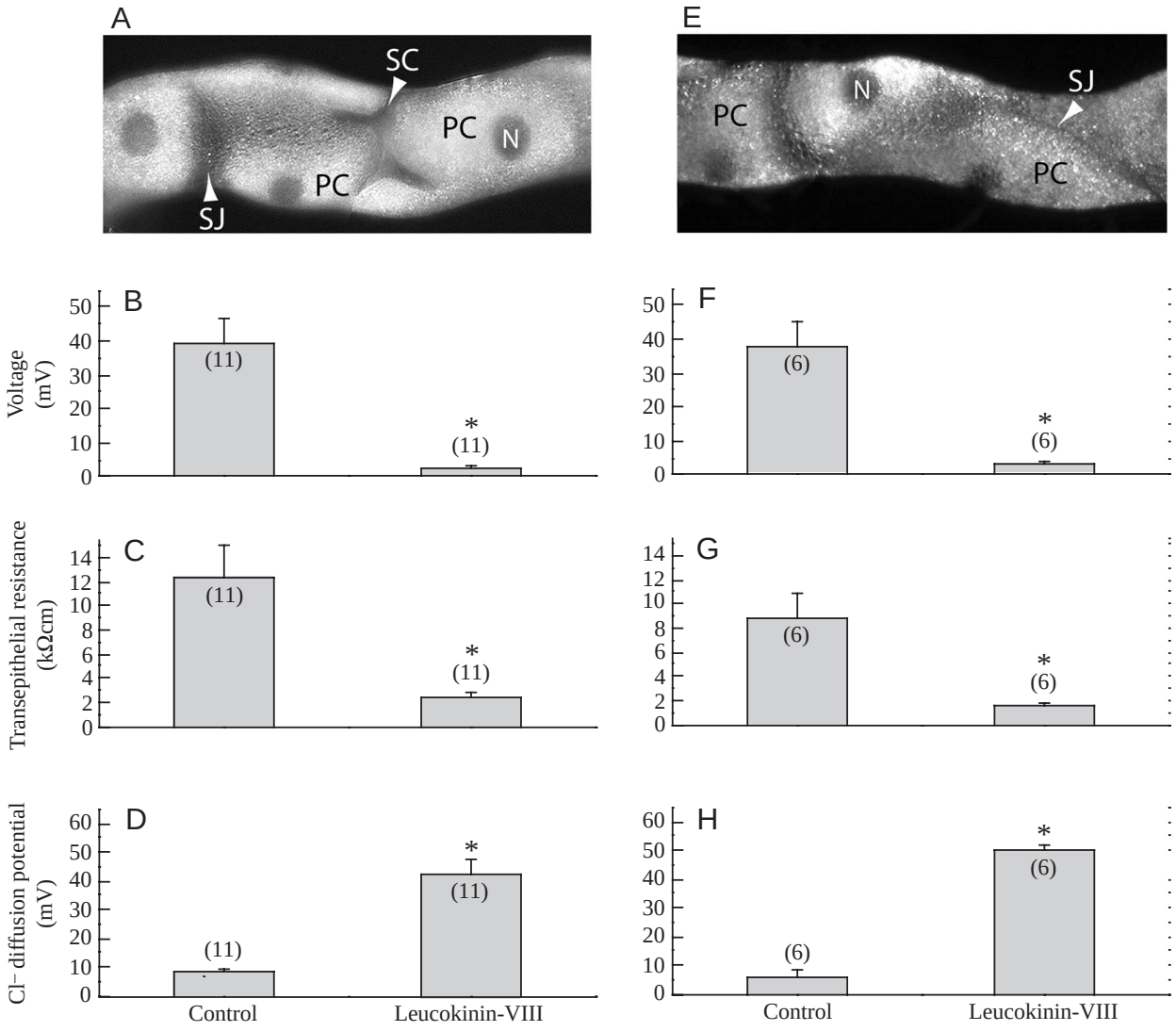

Fig. 3. The electrophysiological effects of leucokinin-VIII (1 μmol l^{-1}) on *Aedes* Malpighian tubule segments with (A–D) or without (E–H) stellate cells. N, nucleus; PC, principal cell; SC, stellate cell; SJ, septate junction. Values are means ± S.E.M. (*N*, number of Malpighian tubules). Asterisks indicate significant difference (P<0.05).

significantly from 12.4 kΩcm to 2.4±0.3 kΩcm (Fig. 3B,C), turning these moderately 'tight' epithelia into 'leaky' epithelia. In parallel with the effects on transepithelial voltage and resistance, leucokinin-VIII significantly increased the transepithelial Cl^- diffusion potential from 8.2 mV to 42.1±5.4 mV (Fig. 3D), indicating the activation of a transepithelial Cl^- conductance.

The above experiments were repeated in six Malpighian tubule segments containing only principal cells and their septate junctions (Fig. 3E–H). The perfused tubule segments were 146.7±15.8 μm long. The leucokinin response of these tubule segments without stellate cells was identical to that of tubule segments composed of principal and stellate cells. Again, leucokinin-VIII significantly decreased the transepithelial voltage from 37.8±7.0 mV to 3.4±0.6 mV, and it reduced the transepithelial resistance from 8.8±2.1 kΩcm to 1.7±0.2 kΩcm (Fig. 3F,G). At the same time, leucokinin significantly increased transepithelial Cl^- diffusion potentials from 5.8±2.6 mV to 50.0±2.1 mV (Fig. 3H). Clearly, the absence of stellate cells did not impair, or in any way diminish, the effects of leucokinin-VIII on epithelial electrophysiology.

One noteworthy feature of the leucokinin effects is the speed of switching the tubule between the 'tight' and the 'leaky' states (Fig. 4). Within seconds of adding leucokinin-VIII to the peritubular bath, transepithelial voltage dropped from 58.5 mV to 5.1 mV and transepithelial resistance dropped from 7.9 to 1.5 kΩcm, producing the 'leaky' epithelium state. The epithelium remained 'leaky' with low values of transepithelial voltage (4.6 mV) and resistance (1.5 kΩcm) as long as leucokinin-VIII was present. However, once leucokinin-VIII was removed from the peritubular bath, the transepithelial voltage and resistance quickly returned towards control levels

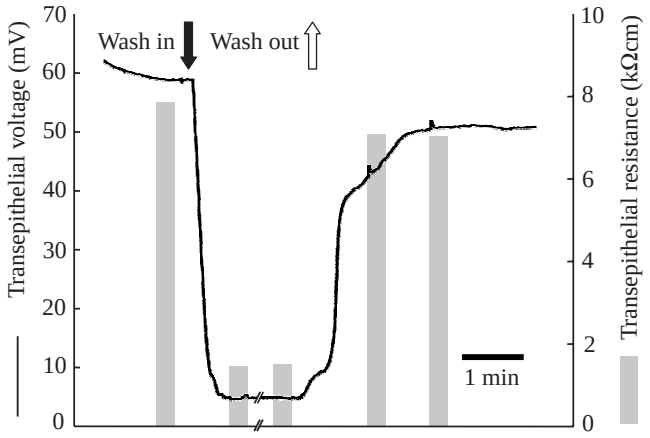

Fig. 4. Continuous time course of transepithelial voltage (solid line) and resistance changes measured at intervals (tinted bars) in the presence of leucokinin-VIII in *Aedes* Malpighian tubules devoid of stellate cells. A representative tubule experiment is shown. The on/off responses were as immediate as the changes in peritubular medium.

(50.1 mV and 7.1 kΩcm), switching the epithelium back to the 'tight' state, albeit somewhat slower than the rapid on-effect of leucokinin.

Discussion

Malpighian tubules and extracellular fluid homeostasis

Malpighian tubules are insect renal tubules that maintain extracellular fluid homeostasis (Beyenbach, 1993) by responding to peritubular ion concentrations (Leyssens et al., 1992; Van Kerkhove et al., 1989) or hormones (Coast et al., 2002). Antidiuretic hormones may assist in renal conservation of water, which is a constant challenge for terrestrial insects (Coast et al., 2002). Diuretic hormones may serve to reduce excess electrolytes and mass of the insect during eclosion or a blood meal. They may also assist in the renal excretion of metabolic water and toxins. One family of diuretic hormones, the leucokinins, were first isolated based on their myotropic activity in the hindgut of the cockroach *Leucophaea maderae* (Holman et al., 1987). Since the discovery of their diuretic potency in *Aedes* Malpighian tubules (Hayes et al., 1989), interest in the leucokinins has extended to signaling pathways and mechanisms of action (Coast, 2001; Coast et al., 1993; O'Donnell et al., 1996; Pannabecker et al., 1993; Pollock et al., 2003; Radford et al., 2002; Yu and Beyenbach, 2002).

Site of the leucokinin signaling pathway

Where their mechanism of action has been studied in Malpighian tubules, the leucokinins consistently increase transepithelial NaCl and KCl secretion, suggesting an effect on the secretion of Cl^-, the counterion common to transepithelial secretion of Na^+ and K^+ (Coast, 2001; Pannabecker et al., 1993). Either principal or stellate cells, or both, may respond to leucokinin. In *Drosophila* Malpighian tubules, stellate cells mediate the effects of drosokinin, the *Drosophila* leucokinin (Radford et al., 2002; Terhzaz et al., 1999). In the house cricket *Acheta domesticus*, principal cells must by default mediate the effects of achetakinins (the *Acheta* leucokinin) because *Acheta* Malpighian tubules do not possess stellate cells (Coast et al., 1990; Hazelton et al., 1988). However, Malpighian tubules of the blood-sucking bug *Rhodnius prolixus*, which also lack stellate cells, do not respond to leucokinin (Bradley, 1983; Te Brugge et al., 2002). Thus, responsiveness to leucokinin is not a universal property of Malpighian tubules, and in those Malpighian tubules that do respond to leucokinin, the presence of stellate cells is not necessary.

The large size of principal cells in Malpighian tubules of *Aedes aegypti* has allowed us to study signaling and transport processes in these cells. In contrast, the small size of stellate cells has precluded their study. For this reason we must infer the functions of stellate cells indirectly, by comparing the effects of leucokinin in the presence or absence of stellate cells. Since leucokinin elicited similar qualitative and quantitative effects in tubules regardless of the presence of stellate cells (Fig. 3), it is clear that stellate cells are not required to express the effects of leucokinin in Malpighian tubules of *Aedes aegypti*. Indeed, in a previous study that probed principal cells with intracellular microelectrodes we found that leucokinin activates a Ca^{2+}-signaling pathway (Yu and Beyenbach, 2002). In particular, leucokinin activated Ca^{2+} channels in the basolateral membrane of principal cells, allowing the entry of Ca^{2+} into the cell from the peritubular medium as one critical step in the signaling pathway.

Site of the transepithelial Cl^- conductance activated by leucokinin

Like the Ca-signaling pathway of leucokinin, the site of the transepithelial Cl^- conductance activated by leucokinin is species-specific. Both transcellular and paracellular pathways have been proposed. The evidence for a transcellular Cl^- pathway is strongest in Malpighian tubules of the fruit fly, whereas the evidence for a paracellular Cl^- conductance is strongest in Malpighian tubules of the yellow fever mosquito.

In *Drosophila* Malpighian tubules, stellate cells are thought to provide a transcellular route for Cl^- secretion in the presence of leucokinin for the following reasons: (1) the location of the drosokinin receptor in stellate cells and (2) the response of stellate cells to drosokinin with elevated intracellular Ca^{2+} concentration (Radford et al., 2002; Terhzaz et al., 1999). Other observations by O'Donnell et al. (1998) are consistent with a Cl^- transport pathway through stellate cells but do not prove it: (1) the identification of maxi-Cl^- channels in unspecified apical membrane domains of the tubule, (2) the paucity of maxi-Cl^- channels in only 5% of apical membrane patches and (3) the measurement of high currents sensitive to low extracellular Cl^- concentrations and Cl^- channels blockers in the vicinity of stellate cells. Together, these observations support the conclusion that leucokinin activates apical maxi-Cl^- channels of stellate cells *via* intracellular Ca^{2+}, thereby increasing transepithelial Cl^- secretion by *Drosophila* Malpighian tubules.

Studies of *Aedes* Malpighian tubules in our laboratory have revealed a dense population of low-conductance Cl^- channels in the apical membrane of stellate cells, which could mediate transepithelial Cl^- secretion in Malpighian tubules under control conditions (O'Connor and Beyenbach, 2001). However, in the presence of leucokinin a paracellular Cl^- conductance is activated, which overpowers any contribution that stellate cells might make to transepithelial Cl^- secretion. Our evidence supporting a paracellular Cl^- conductance activated by leucokinin is as follows: (1) leucokinin doubles the rate of transepithelial fluid secretion *via* a non-selective increase in NaCl and KCl secretion, suggesting the stimulation of the transport pathway for Cl^-, the counterion of Na^+ and K^+ (Pannabecker et al., 1993), (2) leucokinin drops both transepithelial resistance and voltage to values close to zero, turning the tubule into 'leaky' epithelium with high paracellular Cl^- conductance that allows transepithelial Cl^- diffusion potentials to reach 80% of the Cl^- Nernst potential (Pannabecker et al., 1993; Yu and Beyenbach, 2001), (3) the large transepithelial Cl^- diffusion potentials induced by leucokinin are similar for lumen-to-bath and bath-to-lumen transepithelial Cl^- gradients, pointing to diffusion potentials across a single barrier such as that of septate junctions (Pannabecker et al., 1993), (4) leucokinin depolarizes the apical membrane voltage and hyperpolarizes the basolateral membrane voltage, consistent with an increased paracellular conductance (Pannabecker et al., 1993; Yu and Beyenbach, 2002), and (5) leucokinin decreases the transepithelial resistance 4.3-fold but the input resistance of the principal cells only 1.7-fold (Masia et al., 2000; Yu and Beyenbach, 2001), pointing to a major resistance change outside principal cells. Thus, experimental data collected in studies employing four different experimental methods and conceptual approaches are internally consistent with leucokinin increasing the Cl^- conductance of the paracellular pathway in Malpighian tubules of the yellow fever mosquito.

Observing the full effects of leucokinin on transepithelial voltage, resistance and Cl^- diffusion potentials in tubule segments without stellate cells narrows the site of the activated Cl^- conductance to principal cells and/or the paracellular pathway associated with principal cells (Fig. 3). Principal cells can be ruled out because only an increase in paracellular Cl^- conductance can account for all the experimental voltage changes that are induced by leucokinin (Pannabecker et al., 1993). Furthermore, analysis of the epithelial circuit model yields only one conclusion that is supported by the experimental data: the increase in paracellular Cl^- conductance in the presence of leucokinin.

The strongest evidence that leucokinin activates a paracellular rather than a transcellular Cl^- conductance in *Aedes* tubules is obtained by examining the effect of leucokinin on transepithelial resistance (Pannabecker et al., 1993). Leucokinin lowers the shunt resistance from 52.5 Ωcm^2 to 5.8 Ωcm^2. The unilateral reduction of the Cl^- concentration to 5 mmol l^{-1} in the peritubular bath or tubule lumen increases the transepithelial resistance to only 16.9 and 20.1 Ωcm^2, respectively. Apparently, the reduction of the Cl^- concentration on just one side of the epithelium leaves sufficient Cl^- in the Cl^- conductive pathway to elicit only a partial increase in transepithelial resistance. However, lowering Cl^- concentration on both sides of the epithelium to 5 mmol l^{-1} increases the transepithelial resistance to 55.8 Ωcm^2, fully reversing the effect of leucokinin on transepithelial resistance. Thus, the epithelial conductance activated by leucokinin is an extracellular conductance, as would be expected from a septate or tight junction.

Dynamic regulation of the paracellular pathway

Like tight junctions in vertebrate tissues, septate junctions in invertebrate tissues provide contacts between epithelial cells that not only prevent the lateral mixing of apical and basolateral membrane domains but also define the permselectivity and magnitude of the paracellular transport pathway (Matter and Balda, 2003). For a long time, tight junctions were thought to be rather fixed structures locked into strict functional limits of differentiated epithelia. However, the identification of three types of tight junction proteins, occludin, claudins and junctional adhesion molecule, as well as their associated adaptor proteins, has begun to unveil the dynamic regulation of tight junctions with various speeds. Changes in tight junction properties involving gene expression or molecular remodeling of tight junction proteins occur in days or hours (Van Itallie et al., 2001). Dynamic formation and reorganization of tight junctions between claudin-transfected fibroblasts can occur within minutes (Sasaki et al., 2003). In Malpighian tubules of the yellow fever mosquito, we observe the regulation of the paracellular Cl^- conductance with switch-like speed that has not been observed in other epithelia (Beyenbach, 2003). Malpighian tubules, in particular, may have developed the regulation of tight junction permeability to an extraordinary degree. In the absence of glomerular filtration, Malpighian tubules must rely entirely on tubular mechanisms of renal regulation. Rapid, reversible changes in paracellular Cl^- conductance may endow the tubule with powers of diuresis not unlike those of glomerular kidneys.

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