The single kinin receptor signals to separate and independent physiological pathways in Malpighian tubules of the yellow fever mosquito


Departments of 1Biomedical Sciences and 2Molecular Biology and Genetics, Cornell University, Ithaca, New York; 3Department of Biology, Division of Animal Physiology, Universität Osnabrück, Osnabrück, Germany; and 4Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas

Submitted 26 January 2010; accepted in final form 8 June 2010

EPITHELIAL SECRETION AND ABSORPTION are the two mechanisms for regulating the volume and composition of the hemolymph in insects (4, 16). In the case of Malpighian tubules, epithelial secretion delivers a fluid to the lumen in upstream (distal) segments of the Malpighian tubule, creating a urinary space into which excess solutes, metabolic wastes, and toxins can subsequently be dumped for excretion. In downstream (proximal) segments of the tubule, in the hindgut and the rectum, reabsorptive mechanisms recover the electrolytes and water that initially served to produce the urinary space, while concentrating unwanted solutes for excretion.

Transport mechanisms of epithelial secretion and absorption are under hormonal control as Malpighian tubules execute the tasks of extracellular fluid homeostasis (4, 20). Diuretic hormones stimulate tubular secretion and urine flow, whereas antidiuretic hormones stimulate tubular reabsorption and decrease the excretion of urine.

Four families of diuretic peptides have been identified in insects: the CRF-like peptides, the calcitonin-like peptides, the CAPA peptides, and the kinin peptides (20, 22, 26). Holman et al. (30) isolated the first kinin peptides from head extracts of the cockroach Leucophaea maderae by using the motility of the cockroach hindgut as a bioassay. Thus, the isolated peptides were considered smooth muscle stimulants, i.e., myokins, and called leucokinsins (30). Agonists of smooth muscle are often secretagogues, which led our laboratory to examine the effect of leucokinin on fluid secretion in Malpighian tubules of the yellow fever mosquito, Aedes aegypti (27).

We found that leucokinin-VIII from the cockroach stimulated fluid secretion in Aedes Malpighian tubules, suggesting the wide distribution of kinins in insects. Indeed, the stimulation of fluid secretion by leucokinin and other kinins has now been observed in Malpighian tubules of the house cricket (23), locust (21, 55), corn earworm (10), fruit fly (36, 54), and housefly (32). The insect subclass Pterygota, the winged insects, includes 97% of all insects, and the kinins have been found in every order of this subclass that has been examined for kinin peptides.

In the past, we have used the kinins of the cockroach (the leucokinsins) to elucidate the mechanism of kinin action in Malpighian tubules of the yellow fever mosquito (5, 27, 39, 65–67). As early as 1994, the laboratory of Veenstra (57) has identified three kinins of the mosquito Aedes aegypti, the aedeskinins. In his laboratory, all three aedeskinins depolarized the transepithelial voltage in Aedes Malpighian tubules like leucokinin (58). However, only aedeskinin-I and -III stimulated fluid secretion in his laboratory, whereas aedeskinin-II had no detectable effect on fluid secretion (58).

In contrast to Veenstra’s earlier work, we report here that all three aedeskinins (including aedeskinin-II) significantly increase the rate of transepithelial fluid secretion. Moreover, aedeskinin-III increases the transepithelial secretion of NaCl...
and KCl, like leucokinin-VIII (5, 39). Furthermore, all three aedeskinins hyperpolarize the basolateral membrane voltage (V_{bl}) of principal cells, while decreasing the input resistance (R_{in}) of principal cells, like leucokinin-VIII (5, 39). Thus, aedeskinins and leucokinins share similar, if not the same, mechanisms of action.

Our study of five additional synthetic kinin analogs that possess single or multiple substitutions of β- and/or α-α-disubstituted amino acids, both within and just outside the COOH-terminal pentapeptide core sequence critical for biological activity, reveal separate and independent signaling pathways that mediate the effects on fluid secretion (V\_S) and electrophysiology (V\_bl, R\_in). Some kinin analogs increase V\_S without affecting V\_bl and R\_in. Other analogs show the opposite: no effect on V\_S but clear effects on V\_bl and R\_in. Since the Malpighian tubule of Aedes aegypti is thought to express only one kinin receptor (42), different physiological effects that originate from a single receptor may reflect mechanisms of ligand-dependent signaling and/or different glycosylation states of the receptor. Thus, the G protein-coupled kinin receptor is functionally more diverse than previously assumed.

Finally, we find that some tubules do not respond to aedeskinins at all (natural or the analogs). Preliminary observations point to differences in individual mosquitoes and in differences in populations of mosquitoes.

**MATERIALS AND METHODS**

**Mosquitoes and Malpighian Tubules**

The method for rearing mosquitoes (Aedes aegypti) is described in Ref. 39, with the exception that we now feed larval mosquitoes Tetramin flakes (tropical fish food) finely ground with mortar and pestle. On the day of the experiment a female mosquito, 4–7 days posteclosion, was cold anesthetized and decapitated. Malpighian tubules were removed from the mosquito under Ringer solution by pulling gently on the rectum which freed the gut and Malpighian tubules from the abdominal cavity. A Malpighian tubule was then removed with forceps from its attachment to the gut. The proximal end of the tubule (crushed by the forceps) was subsequently teased open with sharp jeweler’s broaches.

**Ringer Solution and Diuretic Peptides**

Ringer solution contained the following (in mM): 150 NaCl, 3.4 KCl, 25 HEPES, 1.8 NaHCO\_3, 1.0 MgSO\_4 (or MgCl\_2), 1.7 CaCl\_2, and 5.0 glucose. The pH was adjusted to 7.1 using 1 M NaOH. Table 1 lists the peptides used in the present study. Leucokinin-VIII, aedeskinins, and kinin analogs were synthesized in the laboratory of Nachman (50, 69). The identity of each peptide was confirmed by MALDI-TOF mass spectrometry, and the amount was quantified by amino acid analysis. The three aedeskinins are splice variants of the same gene (58).

In one set of experiments, we dissolved the peptides directly in Ringer solution and stored them at −20°C until use. In another set of experiments, the peptides were dissolved in 80% acetonitrile and 0.01% trifluoroacetic acid and stored at 4°C. On the day of the experiment, an aliquot was lyophilized and then dissolved in Ringer solution.

**Ramsay Method of Fluid Secretion**

The V\_S was measured at room temperature in isolated Malpighian tubules as developed by Ramsay (45) and adopted by us (29). In brief, the distal (blind) end of the tubule was bathed in a Ringer droplet of 50 µl under light mineral oil. The open end of the tubule was pulled into the oil with a glass hook so that fluid secreted by the tubule exited into the oil (Fig. 1A). The glass hook was formed on a microforge (Stoelting, Wood Dale, IL) using soft glass (R-6; Drummond Scientific, Broomall, PA). After the glass hook was acid-washed with a mixture of K\_2CrO\_4 and H\_2SO\_4, the hook was exposed to the vapor of 20 µl dimethyl-dichlorosilanol for 90 s (Fluka, Buchs, Switzerland) and then baked overnight at 110°C. The silanization prevented secreted fluid from spreading out along the hook.

Each tubule was used as its own control. The initial 30 min marked the control fluid secretion period. Thereafter, the control droplet (nanoliter volumes, 10\(^{-9}\) liter) was removed and prepared for the analysis of its ionic composition with the electron probe. Subsequently, 5 µl of Ringer solution was removed from the peritubular bath and replaced with 5 µl of Ringer solution containing one of the peptides of Table 1.

The 30-min experimental period began as soon as the peptide had been added to the peritubular bath. Rates of fluid secretion were determined by plotting cumulative volume (nl) secreted by the tubule as a function of time (30 min), each for the control and the experimental period. The plots were usually linear, yielding the rate of fluid secretion (V\_S) as the slope of the line (Microsoft Excel 2007; Microsoft, Redmond, WA).

**Electron Probe Analysis of Fluid Electrolyte Composition**

The ionic composition of fluid secreted by Malpighian tubules was analyzed with the electron probe as described previously (7, 62), except that we used the JEOL JXA 8900 EPMA Microprobe (Tokyo, Japan). Control and experimental fluid droplets collected in the Ramsay assay were prepared for electron probe analysis within 1 h of collection. A picoliter pipette was used to deposit a constant volume of unknowns (secreted fluid droplets) and standard solutions of known Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) concentrations onto a polished beryllium block under light mineral oil.

**Table 1. Kinins and synthetic analogs (28)**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Primary Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conserved kinin sequence</td>
<td>Phe-X(^2)-X(^3)-Trp-Gly-NH(_2)</td>
<td>(56)</td>
</tr>
<tr>
<td>Leucokinin-VIII</td>
<td>Gly-Ala-Asp-Phe-Tyr-Ser-Trp-Gly-NH(_2)</td>
<td>(30)</td>
</tr>
<tr>
<td>Aedeskinin I</td>
<td>Asn-Ser-Lys-Tyr-Val-Ser-Lys-Gln-Lys-Phe-Tyr-Ser-Trp-Gly-NH(_2)</td>
<td>(57)</td>
</tr>
<tr>
<td>Aedeskinin II</td>
<td>Asn-Pro-Phe-His-Ala-Trp-Gly-NH(_2)</td>
<td>(57)</td>
</tr>
<tr>
<td>Aedeskinin III</td>
<td>Asn-Asn-Pro-Asn-Val-Phe-Tyr-Pro-Trp-Gly-NH(_2)</td>
<td>(57)</td>
</tr>
<tr>
<td>Analog 1460 II</td>
<td>Ac-Arg-Phe-[(\beta)-Pro]-Trp-Gly-NH(_2)</td>
<td>(52)</td>
</tr>
<tr>
<td>Analog 1457</td>
<td>Phe-Phe-Phe-Ser-Trp-Gly-NH(_2)</td>
<td>(51)</td>
</tr>
<tr>
<td>Analog 1577</td>
<td>Ac-Arg-[(\beta)-Phe]-Phe-[(\beta)-Pro]-Trp-Gly-NH(_2)</td>
<td>(52)</td>
</tr>
<tr>
<td>Analog 1578</td>
<td>Ac-Arg-[(\beta)-Phe]-Phe-[(\beta)-Pro]-Trp-Gly-NH(_2)</td>
<td>(52)</td>
</tr>
<tr>
<td>Analog 1708, K-Aib-4</td>
<td>Ac-Arg-[(\beta)-Phe]-Phe-Phe-[(\beta)-Pro]-Trp-Gly-NH(_2)</td>
<td>(50)</td>
</tr>
</tbody>
</table>

Bold print identifies the critical pentapeptide sequence for activity in natural kinins. Ac, acetyl group; \(\beta\)-3-amino acid (with methylene group preceding the carbonyl group in the backbone); X\(^2\) can be His, Asn, Ser, Phe or Tyr; X\(^3\) can be Ser, Pro or Ala; Aib, \(\alpha\)-aminoisobutyric acid (31, 52).
Volumetric picoliter (10⁻¹² liter) pipettes were produced using soft glass (R6; Drummond Scientific) and a microforge (Stoelting) to form a volumetric chamber of ~60 pl from the pipette opening to an internal constriction. The exact pipette volume is not important as long as the same pipette is used for both standards and unknowns. Prior to use, the volumetric part of the pipette was vapor-silanized as described above. The silanization prevents fluid from sticking to the walls of the volumetric chamber.

After standards and unknowns were deposited on the beryllium block under oil (using the same picoliter pipette), the oil was removed in three washes with chloroform. The chloroform wash also removes water from the droplets, leaving salt crystals behind. The block is then stored in a desiccator under vacuum until analysis with the electron probe.

Wavelength dispersive spectroscopy is based on X-ray emissions specific to each element in the sample. The JEOL JXA 8900 EPMA Microprobe allows the measurement of as many as three elements in a single sample: Na⁺, K⁺, and Cl⁻ in the present study. Seven standard solutions containing a range of Na⁺, K⁺, and Cl⁻ concentrations were used to produce standard curves. X-ray counts from the unknowns (secreted fluid droplets) were read against the appropriate standard curve to yield the Na⁺, K⁺, and Cl⁻ concentrations in secreted fluid. Transepithelial secretion rates of Na⁺, K⁺, and Cl⁻ were calculated as the product of the ion concentration and the fluid secretion rate for both control and stimulated conditions.

Electrophysiological Effects of Aedeskinin and Analogs

The Vbd and the Rm of a principal cell of an isolated Malpighian tubule were measured as shown in Fig. 1B and as described previously (8, 34). In brief, Malpighian tubules were isolated and deposited on a sheet of Parafilm (American National Can, Menasha, WI) that had been stretched over the bottom of the Ringer perfusion bath of 0.5 ml volume. Malpighian tubules stick to stretched Parafilm, which secures them in place for the impalement of principal cells with microelectrodes and for flushing the Ringer bath in the washout of test substances.

Microelectrodes (Omega dot borosilicate glass capillaries, model 30–30-1; Frederick Haer, St. Bowdoinham, ME; or model 1B100F-4; World Precision Instruments, Sarasota, FL) were pulled on a programmable puller (model P-97; Sutter Instruments, Novato, CA) to yield resistances between 10 and 40 MΩ when filled with 3 M KCl. The microelectrode was bridged to the measuring hardware by using an Ag/AgCl junction. The junction was prepared by first degreasing a silver wire (OD, 0.25 mm) with alcohol, and then by Cl⁻-plating it in either 1) 0.1 M HCl for 20 min at a current of 50 μA or 2) in a concentrated solution of household Clorox for 15 min. The Ag/AgCl wires were inserted directly into the back of voltage and current electrodes (Fig. 1B). An Ag/AgCl wire with an OD of 0.5 mm was inserted into a 4% agar bridge of Ringer solution to serve as the ground electrode.

Vbd was measured with a Geneclamp 500 electrometer (Axon Instruments, Molecular Devices, Sunnyvale, CA) at room temperature. The Rm of the impaled principal cell was measured from current-voltage plots generated by voltage clamping the cell in a series of five increasing voltage-clamp steps: ΔV 5 mV, 400 ms each, starting at (Vbd - 10 mV). The voltage-stepping protocol and data acquisition were executed digitally by using a Digidata 1440 (Molecular Devices) under control of the Clampex module of the pCLAMP software package (version 10; Molecular Devices).

In the typical experiment, a single principal cell near the blind (distal) end of the Malpighian tubule was impaled with both voltage and current electrodes (Fig. 1B). The experiment was continued if both electrodes measured values of Vbd within 10 mV of each other. After recording a stable Vbd and Rm, the kinin of interest was added to the peritubular Ringer solution to yield a concentration of 10⁻⁶ M. New steady-state values of Vbd and Rm were recorded 1–2 min later as shown in Figs. 2, 4, and 5. The kinin was then washed out by flushing the 0.5 ml Ringer bath with Ringer solution at a rate of 5 ml/min.

Statistical Treatment of Data

Statistical significance of the data was evaluated using the paired Student t-test where each tubule was used as its own control. To construct a dose-response curve, the data were fitted using a sigmoid dose-response regression offered by the GraphPad Prism version 4.0 software for Windows (GraphPad Software, San Diego CA).

RESULTS

Effect of Leucokinin-VIII on Transepithelial Fluid Secretion and Tubule Electrophysiology

From a previous study (39), we know that leucokinin-VIII significantly increases the Vbd in isolated Malpighian tubules from 0.49 ± 0.04 nl/min to 0.91 ± 0.08 nl/min (P < 0.0001, n = 10 tubules). In the present study, leucokinin-VIII also had electrophysiological effects on principal cells of the tubule. In the representative experiment shown in Fig. 2, leucokinin-VIII hyperpolarized Vbd from about −90 mV to −108 mV, while lowering Rm from 516 to 330 kΩ. Both effects are reversible upon washout of leucokinin-VIII from the peritubular bath.

Effect of Aedeskinins on Transepithelial Fluid Secretion and Tubule Electrophysiology

All three aedeskinins tested at a concentration of 10⁻⁶ M significantly increased Vbd, hyperpolarized the Vbd, and de-
increased the $R_{in}$ of principal cells in isolated Aedes Malpighian tubules (Fig. 2).

Aedeskinin-I significantly increased $V_S$ from 0.53 ± 0.06 nl/min to 0.84 ± 0.11 nl/min in 21 Malpighian tubules ($P < 0.05$). In electrophysiological studies, the addition of aedeskinin-I to the peritubular medium of 14 Malpighian tubules immediately and significantly hyperpolarized $V_{bl}$ from −61.2 ± 6.6 mV to −69.7 ± 7.2 mV ($P < 0.001$, paired t-test) and significantly decreased $R_{in}$ from 413.0 ± 91.6 kΩ to 326.4 ± 75.3 kΩ ($P < 0.05$, paired t-test). A representative experiment is shown in Fig. 2. The effects on $V_{bl}$ and $R_{in}$ were reversible upon washout of aedeskinin-I (data not shown).

Aedeskinin-II significantly increased $V_S$ from 0.51 ± 0.07 nl/min to 0.93 ± 0.10 nl/min in 21 Malpighian tubules ($P < 0.001$). In electrophysiological studies, the peritubular addition of aedeskinin-II immediately and significantly hyperpolarized $V_{bl}$ from −70.2 ± 8.1 mV to −77.7 ± 8.2 mV in 10 principal cells of as many tubules ($P < 0.001$, paired t-test). In parallel, the $R_{in}$ dropped significantly from 331.4 ± 41.2 kΩ to 307.0 ± 38.7 kΩ ($P < 0.05$, paired t-test). A representative experiment is shown in Fig. 2. The effects on $V_{bl}$ and $R_{in}$ were reversible upon washout of aedeskinin-II.

Aedeskinin-III significantly increased $V_S$ from 0.52 ± 0.06 nl/min to 1.04 ± 0.13 nl/min in 21 Malpighian tubules ($P < 0.001$, paired t-test). In electrophysiological studies, the peritubular addition of aedeskinin-III immediately and significantly hyperpolarized $V_{bl}$ from −64.3 ± 3.7 mV to −87.8 ± 4.3 mV in 13 principal cells of as many tubules ($P < 0.001$, paired t-test). In parallel, the $R_{in}$ dropped significantly from 343.3 ± 54.8 kΩ to 265.2 ± 41.9 kΩ ($P < 0.01$, paired t-test). A representative experiment is shown in Fig. 2. The effects on $V_{bl}$ and $R_{in}$ were reversible upon washout of aedeskinin-III (data not shown).

Invariably, if a principal cell exhibited spontaneous oscillation of $V_{bl}$ under control conditions, then the peritubular addition of aedeskinin-I, -II, or -III substantially diminished or eliminated entirely these oscillations (Fig. 2).

**Aedeskinin-III and Transepithelial Fluid Secretion: a Dose Response Study**

To explore the diuretic mechanism of action of the aedeskinins, we studied in detail the effects of aedeskinin-III on $V_S$ in isolated Aedes Malpighian tubules. Aedeskinin-III was selected for this study because it was the most potent secretagogue in our laboratory (Fig. 2) and in the laboratory of Veenstra et al. (58).

Fig. 3A illustrates a dose response to aedeskinin-III. At a concentration of $10^{-10}$ M, aedeskinin-III had no effect on $V_{bl}$. At concentrations $10^{-8}$ M and higher, aedeskinin-III significantly increased $V_S$. Maximal stimulation was observed at $10^{-6}$ M with a 104.5 ± 14.0% increase in $V_S$ in 21 tubules ($P < 0.001$, paired t-test) and an EC50 value of $1.5 \times 10^{-8}$ M (Fig. 3A). The average control rate of $V_S$ in all 71 Malpighian tubules shown in Fig. 3A was 0.58 ± 0.04 nl/min.

The effect of aedeskinin-III on transepithelial Na$^+$, K$^+$, and Cl$^−$ secretion was tested in 10 separate Malpighian tubules (Fig. 3, B and C). In these tubules, the average control $V_S$ was 0.48 ± 0.10 nl/min (data not shown). It increased significantly to 0.93 ± 0.21 nl/min in the presence of $10^{-6}$ M aedeskinin-III ($P < 0.01$, paired t-test). In the same 10 tubules, aedeskinin-III significantly decreased the concentration of Na$^+$ in secreted fluid from 153.3 ± 13.1 mM to 114.3 ± 12.8 mM ($P < 0.01$, paired t-test), and significantly increased the concentration of K$^+$ from 61.9 ± 7.5 mM to 83.9 ± 6.4 mM ($P < 0.02$, paired t-test). The change in Cl$^−$ concentration from 183.7 ± 13.2 mM to 172.1 ± 6.9 mM was not significant.

The product of the fluid secretion rate and the ion concentration in secreted fluid yields the rate of transepithelial ion secretion. As shown in Fig. 3C, aedeskinin-III significantly increased the tubular secretion rates of Na$^+$, K$^+$, and Cl$^−$. The transepithelial Na$^+$ secretion increased 32%, from 70.9 ± 14.8 pmol/min to 93.4 ± 18.9 pmol/min ($P < 0.02$, paired t-test). The secretion of K$^+$ increased 140%, from 33.5 ± 8.8 pmol/min to 80.5 ± 20.5 pmol/min ($P < 0.01$, paired t-test).
paired t-test), and the secretion of Cl− increased 69%, from 94.1 ± 20.6 pmol/min to 158.7 ± 34.3 pmol/min (P < 0.01, paired t-test).

**Effects of Synthetic Kinin Analogs on Fluid Secretion and Tubule Electrophysiology**

**Analog 1460 II.** In the Ramsay fluid secretion assay, analog 1460 II (10⁻⁶ M) significantly increased Vₛ from 0.76 ± 0.11 nl/min to 1.32 ± 0.24 nl/min in the six tubules studied (P < 0.01, paired t-test; Fig. 4). In the electrophysiological assay, analog 1460 II (10⁻⁶ M) significantly and reversibly hyperpolarized the Vᵦ from −70.0 ± 7.7 mV to −84.4 ± 6.2 mV in 8 principal cells from as many tubules (P < 0.01, paired t-test). In parallel, the Rᵦ decreased significantly and reversibly from 323.0 ± 42.9 kΩ to 281.5 ± 34.3 kΩ (P < 0.01, paired t-test). The representative experiment shown in Fig. 4 illustrates Vᵦ oscillating between −65 and −85 mV under control conditions. The addition of analog 1460 II to the peritubular medium hyperpolarized Vᵦ to a steady-state voltage of −98 mV; in parallel, the Rᵦ of the principal cell dropped from 208 kΩ to 169 kΩ. Upon washout of 1460 II, Vᵦ returned to −74 mV, while Rᵦ increased to 255 kΩ.

![Fig. 3. A: dose-response curve for the effects of aedeskinin-III on transepithelial fluid secretion in isolated Malpighian tubules of Aedes aegypti. The effect of aedeskinin-III (10⁻⁶ M) on the concentrations of ions in secreted fluid and the rates of transepithelial ion secretion are shown in B and C, respectively. Open and shaded bars represent means ± SE with the number of paired tubule experiments in parentheses. *P < 0.02, **P < 0.01, ***P < 0.001, paired t-test.](http://ajpregu.physiology.org/)

**Fig. 4.** Effects of synthetic kinin analogs on the rate of transepithelial fluid secretion and the Vᵦ and Rᵦ of principal cells in isolated Malpighian tubules of female Aedes aegypti. Kinin analogs were used at a concentration of 10⁻⁶ M. Analog 1708 stimulated fluid secretion in some tubules and inhibited it in other tubules, such that, on average in 29 tubules, analog 1708 had no significant effect on fluid secretion. Open and shaded bars represent means ± SE with the number of tubule experiments in parentheses; *P < 0.05, **P < 0.01, paired t-test.
Analog 1547. In the Ramsay fluid secretion assay, analog 1547 (10^{-6} M) significantly increased V_S from 0.47 ± 0.07 nl/min to 1.01 ± 0.20 nl/min in the seven tubules studied (P < 0.01, paired t-test; Fig. 4). In the electrophysiological assay, analog 1547 significantly increased V_bl from −73.2 ± 6.3 mV to −83.0 ± 7.2 mV in nine principal cells from as many tubules (P < 0.01, paired t-test), but the effects on R_in were variable. The R_in increased by as much as 131 kΩ in five tubules, but decreased by as much as 209 kΩ in four tubules even though all nine tubules recorded the hyperpolarization of V_bl. Thus, the change in mean R_in from 337.3 ± 23.4 kΩ to 305.5 ± 58.6 kΩ was not significant. In the experiment shown in Fig. 4, analog 1547 reversibly hyperpolarized V_bl together with a drop in R_in.

Analog 1577. In the Ramsay fluid secretion assay, analog 1577 (10^{-6} M) significantly increased V_S from 0.72 ± 0.18 nl/min to 1.22 ± 0.22 nl/min in the six tubules studied (P < 0.05, paired t-test; Fig. 4). In the electrophysiological assay, analog 1577 significantly hyperpolarized V_bl from −73.8 ± 2.8 mV to −85.9 ± 2.3 mV in eight principal cells from as many tubules (P < 0.01, paired t-test), but the effects on R_in were variable. In six of eight tubules the R_in decreased in the presence of analog 1577, and in two tubules R_in increased. Thus, the small decrease in the mean R_in from 372.4 ± 26.7 kΩ to 356.2 ± 26.0 kΩ did not reach statistical significance. In the experiment shown in Fig. 4, analog 1577 hyperpolarized V_bl from −62 mV to −75 mV; in parallel, the R_in increased from 693 kΩ to 759 kΩ.

Analog 1578. In the Ramsay fluid secretion assay, analog 1578 (10^{-6} M) significantly increased V_S from 0.49 ± 0.05 nl/min to 1.01 ± 0.16 nl/min in the six tubules studied (P < 0.01, paired t-test; Fig. 4). However, in the electrophysiological assay, analog 1578 (10^{-6} M) had no effect on V_bl and R_in. As shown in the representative experiment of Fig. 4, the usual step change in V_bl and R_in upon the addition of aedeskinins did not occur after adding analog 1578 to the peritubular bath. As such, the small change in the mean V_bl from −81.1 ± 5.9 mV to −84.1 ± 6.1 mV in 10 principal cells from as many tubules was not statistically significant. Likewise, the small drop in mean R_in from 401.2 ± 45.8 kΩ to 388.4 ± 47.4 mV kΩ was not significant.

We point out that tubules which did not respond to analog 1578 still responded to aedeskinin-III (and/or to leucokinin-VIII) at concentrations of 10^{-6} M (Fig. 5E). Thus, analog 1578 was remarkable in that it clearly stimulated fluid secretion in Aedes Malpighian tubules without effects on tubule electrophysiology (Fig. 4).

Analog 1708. In the Ramsay fluid secretion assay, analog 1708 (10^{-6} M) is the only one that had no effect on the mean V_S in studies of 29 tubules (Fig. 4), which stems from the fact that in 12 tubules, analog 1708 increased V_S, whereas in 17 others it decreased V_S. In contrast, analog 1708 significantly hyperpolarized V_bl from −73.3 ± 4.6 mV to −95.0 ± 5.0 mV in nine principal cells from as many tubules (P < 0.001, paired t-test) and significantly decreased R_in from 439.1 ± 60.0 kΩ to 351.1 ± 38.1 kΩ (P < 0.02, paired t-test). In the representative experiment shown in Fig. 4, analog 1708 hyperpolarized V_bl from −92 mV to −110 mV, while decreasing R_in from 292 kΩ to 274 kΩ. In this and another tubule experiment, the effects of analog 1708 on V_bl and R_in were irreversible upon washout of analog 1708. In the remaining seven tubules, the effects on principal cell voltage and resistance were slowly reversible upon washout of analog 1708. Thus, analog 1708 was remarkable in that it clearly affected the electrophysiology of Aedes Malpighian tubules without consistent effects on fluid secretion (Fig. 4).

Spontaneous Voltage Oscillations

Malpighian tubules of Aedes aegypti often display oscillating membrane voltages under control, unstimulated conditions (Figs. 2, 4, and 5). From previous studies, we know that oscillations of the V_bl can come about from oscillating changes in the paracellular Cl− conductance (6, 8). As the paracellular Cl− conductance increases, V_bl hyperpolarizes.

The natural aedeskinins and those synthetic kinin analogs with effects on tubule electrophysiology not only caused the hyperpolarization of V_bl together with the drop in R_in (Figs. 2 and 4), they also substantially diminished or abolished the oscillations of V_bl. Upon washout of the kinin, the oscillations usually reappeared.

Variability in the Response to Kinins

We make the occasional observation that Malpighian tubules do not respond to kinins, neither the natural aedeskinins nor the synthetic analogs (Fig. 5). We encounter nonresponding tubules under three conditions. First, Malpighian tubules with control values of V_bl more negative than −75 mV and non-oscillating, are less likely to respond to kinins than tubules with the usual control values of V_bl in the vicinity of −60 mV. In the example shown in Fig. 5B, V_bl is about −80 mV, and it does not oscillate. Aedeskinin-I (10^{-6} M) had no effect on V_bl and R_in in this tubule. Since kinins hyperpolarize V_bl, we hypothesize that the tubule was already kinin-activated in the
mosquito from which it had been isolated. Second, the failure to respond to kinins is also observed in tubules with low and oscillating values of V_{bl}. In the example illustrated in Fig. 5C, V_{bl} oscillates around values of −50 mV. The tubule did not respond to aedeskinin-I. Third, we observe receptor desensitization in the presence of kinins. As shown in Fig. 5D, the isolated Malpighian tubule displayed the typical electrophysiological response in the presence of kinin: 1) the hyperpolarization of the V_{bl}, 2) the drop in the cell R_{m}, and 3) the cessation of V_{bl} oscillations. However, after 5 min in the presence of aedeskinin-III, V_{bl} started to repolarize and voltage oscillations returned after ~10 min. As V_{bl} continued to repolarize and as voltage oscillations increased with time, the cell R_{m} returned to control values in the presence of aedeskinin-III. The return to control values in the presence of agonist indicates receptor desensitization.

To rule out the study of nonresponding tubules when a kinin analog had no effect, we routinely pulsed the tubule with aedeskinin-III. For example, as shown in Fig. 5E, the tubule responded to aedeskinin-III but not to kinin agonist 1578.

DISCUSSION

Aedeskinin-II Stimulates Fluid Secretion

Our experiments on the effects of the three natural aedeskinins on the fluid secretion rates and electrophysiology of isolated Aedes Malpighian tubules have confirmed an analogous study by Veenstra et al. (58) with one glaring exception: in the laboratory of Veenstra, aedeskinin-II elicits electrophysiological effects but has no effect on fluid secretion (58). In our study, we find that aedeskinin-II stimulates both fluid secretion and electrophysiology in a similar manner as aedeskinin-I and -III (Fig. 2).

The discrepancy between the results of our study and those of Veenstra may relate to our observation that occasionally Malpighian tubules do not respond to kinins at all. For example, in one series of experiments using the same hatch of mosquitoes, two out of nine tubules did not respond to aedeskinin-I in the fluid secretion assay and five of nineteen Malpighian tubules did not respond in the electrophysiological assay (Fig. 5B and C). Similar observations can be made with aedeskinin-II and -III, as if the kinin receptor is not expressed in these Malpighian tubules.

In a review of our data back to 1989 when we first started to study insect kinins (27), the observation of nonresponding Malpighian tubules is rare, <1% of all studies when tubules were obtained from adult mosquitoes 3–5 days old. However, that percentage can increase to 100% within some mosquito hatches (populations) where the responsiveness of the tubules to kinins is delayed for unknown reasons. Since these nonresponding tubules are not the norm, we exclude them from the present study.

Nonresponsiveness to kinins may have a developmental and/or nutritional causation. Tubules taken from imago mosquitoes shortly after eclosion may not express the kinin receptor. For example, the tubule shown in Fig. 5C had a normal, oscillating V_{bl}, which did not change upon stimulation with aedeskinin-I. This tubule was taken from a mosquito 2 days after eclosion, when tubules from this hatch of mosquitoes did not respond to kinins. However, a few days later, hatch mates of this mosquito responded to kinin stimulation. As a first hypothesis, the time lag in receptor expression may reflect 1) the nutritional history of the mosquito in the larval stage, 2) the continued development of Malpighian tubules after eclosion, 3) the coupling of kinin receptor expression to reproduction, blood feeding, and/or other factors. For example, the quality of larval nutrition is known to affect ovarian development in Aedes aegypti (19, 25). Females with insufficient nutrition as larvae (due to a limited food supply or larval overcrowding) emerge as undersized gono-inactive adults. These gono-inactive females require additional feeding after emerging to develop their eggs to the previtellogenic resting stage of development. In contrast, females that are sufficiently nourished as larvae emerge as large gono-active females with ovarian follicles capable of commencing vitellogenesis upon feeding on blood. Given that the diuretic responses triggered by the kinins would physiologically be most relevant to gono-active, blood-fed females, it is tantalizing to speculate that the expression of the kinin receptor in Malpighian tubules is linked to an endocrine and/or developmental axis of ovarian development.

Accordingly, development, nutrition, reproduction, bloodfeeding, and other physiological processes may all influence the expression of the kinin receptor in Malpighian tubules. It is therefore conceivable that Veenstra et al. (58) observed electrophysiological effects of all three aedeskinins when he used tubules from mature and well-developed mosquitoes, but he failed to observe the effects of aedeskinin-II on fluid secretion in tubules isolated from developmentally or nutritionally compromised mosquitoes. Using mature mosquitoes in the present study, we have observed consistent effects of all three aedeskinins on both fluid secretion and electrophysiology (Fig. 2). Furthermore, Cady and Hagedorn (17) report that the injection of aedeskinin-II into the hemolymph of female Aedes aegypti increases urine production.

Aedeskinin-III Duplicates the Effects of Leucokinin

Before the aedeskinins became available, our laboratory studied the mechanism of action of the cockroach kinin, leucokinin-VIII, on Malpighian tubules of Aedes aegypti, where we made the first observation of the diuretic activity of the invertebrate family of kinins (27). We found that leucokinin-VIII increases the transepithelial secretion of water by increasing the transepithelial secretion of both NaCl and KCl (39). We have attributed the nonselective increase of both NaCl and KCl secretion to the activation of a large paracellular Cl⁻ conductance (5, 39, 59, 65).

Like leucokinin-VIII, aedeskinin-III increases the transepithelial secretion of NaCl and KCl, and both kinins significantly decrease the Na⁺ concentration and increase the K⁺ concentration in secreted fluid with no effect on the concentration of Cl⁻ (Fig. 3). Opposite effects on luminal Na⁺ and K⁺ concentrations (Fig. 3B) stem from the nearly complete transepithelial short circuit caused by the large increase in the paracellular Cl⁻ conductance in the presence of kinins (5, 65). The transepithelial short circuit depolarizes the transepithelial voltage to values close to zero (39, 58) with the effect of depolarizing the apical membrane voltage and hyperpolarizing the V_{bl} of principal cells (Fig. 2). Since the K⁺ conductance of the basolateral membrane of principal cells is four times greater than the Na⁺ conductance (8), it follows that the hyperpolar-
ization of $V_{h}$ increases the influx of $K^+$ from the peritubular medium to the cytoplasm of principal cells. As a result, the concentration of cytoplasmic $K^+$ is expected to rise, improving its competition with $Na^+$ for extrusion across the apical membrane into the tubule lumen. Consequently, the concentration of $K^+$ in the secreted fluid increases, while that of $Na^+$ falls (Fig. 3B). The concentration of $Cl^-$ in secreted fluid does not change because the luminal and peritubular $Cl^-$ concentrations are already similar and high under control conditions (9, 20, 37, 61, 62). Accordingly, the kinin-induced increase in paracellular $Cl^-$ conductance merely collapses the transepithelial voltage without a change in luminal or peritubular $Cl^-$ concentration.

Voltage and Resistance Oscillations and Evidence for a Role of Ca$^{2+}$

As shown in Figs. 2, 4, and 5, Malpighian tubules of Aedes aegypti often display oscillations of voltage and resistance under control conditions (6, 60, 63, 65, 67). Significantly, these oscillations are dependent on the $Cl^-$ concentrations in the peritubular medium (6, 8). Oscillations of the transepithelial voltage that are greater than the simultaneous voltage oscillations of the basolateral and apical membranes (6, 47) point to the paracellular pathway as the source of oscillations. Indeed, the oscillations derive from spontaneous cyclical $Cl^-$ conductance changes of the paracellular pathway (6). As the paracellular $Cl^-$ conductance increases, the transepithelial voltage goes to values close to 0 mV (approaching transepithelial short circuit). Consequently, the $V_{h}$ hyperpolarizes and the apical membrane voltage depolarizes (6, 47).

Studies in Drosophila Malpighian tubes have added importantly to our understanding of the spontaneous voltage and resistance oscillations observed in Malpighian tubules. Blumenthal (14) has found that the biogenic amine tyramine is a potent stimulator of transepithelial $Cl^-$ transport and a diuretic agent. The mechanism of action of tyramine, i.e., the sudden activation of a Ca$^{2+}$-dependent transepithelial $Cl^-$ conductance and its kinin-like change in transepithelial voltage (depolarization) is strikingly similar to that of the kinin diuresis we have elucidated in Aedes Malpighian tubules (5, 39, 65–67). Moreover, low doses of tyramine (1–10 nM) induce oscillations of voltage and resistance in Drosophila Malpighian tubules (12, 13). What is remarkable is that Drosophila Malpighian tubules generate their own tyramine from tyrosine (12), suggesting the autoregulation of fluid secretion in Malpighian tubules. The kinins and other diuretic agents, as well as antidiuretic agents, could have overriding influences on this autoregulation. Consistent with this hypothesis is the cross-desensitization of tyramine and leucokinin that Blumenthal (11, 13) reports in Drosophila Malpighian tubules. Whether tyramine is responsible for the spontaneous oscillations in Aedes Malpighian tubules remains to be investigated.

Changes in intracellular [Ca$^{2+}$] are likely to 1) mediate the spontaneous voltage oscillation and 2) signal the electrophysiological effects of kinins in Aedes and perhaps also Drosophila Malpighian tubules (11, 67). First, the Ca$^{2+}$ ionophore A23187 duplicates the effects of leucokinin in Aedes Malpighian tubules by lowering the transepithelial voltage and resistance, provided that Ca$^{2+}$ is present in the extracellular Ringer bath (18, 67). Second, thapsigargin, the inhibitor of Ca$^{2+}$ uptake by intracellular Ca$^{2+}$ stores, duplicates the electrophysiological effects of leucokinin-VIII (67). Third, the voltage oscillations disappear in the presence of kinins (Fig. 2), which are known to activate nifedipine-sensitive Ca$^{2+}$ channels in the basolateral membrane of principal cells (67). Fourth, analog 1460 II is the most potent synthetic kinin in an intracellular Ca$^{2+}$ fluorescence assay (aequorin) of CHO-K1 cells expressing the recombinant kinin receptor of Aedes aegypti (52) and a potent activator of the electrophysiological response in Aedes Malpighian tubules (Fig. 4). Fifth, analog 1578 elicits a weak Ca$^{2+}$ response in CHO-K1 cells expressing the kinin receptor of Aedes aegypti (52), and in the present study, analog 1578 has no electrophysiological effect (Fig. 4). Furthermore, we found that analogs 1547, 1577, and 1578 progressively lose the ability to activate electrophysiological effects (Fig. 4), which is the rank order similar to that observed by Taneja-Bageshwar et al. (52) using the intracellular Ca$^{2+}$ bioassay in CHO-K1 cells expressing the kinin G protein-coupled receptor (GPCR). Taken together, the above observations suggest Ca$^{2+}$ mediates both the spontaneous voltage and resistance oscillations under control conditions and the electrophysiological effects of kinins. The signaling pathway(s) kinins use to increase fluid secretion are unknown.

One Receptor, Two or More Signaling Pathways

Whereas all three aedekinsins exert effects on both tubule electrophysiology and fluid secretion, not every synthetic kinin analog displays this twin effect. Analog 1708 has no effect on transepithelial fluid secretion but consistent effects on tubule electrophysiology (Fig. 4). In contrast, analogs 1547, 1577, and 1578 consistently stimulate fluid secretion, but they lose the ability to activate electrophysiological effects with increasing length of the COOH-terminal pentapeptide (Fig. 4). Since Drosophila and Aedes express only one transcript of the single kinin receptor gene, which encodes a GPCR (41–43), the question arises as to how this single GPCR can give rise to the twin effects on fluid secretion and electrophysiology on the one hand, and to separate and independent effects on fluid secretion and electrophysiology on the other hand.

As a first hypothesis, we consider ligand-dependent selectivity of receptor signaling where a single receptor can give rise to more than one physiological response (Fig. 6). The phenomenon is also known as agonist-directed signaling, ligand-directed trafficking, conformation-specific agonism, or functional selectivity (for reviews see Refs. 2 and 48). Ligand-dependent signaling is particularly well known forGPCRs (48). For example, the single mammalian serotonin GPCR can activate 1) a phospholipase C-mediated increase in inositol phosphates, and/or 2) a phospholipase A2-mediated release of arachadonic acid, depending on the ligand that binds to it (3, 49). Each ligand is thought to induce a distinct receptor conformation that targets a specific signaling pathway. For example, the binding of serotonin to the serotonin GPCR may induce one conformation that activates the inositol phosphate and arachadonic acid pathways equally, the binding of agonist (6–1-(2,5-dimethoxy-4-iodophenyl)-2-amino propyl may induce a different receptor conformation that preferentially activates the arachadonic acid pathway, and the binding of agonist 3-trifluromethylphenyl-piperazine may trigger a receptor conformation that preferentially activates the inositol phos...
Fig. 6. Models of ligand/receptor interactions. A: ligand-dependent signaling via a single receptor; [model adapted from Berg and Clarke (2)]. B: glycosylation-dependent signaling, where R1 and R2 represent glycosylated and nonglycosylated forms of the same receptor.


te pathway (48). Similarly, the three aedeskinins, leucokinin, and analog 1460 II may activate equally those signaling pathways that increase rates of fluid secretion and induce electrophysiological changes (Figs. 2 and 6A). In the case of the other synthetic analogs, the amino acid substitutions produce conformations in the ligand that allow only partial binding to the receptor, thereby activating one physiological effect but not the other (Fig. 6A). Thus, the spatial modification found in analog 1708 may allow this analog to interact with the region of the receptor, which activates the electrophysiological response while preventing interactions with the receptor region-associated fluid secretion (Fig. 6A). In contrast, the spatial modifications of analog 1578 may allow the interaction with the receptor binding sites associated with the activation of fluid secretion, but prevent the interaction with binding sites that signal electrophysiological effects (Fig. 6A).

As a second hypothesis, we propose that the single kinin GPCR may control the access of ligands by way of glycosylation (Fig. 6B). GPCRs are typically glycoproteins with the glycosylation sites existing within the extracellular NH2-terminal region (46). The consensus sequence Asn-X-Ser/Thr provides for N-linked glycosylations. Although Drosophila Malpighian tubules express a single transcript encoding a kinin GPCR (consistent with one receptor), two immunochemical forms of the drosokinin GPCR are detected: one is N-glycosylated and the other is not (43). Likewise, Malpighian tubules of the Anopheles mosquito express two analogous immunochemical forms of the kinin GPCR (44). If we assume that Aedes Malpighian tubules also express two varieties of the kinin GPCR, R1 and R2, then the glycosylated receptor may signal one physiological event and the nonglycosylated receptor may signal the other. Thus, the three aedeskinins, leucokinin-VIII, and 1460 II may bind to both GPCRs, thereby triggering the twin physiological response of fluid secretion and tubule electrophysiology (Fig. 6B). However, in the case of analogs 1708 and 1578, the chemical modifications to the peptides may favor binding to the glycosylated form of the GPCR over the nonglycosylated form (or vice versa), resulting in one physiological response that dominates over the other.

Glycosylation-dependent ligand binding of GPCRs and signal-transduction response (1, 35).

The phenomena of agonist-directed or glycosylation-dependent signaling now sheds light on an observation that has been enigmatic to us for the past 21 years: leucokinin-VIII produces different dose response curves of its effects on 1) the transepithelial voltage, and 2) rates of fluid secretion in Aedes Malpighian tubules (27). The voltage dose-response curve is steep with an EC50 of 2.4 × 10–9 M, whereas the fluid secretion dose-response curve is shallow with an EC50 of 3.0 × 10–8 M (27), similar to that observed in the present study using aedeskinin-III (Fig. 3A). Veenstra and colleagues (58) made similar observations finding that the aedeskinins have different threshold concentrations when assayed for the depolarizing activity of the transepithelial voltage and also different potencies on fluid secretion. Veenstra et al. (58) suggested more than one type of kinin receptor to account for the differences observed in electrophysiological and fluid secretion assays. The present study suggests agonist-directed signaling of kinins, or alternatively, a differentially glycosylated receptor that can activate electrophysiological or fluid secretion responses.

Perspectives and Significance

In the present study, we have used two bioassays (electrophysiology and fluid secretion) to probe kinin/receptor interactions in Malpighian tubules of the yellow fever mosquito Aedes aegypti. We have observed that the three natural aedeskinins activate both physiological responses. In contrast, synthetic kinin analogs containing spatial modifications in the critical pentapeptide core region may trigger just one or both effects. Separate and independent effects on electrophysiology and fluid secretion may reflect ligand-directed signaling and/or glycosylation-dependent functional states of the single kinin receptor expressed in Aedes Malpighian tubules. Separate effects of kinin analogs on electrophysiology and fluid secretion suggest that natural kinins may be physiologically more diverse than previously assumed. For example, dose-dependent
effects on tubule electrophysiology without an effect on fluid secretion may indicate the renal response to a purely ionic challenge, whereas effects on fluid secretion without an effect on electrophysiology may indicate the renal (electronutral) response to a volume load. More than one bioassay should be consulted to fully evaluate the structure/function relations of insect kinins and their GCPRs. The reliance on only one bioassay (as in high-throughput screens of voltage effects or changes in intracellular Ca\(^{2+}\) concentrations) would have excluded analog 1578 as a potential diuretic agent. By incorporating the Ramsay fluid secretion assay, we have proven otherwise.

**APPENDIX: ON CHANGES IN VOLTAGE AND FLUID SECRETION**

In response to a reviewer’s inquiry about how voltage effects can be independent from effects on ion transport and fluid secretion in Malpighian tubules, it is instructive to compare the quantities of ions (charge) needed to change a voltage with the quantities of ions secreted across the epithelium into the tubule lumen. Figure 3 illustrates that in the presence of aedeskinin-III the tubule secretes cations or anions at a rate of \(\sim 165\) pmol/min. The secretion is mediated by the 1.5-mm tubule segment suspended in the Ringer droplet of the Ramsay fluid secretion assay (Fig. 1A). Since the average electrical diameter of the *Aedes* Malpighian tubule determined by cable analysis is \(\sim 22\) \(\mu\)m (38), the transepithelial cation or anion secretion can be normalized to an epithelial surface area, i.e., of \(2.7 \times 10^{-9}\) mol/s – cm\(^{-2}\). Applying the Faraday constant yields an electrical equivalent of \(2.6 \times 10^{-4}\) coul/s – cm\(^{-2}\), or \(260\) \(\mu\)A/cm\(^{2}\).

The electrical capacitance of epithelia is \(\sim 3.5\) \(\mu\)F/cm\(^{2}\) (33). The quantity of charge \(q\) is the product of capacitance (C) and voltage (V). Accordingly, the kinin-induced depolarization of the transepithelial voltage of *Aedes* Malpighian tubule from 59 mV to 6 mV (39) requires a charge redistribution of only \(1.9 \times 10^{-7}\) coul/cm\(^{2}\) which takes only 0.72 ms at the transepithelial charge secretion rate of \(2.6 \times 10^{-4}\) coul/s – cm\(^{-2}\). In that short time the tubule secretes a volume of only \(1.2 \times 10^{-14}\) liters of fluid, far too small for detection in the Ramsay fluid secretion assay. The above calculations illustrate that a change of voltage requires very little ion flux. Accordingly, a change in voltage may not reflect a significant change in ion and fluid secretion.

The kinin-induced drop in transepithelial voltage from 59 mV to 6 mV together with the drop in transepithelial resistance from 58 \(\Omega\)/cm\(^{2}\) to 10 \(\Omega\)/cm\(^{2}\) (39) indicates the switch from a moderately tight epithelium to a leaky epithelium. At the extremes, leaky epithelia are specialized for massive transepithelial transport of solute and water (often in isosmotic proportions as in renal proximal tubules), and tight epithelia (with low rates of transepithelial transport) are specialized for storage functions (such as the urinary bladder). In tight epithelia, changes in transepithelial ion transport are reflected in changes in voltage and resistance; however, in leaky epithelia large changes in transepithelial ion transport may take place with little effect on voltage and resistance, because leaky epithelial function is state-dependent condition (where short-circuit is the condition of zero voltage).

**ACKNOWLEDGMENTS**

We thank Dr. Marcus Huss at the University of Osnabrück for erudite discussions.

**GRANTS**

The National Science Foundation Award IBN-0078058 made this work possible in the Beyenbach laboratory, and the U.S. Department of Agriculture/Department of Defense, Deployed War Fighter Protection Initiative No. 0500-32000-001-01R supported work in the Nachman laboratory.


