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Leucokinin activates Ca^{2+}-dependent signal pathway in principal cells of *Aedes aegypti* Malpighian tubules

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Yu, Ming-Jiun, and Klaus W. Beyenbach. Leucokinin activates Ca^{2+}-dependent signal pathway in principal cells of *Aedes aegypti* Malpighian tubules. *Am J Physiol Renal Physiol* 283: F499–F508, 2002. First published April 10, 2002; 10.1152/ajprenal.00041.2002.—The role of Ca^{2+} in mediating the diuretic effects of leucokinin-VIII was studied in isolated perfused Malpighian tubules of the yellow fever mosquito, *Aedes aegypti*. Peritubular leucokinin-VIII (1 μM) decreased the transepithelial resistance from 11.2 to 2.6 kΩ·cm, lowered the transepithelial voltage from 42.8 to 2.7 mV, and increased transepithelial Cl^- diffusion potentials 5.1-fold. In principal cells of the tubules, leucokinin-VIII decreased the fractional resistance of the basolateral membrane from 0.733 to 0.518. These effects were reversed by the peritubular Ca^{2+}-channel blocker nifedipine, suggesting a role of peritubular Ca^{2+} and basolateral Ca^{2+} channels in signal transduction. In Ca^{2+}-free Ringer bath, the effects of leucokinin-VIII were partial and transient but were fully restored after the bath Ca^{2+} concentration was restored. Increasing intracellular Ca^{2+} with thapsigargin duplicated the effects of leucokinin-VIII, provided that peritubular Ca^{2+} was present. The kinetics of the effects of leucokinin-VIII is faster than that of thapsigargin, suggesting the activation of inositol-1,4,5-trisphosphate-receptor channels of intracellular stores. Store depletion may then bring about Ca^{2+} entry into principal cells via nifedipine-sensitive Ca^{2+} channels in the basolateral membrane.

nifedipine; thapsigargin

HORMONES AND NEUROPEPTIDES are known to regulate transepithelial electrolyte transport in Malpighian tubules of insects (12, 39). So far, five classes of diuretic hormones have been identified: serotonin (8, 10, 29), the corticotropin-releasing factor (CRF)-like diuretic peptides (27), the leucokinins (20), the cardioacceleratory peptides (17), and the calcitonin-like diuretic peptides (18).

The leucokinins have been named after *Leucophaea*, the cockroach from which these neuropeptides were first isolated, sequenced, and synthesized in the laboratory of Holman et al. (23). Holman et al. considered all eight leucokinins myotropic peptides because they stimulate contractions of the cockroach hindgut. Curious that neuropeptides stimulating excretory functions in the gut might also stimulate epithelial transport in Malpighian tubules upstream, we discovered that leucokinins increased the rate of fluid secretion in isolated Malpighian tubules of the yellow fever mosquito, *Aedes aegypti* (20). Since then, the diuretic effects of leucokinins have been observed in the house cricket (14), locust (37), tobacco hornworm (5), fruit fly (31), and housefly (26). As many as 30 leucokinins have now been isolated and sequenced in 4 orders and 9 species of insects (24, 38, 39), including 3 leucokinin-like peptides of the yellow fever mosquito (40).

Ca^{2+} is widely believed to mediate the diuretic effects of leucokinin in Malpighian tubules (12). However, the relative roles of extra- and intracellular Ca^{2+} in signal transduction have not been clearly defined. In the present study of the effects of leucokinin-VIII on Malpighian tubules of the yellow fever mosquito, we report that principal cells mediate the signaling pathway, which involves the release of Ca^{2+} from intracellular stores and, more importantly, the entry of Ca^{2+} into the cell via nifedipine-sensitive Ca^{2+} channels in the basolateral membrane.

MATERIALS AND METHODS

Mosquitoes. The mosquito colony was maintained in a controlled environment at 26°C, 45% humidity, and a 14:10-h light-dark cycle. Mark Brown (Univ. of Georgia, Athens, GA) kindly provided eggs of the yellow fever mosquito, *A. aegypti*. After the eggs were submerged (~200) in dechlorinated tap water, they were exposed to a vacuum of ~610 Torr for 20 min to induce hatching. The hatched larvae were transferred to a flat pan (30 cm × 22 cm) containing 1 liter of dechlorinated tap water and fed "mosquito chow" every day. Mosquito chow contained equal volumes of lactalbumin (Sigma, St. Louis, MO), yeast hydrolysate (ICN Biochemicals, Cleveland, OH), and RMH-3200 chow (Agway, Ithaca, NY). The larvae started to pupate after 5–7 days. The pupae were transferred to 200 ml of dechlorinated tap water in a flask equipped with a netted bucket to capture adult mosquitoes emerging from the water (eclosion). Adult mosquitoes were offered 3% sucrose ad libitum. On the day of experiments, a female mosquito (3–7 days posteclosion) was cold anesthetized and decapitated. Malpighian tubules were then removed from the abdominal cavity under Ringer solution. Only tubule segments near the blind end of the tubule, between 0.2 and 0.3 mm long, were used for study. Experiments on blood-fed mosquitoes were not conducted.

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Ringer solution, leucokinin-VIII, and chemicals. Ringer solution contained the following (in mM): 150 NaCl, 3.4 KCl, 1.8 $NaHCO_3$, 1.7 $CaCl_2$, 1.0 $MgSO_4$, 25 HEPES, and 5.0 glucose. The pH was adjusted to 7.1 with 1 M NaOH. $CaCl_2$ was omitted in Ca^{2+}-free Ringer, and, in addition, 1.0 mM EGTA was added to buffer trace Ca^{2+} in some experiments. The free Ca^{2+} concentration in Ringer solution was calculated using an online program (WEBMAXC v2.10. http://www.stanford.edu/~cpatton/webmaxc2.htm).

Synthetic cockroach leucokinin-VIII was a kind gift from Ronald Nachman (US Dept. of Agriculture, College Station, TX). Although the sequences of mosquito leucokinins are known (40), we preferred to study the cockroach leucokinin for the following reasons. As shown in Table 1, the cockroach leucokinin-VIII used in the present study shares high structural similarity with the mosquito leucokinins, *Aedes* leucokinin 1 and 3, in the last 5 amino acids that are required for bioactivity in all 30 leucokinins (24, 38, 39). In addition, the cockroach and mosquito leucokinins share close functional similarities (20, 40). The EC_{50} of the cockroach leucokinin-VIII on transepithelial voltage (V_t) of the mosquito Malpighian tubule is 2×10^{-9} M (20), comparable to that of the mosquito leucokinins (40). Similarly, low concentrations of both cockroach and mosquito leucokinin cause only partial, oscillating depolarizations of V_t in mosquito Malpighian tubules (20, 40). Stable, sustained depolarizations of V_t require high concentrations (1×10^{-6} M) of cockroach or mosquito leucokinins (20, 40). Moreover, diuretic effects are not observed at low concentrations of leucokinin, regardless of origin; they are observed only at concentrations $>1 \times 10^{-8}$ M in the case of cockroach and mosquito leucokinins (20, 40). In the absence of major physiological or pharmacological differences between cockroach and mosquito leucokinins, we continued to study the effects of the cockroach peptide in view of our previous studies of this peptide in mosquito Malpighian tubules (20, 28, 33, 41, 43). To study the electrophysiological correlates of diuresis, we used leucokinin-VIII at a concentration of 1×10^{-6} M, as in previous studies (28, 33, 41, 43) and as required of the native leucokinin in the mosquito (40).

Thapsigargin and nifedipine were dissolved in dimethylsulfoxide (DMSO) and diluted in Ringer solution to the desired concentration. The final concentration of DMSO in Ringer solution was <0.1%, which had no measurable effects on V_t and transepithelial resistance (data not shown). Chemicals were purchased from Sigma, Fisher Scientific (Fair Lawn, NJ), and VWR Scientific (Willard, OH).

In vitro microperfusion of Malpighian tubules. Figure 1*A* illustrates the method for measuring transepithelial and membrane voltages and resistances in isolated perfused Malpighian tubules (7). The tubule lumen was cannulated with a double-barreled perfusion pipette with an outer diameter of ~10 μm (Theta-Borosilicate glass, no. 1402401; Hilgenberg, Germany). One barrel of this pipette was used to perfuse the tubule lumen with Ringer solution and to measure the V_t with respect to ground in the peritubular Ringer bath. The other barrel was used to inject current (I = 50 nA) into the tubule lumen for the measurement of the transepithelial resistance (R_t) by cable analysis (21). The peritubular bath (500 μl) was perfused with Ringer solution at a rate of 6 ml/min. V_t was recorded continuously, and R_t was measured periodically when of interest.

To measure voltage and the fractional resistance of basolateral and apical membranes of principal cells, we impaled one principal cell of the perfused tubule with a conventional microelectrode (Fig. 1*A*). Referenced to ground in the peritubular Ringer bath, this microelectrode measured the basolateral membrane potential (V_{bl}). Because apical and basolateral membranes of principal cells are in series, the apical membrane voltage (V_a) can be calculated as the difference between V_t and V_{bl}. Current injected into the tubule lumen gives rise to membrane voltage deflections across both apical and basolateral membranes (ΔV_a and ΔV_{bl}) that are proportional to their respective resistances (R_a and R_{bl}). The fractional resistance of the basolateral membrane of the principal cell (fR_{bl}) is therefore

$$fR_{bl} = R_{bl}/(R_a + R_{bl}) = \Delta V_{bl}/(\Delta V_a + \Delta V_{bl}) = \Delta V_{bl}/\Delta V_{t,x} \quad (1)$$

where $\Delta V_{t,x}$ is the change in V_t at the site where the microelectrode impales the principal cell "x" cm away from the opening of the current pipette. $\Delta V_{t,x}$ decreases along the length of the tubule according to *Eq. 2*

$$\Delta V_{t,x} = \Delta V_t\{\cosh[(l - x)/\lambda]/\cosh(l/\lambda)\} \quad (2)$$

where l is the length of the perfused tubule segment, λ is the tubule length constant, and cosh is the hyperbolic function of cosine.

All voltage measurements were made with custom-made high-impedance amplifiers (Burr-Brown, 10^{11} Ω). A permanent record of voltages was produced with a strip-chart recorder (model BD 64; Kipp and Zonen, Bohemia, NY). Data were also collected in a digital form using a Macintosh computer (model 7300) equipped with data-acquisition hardware (Multifunction I/O Board PCI-1200 and Termination Board model SC-2071) and software (LabView for Macintosh, v. 4.1; National Instruments Manufacturer, Austin, TX).

Transepithelial Cl^- diffusion potentials. The amplitude of transepithelial Cl^- diffusion potentials was measured as the change in V_t in response to the 10-fold replacement of Cl^- with isethionate in the peritubular Ringer as in previous studies (43). The Cl^- concentration in the tubule lumen was held constant by perfusion of the tubule lumen with normal Ringer at rates <5 nl/min. In the presence of a constant luminal Cl^- concentration, the 10-fold step reduction of the peritubular Cl^- concentration drove Cl^- to diffuse from the tubule lumen to the peritubular bath, generating lumen-positive transepithelial potentials with magnitudes proportional to the shunt Cl^- conductance.

Table 1. *Primary sequences of leucokinin-VIII from the cockroach and Aedes leucokinins from the yellow fever mosquito*

Species	Peptide	Sequence	Reference
Leucophaea maderae	Leucokinin-VIII	GAD**FYSWG**-NH_2	(22)
Aedes aegypti	*Aedes* leucokinin 1	NSKYVSKQK**FYSWG**-NH_2	(40)
	Aedes leucokinin 2	NP**FHAWG**-NH_2	(40)
	Aedes leucokinin 3	NNPNV**FYPWG**-NH_2	(40)

Primary sequences of leucokinin-VIII from the cockroach (*Leucophaea maderae*) and *Aedes* leucokinins from the yellow fever mosquito (*Aedes aegypti*). Bold letters indicate the COOH-terminal pentamer required for bioactivity in all leucokinins.

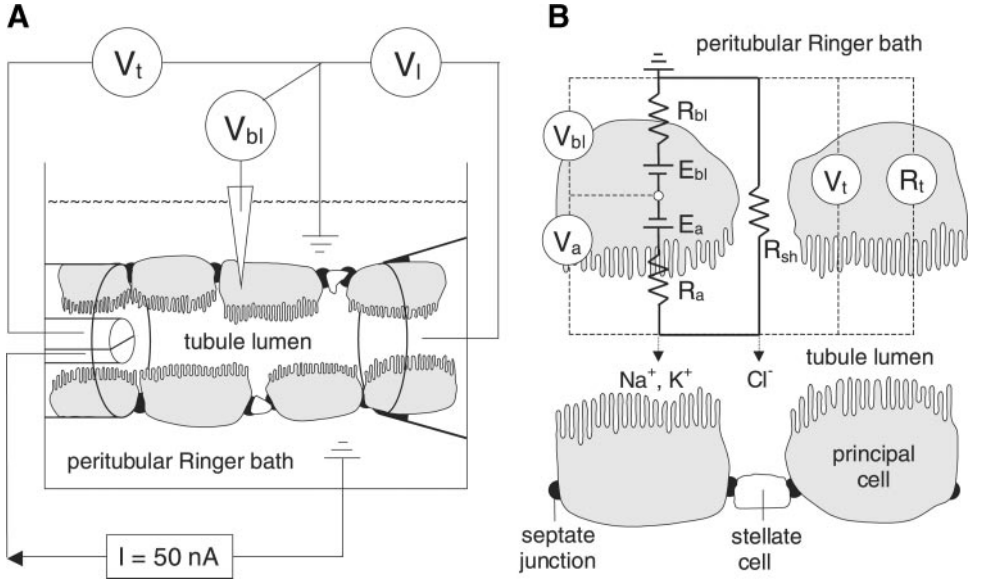

Fig. 1. *A*: electrophysiological measurements in Malpighian tubules of *Aedes aegypti* perfused in vitro by the method of Burg et al. (7). Transepithelial voltage (V_t) is measured through 1 barrel of the perfusion pipette lodged in the tubule lumen. The other barrel serves to inject current (I = 50 nA) into the tubule lumen for measurement of the transepithelial resistance (R_t) by cable analysis (21). The membrane voltage (V_{bl}) and the fractional resistance of the basolateral membrane are measured with a conventional microelectrode impaling a principal cell. V_l is the voltage measured in the collecting pipette. All measurements are referenced to ground in the peritubular bath. *B*: electrical equivalent circuit of transepithelial electrolyte secretion. *V*, voltage; *R*, resistance; E, electromotive force. Subscript letters: t, transepithelial; a, apical membrane; bl, basolateral membrane; sh, shunt.

Equivalent electrical circuit of transepithelial ion transport in Malpighian tubules. Because the secretion of NaCl and KCl by Malpighian tubules of *A. aegypti* generates voltages (2), transepithelial ion transport can be modeled with an electrical equivalent circuit, illustrated in Fig. 1*B*. The circuit distinguishes between the active transport pathway through principal cells and the passive transport pathway through a shunt (R_{sh}) located outside principal cells (Fig. 1*B*). The active transport pathway is further defined by electromotive forces (E) and resistance of the apical (a) and basolateral (bl) membranes. V_{bl} is the sum of the electromotive force of the basolateral membrane (E_{bl}) and the voltage drop ($I_{oc} \times R_{bl}$) across the basolateral membrane resistance, where I_{oc} is the intraepithelial current during epithelial transport under "open-circuit" conditions. Likewise, the V_a is the sum of E_a and $I_{oc} \times R_a$.

Current passing through principal cells is carried largely by Na^+ and K^+ in a secretory direction from the peritubular bath to the tubule lumen. Current passing through the shunt is carried by Cl^-, also in a secretory direction (Fig. 1*B*). Accordingly, the active transport pathway through principal cells is electrically coupled to the passive transport pathway of the shunt, such that an anion (Cl^-) is secreted into the lumen for every cation (Na^+ and K^+) transported through principal cells. Indeed, rates of transepithelial cation secretion come close to, or equal, rates of transepithelial Cl^- secretion in *Aedes* Malpighian tubules (33).

Statistical evaluation of data. Each tubule served as its own control. Accordingly, the data were analyzed for the differences between paired samples, control vs. experimental, with the use of the paired Student's *t*-test.

RESULTS

Leucokinin-VIII decreases the V_t and R_t in Malpighian tubules. Isolated Malpighian tubules generated lumen-positive V_t when the same Ca^{2+}-containing Ringer solution was present on both sides of the epithelium (symmetrical perfusion). In the representative experiment shown in Fig. 2*A*, V_t was 70.7 mV (lumen positive), and the R_t was 18.0 kΩ·cm. After 1 μM leucokinin-VIII was added to the peritubular bath, V_t depolarized sharply to 5.0 mV together with the drop of R_t to 2.9 kΩ·cm. These effects were fully reversible on washout of leucokinin-VIII. In a total of seven Malpighian tubules, leucokinin-VIII significantly depolarized V_t from 40.6 ± 9.0 to 0.5 ± 1.3 mV and signifi-

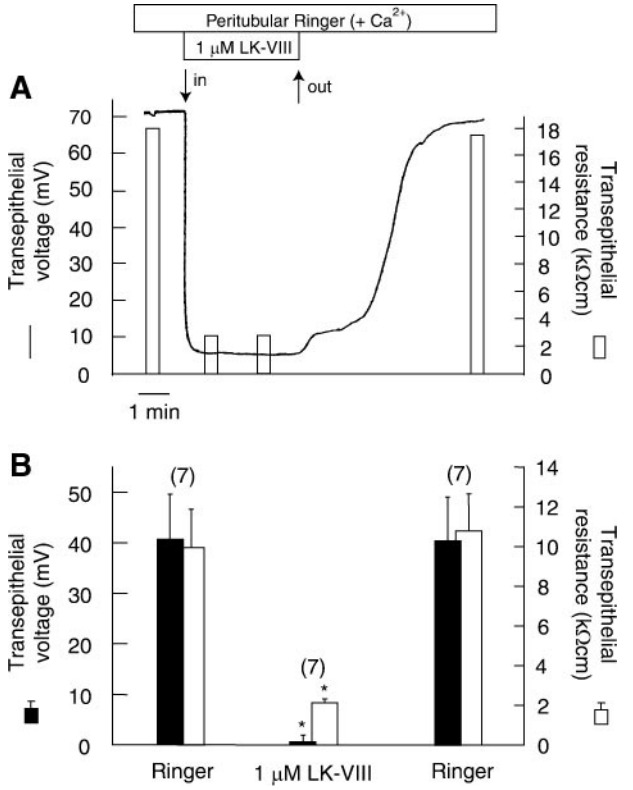

Fig. 2. Effects of leucokinin (LK)-VIII on the V_t and R_t in isolated perfused Malpighian tubules of the yellow fever mosquito, *A. aegypti*. The tubule was bathed and perfused with normal Ringer solution containing 1.7 mM Ca^{2+}. *A*: reversible effects of LK-VIII in a representative Malpighian tubule. LK-VIII was washed into and removed from the peritubular Ringer as indicated by arrows. *B*: means ± SE of 7 tubule experiments.*$P < 0.01$.

cantly decreased R_t from 9.9 ± 1.9 to 2.1 ± 0.2 kΩ·cm (Fig. 2*B*).

Nifedipine reverses the effects of leucokinin-VIII. To explore the effects of leucokinin-VIII and nifedipine on principal cells of the tubule, we measured V_{bl} and fR_{bl} with a conventional microelectrode impaling a principal cell (Fig. 1*A*). Data from 8–18 tubule experiments are summarized in Fig. 3. Under control conditions, V_t was 42.8 ± 5.3 mV, V_{bl} was −69.8 ± 4.3 mV, and V_a was 109.5 ± 7.2 mV, lumen positive. The control R_t was 11.2 ± 1.7 kΩ·cm. The control fR_{bl}, 0.733 ± 0.038, indicated that 73.3% of the transcellular resistance resided at the basolateral membrane and 26.7% at the apical membrane. In the absence of leucokinin-VIII, the 10-fold reduction of the peritubular Cl^- concentration yielded a transepithelial Cl^- diffusion potential (DP_{Cl^-}) of 8.2 ± 1.2 mV, indicating a modest transepithelial Cl^- conductance under control conditions.

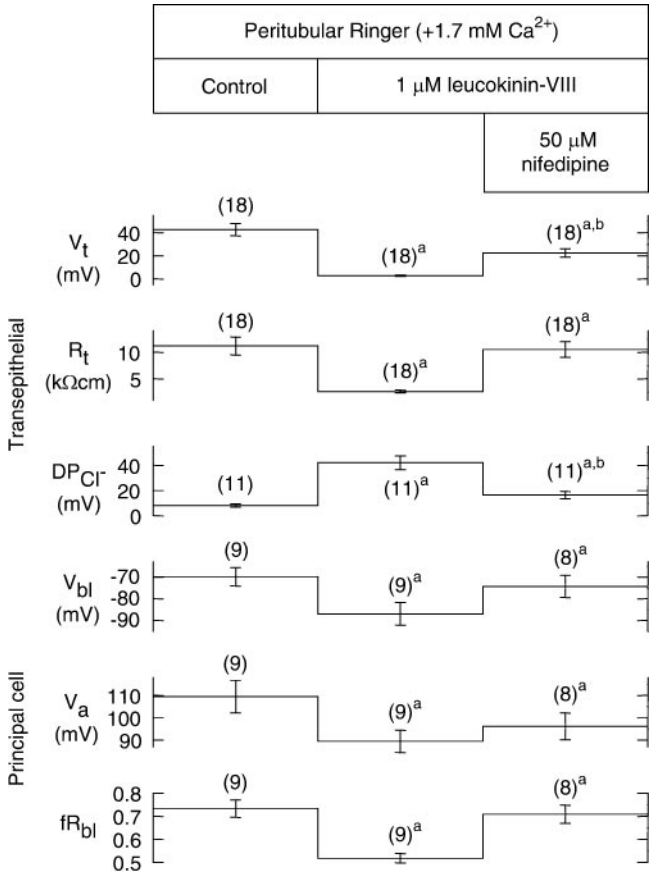

Fig. 3. Effects of LK-VIII on electrophysiological variables of isolated perfused Malpighian tubules of *A. aegypti* and the reversal of these effects by nifedipine. Control data were obtained in the presence of normal Ringer (1.7 mM Ca^{2+}) on both sides of the epithelium (5–10 min). Peritubular bath flow was then switched to include LK-VIII (1 μM) for 5 min and then LK-VIII plus nifedipine (50 μM) for 20 min. Steady-state values are shown for each treatment period. DP_{Cl^-}, transepithelial Cl^- diffusion potential in response to a 10-fold reduction of the peritubular Cl^- concentration; V_{bl}, basolateral membrane voltage of the principal cell; fR_{bl}, fractional resistance of the basolateral membrane. Values are means ± SE (no. of tubules). [a]Statistical significance ($P < 0.05$) is referenced to the previous condition. [b]Statistical significance ($P < 0.05$) is referenced to the control condition.

The addition of leucokinin-VIII to the peritubular bath profoundly and significantly affected all measured variables (Fig. 3). V_t dropped to 2.7 ± 0.6 mV within a few seconds. In parallel, V_{bl} hyperpolarized to −86.9 ± 5.2 mV, and V_a depolarized to 89.4 ± 5.0 mV. Inspection of the equivalent circuit of Fig. 1*B* shows that the hyperpolarization of V_{bl} concomitant with the depolarization of V_a is expected from a decrease in the R_{sh}. Consistent with a decrease in R_{sh} in the presence of leucokinin-VIII was the large drop of R_t from 11.2 to 2.6 ± 0.3 kΩ·cm together with the significant 5.1-fold increase in DP_{Cl^-} to 42.1 ± 5.4 mV. Leucokinin-VIII also affected the transcellular transport pathway through principal cells as indicated by the decrease of fR_{bl} from 0.733 to 0.518 ± 0.020.

Nifedipine (50 μM) reversed the effects of leucokinin-VIII. All variables returned toward control values, some completely and others incompletely (Fig. 3). Although the return of V_t from 2.7 to 22.5 ± 3.7 mV is highly significant ($P < 0.00005$), V_t did not fully return to the control mean value of 42.8 mV. Likewise, DP_{Cl^-} significantly ($P < 0.00005$) decreased from 42.1 to 16.4 ± 2.9 mV but did not reach control diffusion potentials of 8.2 mV. All other variables fully returned to control values. In particular, nifedipine completely reversed the effects of leucokinin-VIII on *1*) R_t, which returned to 10.5 ± 1.5 kΩ·cm; *2*) V_{bl}, which repolarized to −74.3 ± 5.1 mV; *3*) V_a, which repolarized to 96.2 ± 6.0 mV; and *4*) fR_{bl}, which returned to 0.709 ± 0.039 (Fig. 3). It took between 5 and 12 min for nifedipine to reverse the effects of leucokinin-VIII on both principal cells and transepithelial shunt conductance.

In the absence of leucokinin, i.e., under control conditions, nifedipine had no significant effects on V_t (control, 51.6 ± 6.0 mV; nifedipine, 53.2 ± 7.6 mV) and R_t (control, 12.1 ± 2.1 kΩ·cm; nifedipine, 9.9 ± 1.1 kΩ·cm) in seven isolated perfused Malpighian tubules.

Effects of leucokinin-VIII are independent of barium-sensitive K^+ channels. The addition of Ba^{2+} (5 mM) to the peritubular Ringer solution had immediate and significant effects on the electrophysiology of the tubule and its principal cells (Fig. 4). V_t depolarized from 19.0 ± 4.2 to 17.1 ± 4.0 mV, V_{bl} hyperpolarized from −64.6 ± 6.5 to −71.0 ± 6.9 mV, V_a hyperpolarized from 83.7 ± 7.6 to 92.3 ± 7.7 mV, and fR_{bl} increased from 0.710 ± 0.030 to 0.800 ± 0.024, consistent with the block of basolateral membrane K^+ channels observed previously in principal cells (28). The Ba^{2+} blockade of K^+ channels did not significantly increase R_t, although a trend to higher values was observed (Fig. 4).

Ba^{2+} did not impair the efficacy of leucokinin-VIII (Fig. 4). On the addition of leucokinin-VIII to the peritubular bath containing 5 mM Ba^{2+}, V_t dropped immediately and significantly from 17.1 ± 4.0 to 0.9 ± 0.7 mV together with the drop in R_t from 6.7 ± 0.9 to 1.8 ± 0.2 kΩ·cm. Furthermore, V_{bl} significantly hyperpolarized from −71.0 ± 6.9 to −87.8 ± 6.7 mV, and fR_{bl} significantly decreased from 0.800 ± 0.024 to 0.542 ± 0.030. The small depolarization of V_a was not statistically significant.

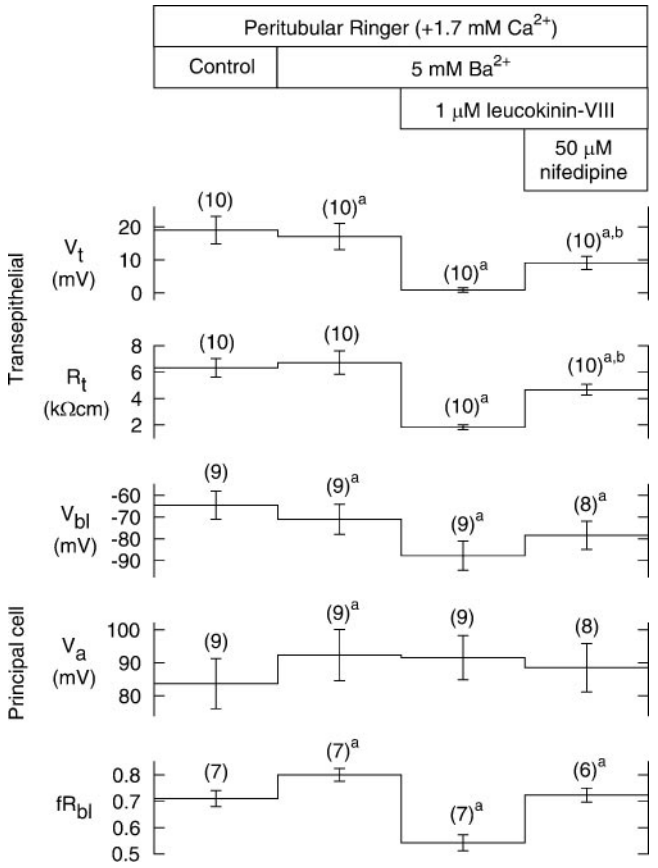

Fig. 4. Effects of LK-VIII in the presence of K^+ channel blocker Ba^{2+}. Experiments began with the perfusion of Malpighian tubules of *A. aegypti* with normal Ringer solution (1.7 mM Ca^{2+}) present in the tubule lumen and the peritubular bath (control). After a control period of 5–10 min, the peritubular Ringer bath was changed to include 5 mM Ba^{2+} for 5 min. Thereafter, the bath was supplemented with LK-VIII in the presence of Ba^{2+} (1 μM) for 5 min and then with nifedipine (50 μM) in the presence of LK-VIII and Ba^{2+} for 20 min. Steady-state values are summarized for each treatment period. Values are means ± SE (no. of tubules). [a]Statistical significance ($P < 0.05$) is referenced to the previous condition. [b]Statistical significance ($P < 0.05$) is referenced to the preLK-VIII with Ba^{2+} condition.

As in the absence of Ba^{2+} (Fig. 3), nifedipine (50 μM) significantly reversed all effects of leucokinin-VIII in the presence of Ba^{2+} (Fig. 4). V_t significantly repolarized to 9.1 ± 2.0 mV, R_t significantly returned to 4.7 ± 0.4 kΩ·cm, V_{bl} significantly repolarized to −78.4 ± 6.5 mV, and fR_{bl} significantly returned to 0.724 ± 0.026. It took 3–9 min for nifedipine to reverse the effects of leucokinin-VIII in the presence of Ba^{2+}.

Effects of leucokinin-VIII depend on the presence of extracellular Ca^{2+}. Because the peritubular addition of the Ca^{2+} channel blocker nifedipine reversed the effects of leucokinin-VIII (Figs. 3 and 4), we explored the role of extracellular Ca^{2+} in signal transduction by testing the effects of leucokinin-VIII in peritubular Ringer solution made Ca^{2+} free by deleting this ion and buffering trace Ca^{2+} with EGTA. As shown in Fig. 5 for a representative tubule experiment, the change from normal peritubular Ringer to Ca^{2+}-free Ringer had no obvious effects on V_t (70.0 mV) and R_t (17.9 kΩ·cm). However, the effects of leucokinin-VIII were markedly diminished in the absence of peritubular Ca^{2+}. On the addition of leucokinin-VIII to the Ca^{2+}-free peritubular Ringer bath, V_t depolarized from 68.9 to 11.2 mV together with the drop of R_t from 18.1 to 4.6 kΩ·cm. However, these effects were only transient. V_t and R_t returned to values approximately one-half of control (44.3 and 61.4%, respectively) and then began to exhibit small oscillations. Characteristic of these oscillations was the parallel change of V_t and R_t. As R_t dropped, V_t depolarized toward zero, and as R_t rose, V_t repolarized (Fig. 5).

The addition of Ca^{2+} to the peritubular bath to yield a free Ca^{2+} concentration of 31 nM only transiently reduced V_t to 13.0 mV and R_t to 5.6 kΩ·cm before a return to oscillations again. A similar response was observed on increasing the peritubular free Ca^{2+} concentration further to 17 μM. But at this Ca^{2+} concentration, the initial parallel effect on V_t and R_t was stronger, and it lasted longer before returning to oscillations that now were more pronounced than those at lower Ca^{2+} concentrations. Only after the Ca^{2+} buffering capacity of peritubular EGTA was exceeded, i.e., in the presence of 1 mM Ca^{2+}, were the full effects of leucokinin-VIII observed: permanent, steady-state low values of V_t (3.6 mV) and R_t (4.0 kΩ·cm). What is shown for the single tubule experiment in Fig. 5 was observed in 10 other Malpighian tubules (data not shown).

Thapsigargin duplicates the effects of leucokinin-VIII, and nifedipine reverses them. The oscillations of V_t and R_t in leucokinin-VIII-treated tubules bathed in Ca^{2+}-free Ringer (Fig. 5) suggest a role of intracellular Ca^{2+} in signal transduction. For this reason, the effects of thapsigargin, a specific inhibitor of Ca^{2+} uptake by the intracellular stores, were of interest. The addition of 1 μM thapsigargin to the normal peritubular Ringer solution containing Ca^{2+} duplicated the effects of leucokinin-VIII (Fig. 6). V_t depolarized from 21.4 ± 4.4 to 3.0 ± 0.3 mV, and R_t decreased from 7.9 ± 1.4 to 2.6 ± 0.2 kΩ·cm. At the same time, V_{bl} hyperpolarized from −70.0 ± 5.7 to −80.6 ± 7.4 mV, V_a depolarized from 91.2 ± 9.9 to 82.0 ± 8.1 mV, and fR_{bl} decreased from 0.628 ± 0.056 to 0.486 ± 0.056. All of these effects were statistically significant (Fig. 6).

The kinetics of the thapsigargin effects was significantly slower than that of leucokinin-VIII. The effects of leucokinin-VIII on electrophysiological variables took only seconds (Figs. 2–5). In contrast, the effects of thapsigargin started with a delay of 2 min and developed slowly, taking 5–7 min with a hyperbolic time course to reach effects comparable with those of leucokinin-VIII.

Nifedipine (50 μM) reversed the effects of thapsigargin (Fig. 6) as it reversed the effects of leucokinin-VIII (Figs. 3 and 4). V_t significantly repolarized to 15.1 ± 4.0 mV but remained significantly different from control. All other variables fully returned to control values in the presence of nifedipine. R_t returned to 7.8 ± 1.5 kΩ·cm, V_{bl} repolarized to −71.5 ± 5.5 mV, V_a repolarized to 87.2 ± 8.7 mV, and fR_{bl} returned to 0.632 ± 0.056 (Fig. 6). It took between 4 and 10 min for nifedipine to reverse the effects of thapsigargin.

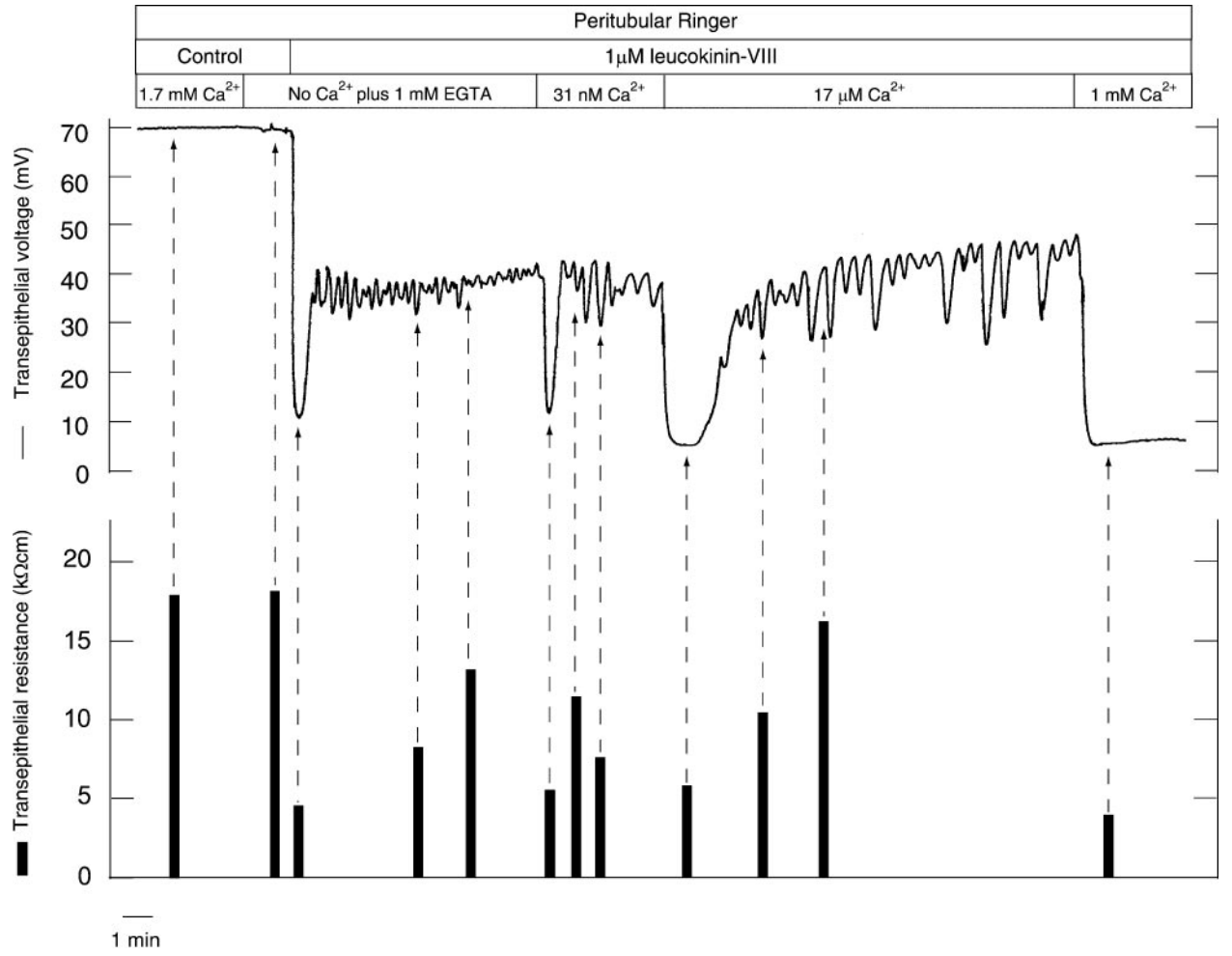

Fig. 5. Dependence of the effects of LK-VIII on peritubular Ca^{2+} concentrations. The representative tubule experiment began in the presence of normal Ringer containing 1.7 mM Ca^{2+} on both sides of the epithelium. The peritubular bathing solution was then changed to Ca^{2+}-free Ringer (no Ca^{2+} plus 1 mM EGTA). LK-VIII (prepared in Ca^{2+}-free Ringer) was subsequently added to the Ca^{2+}-free peritubular bath. Thereafter, $CaCl_2$ was added to the peritubular bath to yield the free Ca^{2+} concentrations indicated. Dashed arrows point to the V_t when R_t was measured.

Effects of thapsigargin depend on extracellular Ca^{2+}. To gain insights into the relative roles of intra- and extracellular Ca^{2+} in the signal transduction of leucokinin-VIII, we explored the effects of thapsigargin in the absence and presence of extracellular Ca^{2+}. As shown in Fig. 7, thapsigargin (1 μM) had no significant effect on any of the six electrophysiological variables in the absence of peritubular Ca^{2+} ($-Ca^{2+}$), not even after 10 min. Moreover, the oscillations of V_t and R_t that were observed with leucokinin-VIII in Ca^{2+}-free Ringer (Fig. 5) were not observed with thapsigargin in Ca^{2+}-free Ringer. However, after exposure to thapsigargin for 10 min, restoration of the Ca^{2+} concentration in the peritubular Ringer to 1.7 mM immediately restored the full effects of thapsigargin with significant effects on all variables: V_t decreased from 32.1 ± 4.4 to 3.2 ± 0.8 mV, R_t decreased from 8.2 ± 1.2 to 2.4 ± 0.4 kΩ·cm, and DP_{Cl^-} increased from 19.2 ± 3.5 to 38.2 ± 3.5 mV (Fig. 7). In parallel with these changes, V_{bl} hyperpolarized from −67.9 ± 6.0 to −86.6 ± 3.1 mV, V_a depolarized from 96.6 ± 5.5 to 89.7 ± 3.6 mV, and fR_{bl} decreased from 0.733 ± 0.043 to 0.451 ± 0.071. Significantly, the effects of thapsigargin were reversed again when the peritubular Ringer solution was switched back to nominally Ca^{2+}-free Ringer. V_t returned to 25.8 ± 4.7 mV, R_t returned to 7.4 ± 1.3 kΩ·cm, and DP_{Cl^-} decreased to 20.4 ± 3.7 mV. Similarly, V_{bl} depolarized to −70.9 ± 5.1 mV, V_a repolarized to 94.7 ± 5.6 mV, and fR_{bl} increased to 0.740 ± 0.040. It took between 2 and 6 min for nominally Ca^{2+}-free Ringer solution to reverse the effects of thapsigargin. Restoring the normal Ca^{2+} concentration in the peritubular bath for a second time elicited the full effects of thapsigargin again (data not shown).

DISCUSSION

Leucokinins and electrophysiological and diuretic effects in Malpighian tubules. The leucokinins were first isolated by Holman et al. (23) from the heads of cockroaches (*Leucophaea)* and named "cephalo-myotropic

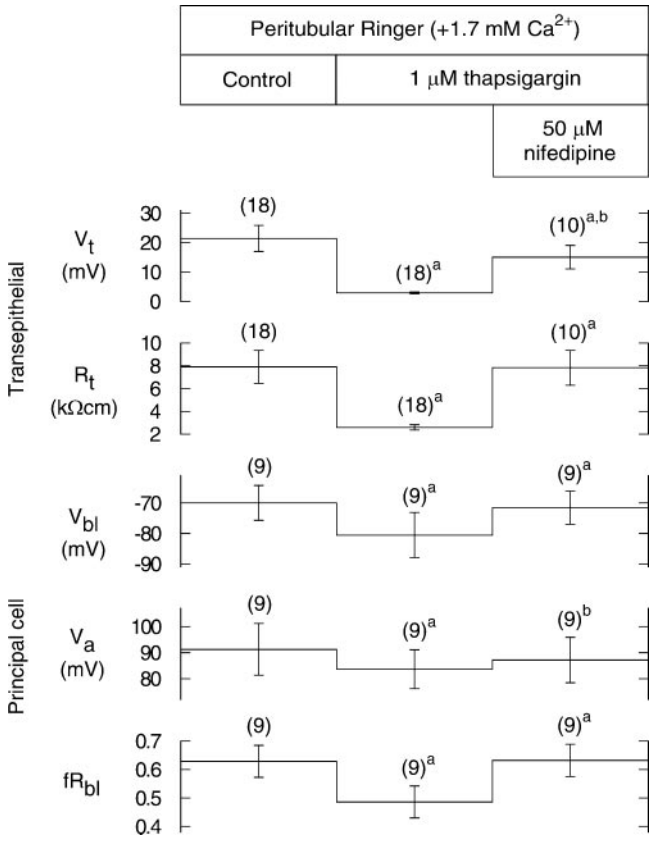

Fig. 6. Effects of thapsigargin on electrophysiological variables of isolated perfused Malpighian tubules of *A. aegypti* and reversal by nifedipine. Tubules were perfused in vitro with normal Ca^{2+}-containing Ringer solution in the tubule lumen and the peritubular bath (control; 5–10 min). The peritubular bath was then switched to include thapsigargin (1 μM) for 5–10 min and then to include thapsigargin plus nifedipine (50 μM) for another 20 min. Only steady-state values are summarized for each treatment period. Values are means ± SE (no. of tubules). [a]Statistical significance ($P < 0.05$) is referenced to the previous condition. [b]Statistical significance ($P < 0.05$) is referenced to the control condition.

peptides" for their anatomical origin and their ability to stimulate contractions in the cockroach hindgut. Diuretic properties of the leucokinins were first found in Malpighian tubules of the yellow fever mosquito, *A. aegypti* (20), and later also in the house cricket, locust, tobacco hornworm, fruit fly, and housefly (5, 14, 26, 31, 37). Because the secretion of electrolytes generates V_t in Malpighian tubules, measures of voltage and resistance have been useful in elucidating transport mechanisms and their regulation (2) and serve as a rapid bioassay in the search and isolation of new diuretic peptides and hormones (19). Although changes in voltage can reflect changes in ion transport, they must not necessarily do so. For example, *Aedes* leucokinin 2 depolarizes the V_t in the isolated Malpighian tubule, but it fails to trigger diuresis even at micromolar concentrations (40). The dissociation of electrophysiological effects from diuretic effects can also be a matter of dose. *Aedes* leucokinin 3 affects the V_t at concentrations as low as 10^{-10} M, but its diuretic effects begin at a concentration around 10^{-8} M and approach maximum at 10^{-6} M (40). Furthermore, at concentrations from 10^{-10} to 10^{-7} M, *Aedes* leucokinin 3 elicits only partial oscillations of the V_t, and low, steady-state depolarizations of the V_t are only observed at the diuretic concentration of 10^{-6} M (40). The situation is similar for the cockroach leucokinin-VIII used in the present study. Like the *Aedes* leucokinins, the cockroach leucokinin-VIII elicits only electrical effects (voltage depolarizations) at low concentrations; concentrations of 10^{-6} M and higher are needed to observe *1*) low, steady-state depolarizations of the V_t and *2*) diuretic effects (20). These functional similarities are not surprising in view of close structural similarities of the cockroach and mosquito leucokinins in the crucial COOH-terminal pentapeptide sequence necessary for bioactivity (Table 1). Kinin-dependent and dose-dependent effects on V_t and fluid secretion suggest multiple receptors and/or signaling pathways in mediating the actions of these peptides.

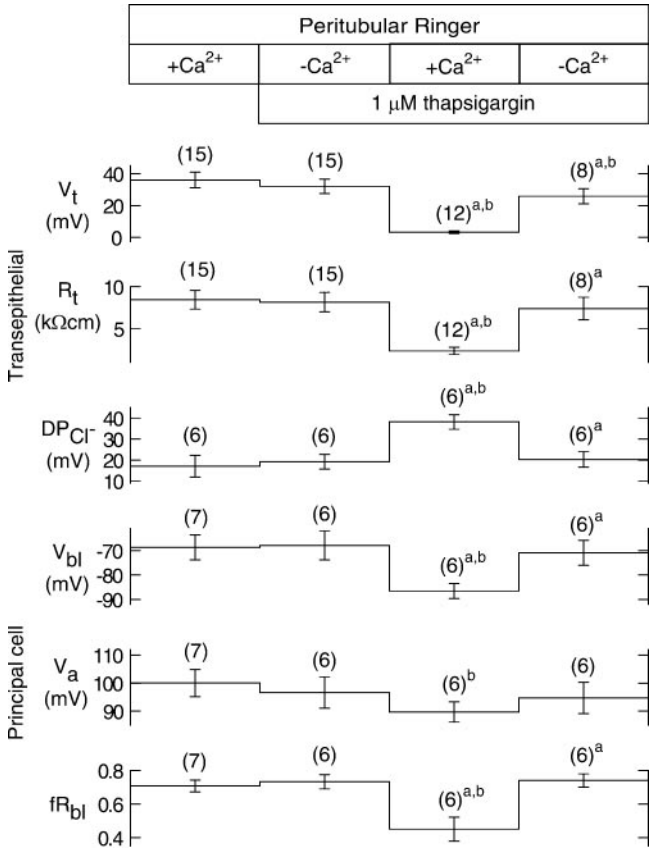

Fig. 7. Dependence of the effects of thapsigargin on peritubular Ca^{2+}. Experiments began with the perfusion of Malpighian tubules of *A. aegypti* with normal Ringer solution containing 1.7 mM Ca^{2+} in the tubule lumen and the peritubular bath ($+Ca^{2+}$). After ~5 min, the peritubular bath was changed to Ca^{2+}-free Ringer ($-Ca^{2+}$) containing 1 μM thapsigargin for 10 min. Then the bath was changed to include 1.7 mM Ca^{2+} in the presence of thapsigargin ($+Ca^{2+}$) for 5 min. Finally, the bath was returned to Ca^{2+}-free Ringer ($-Ca^{2+}$) containing 1 μM thapsigargin for 10 min. Only steady-state values are summarized for each treatment period. Values are means ± SE (no. of tubules). [a]Statistical significance ($P < 0.05$) is referenced to the previous condition. [b]Statistical significance ($P < 0.05$) is referenced to the control condition.

In Malpighian tubules of the fruit fly and the yellow fever mosquito, leucokinin increases the Cl^- conductance of a transepithelial shunt located outside principal cells (32, 33). Two sites of the Cl^- shunt have been proposed, stellate cells in Malpighian tubules of the fruit fly (32) and the paracellular pathway in Malpighian tubules of the yellow fever mosquito (33). In the present study, 1 μM leucokinin-VIII had the following effects: it decreased the R_t 4.3-fold, it short-circuited the V_t to values close to zero, and it increased transepithelial Cl^- diffusion potential >5-fold (Figs. 2–5). These observations confirm the increase in a paracellular shunt Cl^- conductance, with the effects of producing a "leaky epithelium" suitable for high transport rates under conditions of diuresis. The increase in the paracellular conductance is expected to hyperpolarize the V_{bl} of principal cells and to depolarize the V_a that were observed (Figs. 1*B*, 3, and 4). Consistent with the effect on the paracellular pathway is the increase in the transepithelial permeability to two markers of the paracellular pathway, inulin and mannitol, induced by leucokinin in Malpighian tubules of *Locusta* and *Aedes* (12, 41).

Leucokinin and its Ca^{2+}-dependent signal pathway. Wherever the signal transduction pathway of leucokinin has been studied, Ca^{2+} has been found to serve as second messenger (12). Ca^{2+} ionophores that bring Ca^{2+} into the cell from extracellular fluids duplicate the effects of leucokinin by stimulating fluid secretion in Malpighian tubules of the locust (30), house cricket (15), yellow fever mosquito (11), and black field cricket (42). Thapsigargin, which prevents Ca^{2+} uptake by intracellular stores, thereby increasing intracellular Ca^{2+} concentrations, increases fluid secretion in Malpighian tubules of the locust (13), fruit fly (36), housefly (26), house cricket (12), and black field cricket (42). Clamping intracellular Ca^{2+} concentrations at low levels with the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA)-AM abolishes the diuretic effects of leucokinin in Malpighian tubules of the house cricket (15) and the fruit fly (31). Actual measurements of intracellular Ca^{2+} concentrations show that leucokinin increases Ca^{2+} concentrations in stellate cells of the fruit fly Malpighian tubules (32) but increases Ca^{2+} concentration in principal cells of Malpighian tubules of the house cricket (12).

The ability of Ca^{2+} ionophore and thapsigargin to mimic the effects of leucokinin suggests roles of both extracellular and intracellular Ca^{2+} in signal transduction. The present study sought to clarify the relative roles of the two Ca^{2+} sources, extracellular fluid and intracellular stores, in mediating the diuretic effects of leucokinin in Malpighian tubules of the yellow fever mosquito, *A. aegypti*.

Previous studies in our laboratory have shown that Ca^{2+} ionophore A-23187 duplicated the effects of leucokinin-VIII in *Aedes* Malpighian tubules (11). The present study shows duplication of the effects of leucokinin-VIII by thapsigargin, an agent known to increase intracellular Ca^{2+} concentrations (Figs. 6 and 7). Like the effects of leucokinin-VIII, the effects of thapsigargin were critically dependent on peritubular Ca^{2+} as shown by the reversal of both leucokinin-VIII and thapsigargin effects on removal of Ca^{2+} from the peritubular Ringer bath or on the addition of the Ca^{2+} channel blocker nifedipine (Figs. 3–7). Significantly, both leucokinin-VIII and thapsigargin lowered the fR_{bl} of principal cells, an effect that reversed toward control values by removing Ca^{2+} from or adding nifedipine to the peritubular Ringer solution (Figs. 3–7). These observations suggest the activation of basolateral membrane Ca^{2+} channels that are part of the signal transduction pathway of leucokinin. Apparently, a large number of Ca^{2+} channels must be activated in view of the substantial decrease in fR_{bl} on the addition of leucokinin-VIII or thapsigargin. Primary or secondary effects of leucokinin on basolateral membrane K^+ channels were ruled out in the present study, although K^+ channels account for 64% of the basolateral conductance in principal cells of *Aedes* Malpighian tubules (4). After the blocking of K^+ channels with the supramaximal dose of 5 mM Ba^{2+}, the effects of leucokinin-VIII were not blocked (Fig. 4). Again, fR_{bl}, R_t, V_t, and V_{bl} significantly decreased. Moreover, in the presence of Ba^{2+}, the Ca^{2+} channel blocker nifedipine reversed these effects of leucokinin-VIII. Accordingly, leucokinin activates a substantial Ca^{2+} conductance in the basolateral membrane of principal cells.

Roles of intra- and extracellular Ca^{2+}. The present study revealed that intracellular Ca^{2+} initiates and

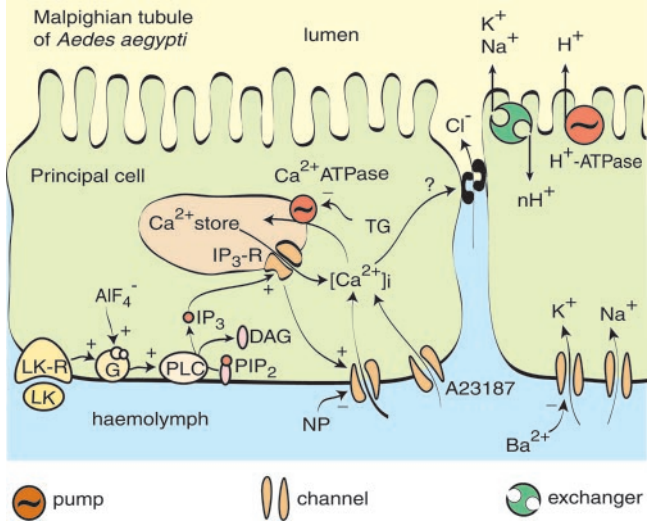

Fig. 8. Hypothetical model of the LK signal transduction pathway in principal cells of Malpighian tubules of the yellow fever mosquito, *A. aegypti*. LK is known to increase rates of the transepithelial secretion of both NaCl and KCl, consistent with the increase in transepithelial Cl^- permeability (33). Water follows by osmosis. Electrophysiological studies indicate the increase of the Cl^- conductance of the paracellular, septate-junctional pathway between principal and/or stellate cells (33). LK-R, LK receptor; G, heterotrimeric G protein; AlF_4^-, aluminum tetrafluoride; PLC, phospholipase C-β; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; IP_3-R, IP_3 receptor; TG, thapsigargin; NP, nifedipine; A-23187, Ca^{2+}/Mg^{2+} ionophore; +, activation; −, inhibition.

peritubular Ca^{2+} sustains the effects of leucokinin in *Aedes* Malpighian tubules. Thapsigargin duplicated the effects of leucokinin only if millimolar Ca^{2+} concentrations were present in the peritubular Ringer bath (Figs. 6 and 7). In the absence of extracellular Ca^{2+}, thapsigargin had no effects on tubule and principal cell electrophysiology (Fig. 7). Thus the increase in cytoplasmic Ca^{2+} from intracellular stores may be sufficient to initiate the effects of leucokinin, but peritubular Ca^{2+} is needed to maintain the effects.

Inositol-1,4,5-trisphosphate (IP_3) takes part in the signaling cascade, as shown in the laboratory of Hagedorn (9), where all three leucokinins of *A. aegypti* were found to increase IP_3 concentration in isolated Malpighian tubules. Because IP_3 is known to release Ca^{2+} from intracellular stores via IP_3 receptor Ca^{2+} channels (1), the depletion of intracellular Ca^{2+} stores could trigger the activation of so-called store-operated Ca^{2+} channels in the basolateral membrane of principal cells (35). The hypothesis of store-operated Ca^{2+} channels in the basolateral membrane is supported by two observations in the present study. First, under conditions of intracellular Ca^{2+} depletion (Figs. 5 and 7), the addition of Ca^{2+} to the peritubular medium of leucokinin- or thapsigargin-treated tubules immediately restored their full effects, as if basolateral membrane Ca^{2+} channels were already open in these Ca^{2+}-depleted cells (Fig. 7). Second, responses mediated via sudden Ca^{2+} store depletion by leucokinin-VIII are expected to be much faster than those mediated via slow Ca^{2+} store depletion by thapsigargin. When IP_3 receptor Ca^{2+} channels of intracellular Ca^{2+} stores are activated by leucokinin-VIII, the electrophysiological responses to the tubule are immediate, within seconds (Figs. 2 and 5). In contrast, when the depletion of intracellular Ca^{2+} stores is achieved by inhibition of the Ca^{2+} pump with thapsigargin, electrophysiological responses of the tubule develop slowly over 10 min, reflecting the slow Ca^{2+} leak from intracellular stores.

In the absence of peritubular Ca^{2+}, thapsigargin had no effect whatsoever on tubule electrophysiology, but leucokinin-VIII had partial and transient effects, consistent with the sudden release of Ca^{2+} from intracellular stores but in quantities insufficient to sustain the full and lasting effects of leucokinin (Figs. 5 and 7). The transients began as large parallel oscillations of the V_t and R_t with brief transitions to the leaky epithelium. These transitions diminished with time, returning the tubule to control values of the moderately "tight epithelium." The transitions had a frequency not uncommon for cyclical changes in intracellular Ca^{2+} concentrations, suggesting temporal displacements of Ca^{2+} release and reuptake by intracellular Ca^{2+} stores.

The oscillations of the V_t and R_t observed in leucokinin-treated tubules in the absence of peritubular Ca^{2+} (Fig. 5) were similar to spontaneous transients seen frequently in *Aedes* Malpighian tubules in the presence of peritubular Ca^{2+} (3). Similar voltage transients are observed in *Aedes* Malpighian tubules in the absence and presence of low *Aedes* leucokinin concentrations (40) and in *Drosophila* Malpighian tubules in the presence and absence of peritubular Ca^{2+} (6). BAPTA-AM, a chelator of cytosolic Ca^{2+}, eliminates the voltage transients in *Drosophila* Malpighian tubules (6). Thus it appears that spontaneous cyclical changes in cytosolic Ca^{2+} concentrations are responsible to oscillating V_t and R_t, even in the absence of peritubular Ca^{2+} (Fig. 5).

Observations made in the present study allow a hypothetical model for the signal transduction pathway activated by high micromolar concentrations of leucokinin (Fig. 8). We propose a leucokinin receptor at the basolateral membrane on the basis of the recent identification of a leucokinin-binding protein in *Aedes* Malpighian tubules (33, 34). The receptor is probably heterotrimeric G protein coupled in view of *1*) predictions from the primary sequences of the known leucokinin receptors from a pond snail (16) and a cattle tick (25) and *2*) duplication of the electrophysiological effects of leucokinin in *Aedes* Malpighian tubules by aluminum tetrafluoride (AlF_4^-), a known activator of G proteins (43). The stimulation of phospholipase C-β to produce diacylglycerol and IP_3 is indicated by the rise in intracellular IP_3 concentrations measured in leucokinin-stimulated *Aedes* Malpighian tubules (9). IP_3 is then thought to trigger the sudden release of Ca^{2+} from intracellular Ca^{2+} stores via IP_3 receptor Ca^{2+} channels (1). The depletion of intracellular Ca^{2+} stores may then activate nifedipine-sensitive Ca^{2+} channels in the basolateral membrane of principal cells. Extracellular Ca^{2+} entering the cells then goes on to produce and hold the epithelium in the "leaky" condition as long as leucokinin is present. Exactly how Ca^{2+} entry leads to the increase in the shunt Cl^- conductance and the maintenance of diuretic transepithelial transport rates is an intriguing question this study leaves unanswered.

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