

Leucokinin mimetic elicits aversive behavior in mosquito *Aedes aegypti* (L.) and inhibits the sugar taste neuron

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Insect kinins (leucokinins) are multifunctional peptides acting as neurohormones and neurotransmitters. In females of the mosquito vector *Aedes aegypti* (L.), aedeskinins are known to stimulate fluid secretion from the renal organs (Malpighian tubules) and hindgut contractions by activating a G protein-coupled kinin receptor designated “*Aedae*-KR.” We used protease-resistant kinin analogs 1728, 1729, and 1460 to evaluate their effects on sucrose perception and feeding behavior. In no-choice feeding bioassays (capillary feeder and plate assays), the analog 1728, which contains α -amino isobutyric acid, inhibited females from feeding on sucrose. It further induced quick fly-away or walk-away behavior following contact with the tarsi and the mouthparts. Electrophysiological recordings from single long labellar sensilla of the proboscis demonstrated that mixing the analog 1728 at 1 mM with sucrose almost completely inhibited the detection of sucrose. *Aedae*-KR was immunolocalized in contact chemosensory neurons in prothoracic tarsi and in sensory neurons and accessory cells of long labellar sensilla in the distal labellum. Silencing *Aedae*-KR by RNAi significantly reduced gene expression and eliminated the feeding-aversion behavior resulting from contact with the analog 1728, thus directly implicating the *Aedae*-KR in the aversion response. To our knowledge, this is the first report that kinin analogs modulate sucrose perception in any insect. The aversion to feeding elicited by analog 1728 suggests that synthetic molecules targeting the mosquito *Aedae*-KR in the labellum and tarsi should be investigated for the potential to discover novel feeding deterrents of mosquito vectors.

neuropeptide GPCR | sucrose taste | sensory neuron | chemical target validation | feeding deterrent

Females of *Aedes aegypti* (L.) mosquitoes (Diptera: Culicidae) are anthropophilic, feeding preferentially on blood from a human host, but both sexes feed on sugar-rich nectar as a source of metabolic energy. The female requires a blood meal for egg production and during that meal can transmit mosquito-borne diseases including dengue, Zika, chikungunya, and yellow fever viruses (1). Sugar feeding begins shortly after adult emergence and continues throughout adulthood. Importantly, sugar feeding influences vectorial capacity by increasing daily survival (2, 3) and can positively affect female reproductive maturation by increasing juvenile hormone synthesis (3).

However, ingesting liquids causes osmotic stress, which insects compensate through diuresis. We targeted this essential mechanism by altering neuropeptides affecting diuresis. Neuropeptide diuretic hormones increase the secretion of primary urine by the Malpighian tubules and increase hindgut contractions, which aid in fluid excretion (4). In *A. aegypti*, three endogenous kinins (aedeskinin I–III) act as diuretic hormones on Malpighian tubule stellate cells (5, 6) by stimulating chloride transport and fluid secretion (6–8). We verified that the aedeskinins activate the

single *Aedes* kinin receptor (*Aedae*-KR), a G protein-coupled receptor (GPCR) that signals through intracellular calcium (9). We designed kinin analogs to be resistant to degrading peptidases and therefore exhibit sustained high potency (10, 11). One biostable kinin peptidomimetic containing aminoisobutyric acid, 1728, has potency similar to or higher than the aedeskinins on recombinant receptors (12). Such biostable kinin analogs have potential in the control of insect pests because they reduce feeding in lepidopteran larvae (10, 13) and increase aphid mortality (14, 15).

Here, we examined whether three biostable insect kinin analogs, 1728, 1729, and 1460 (Fig. S1), affect feeding in female mosquitoes and/or have a direct impact on the gustatory detection of sugars. First, we demonstrate through feeding assays that the kinin analog 1728 significantly reduces the time females spend in contact with a sucrose solution and displays potent antifeedant activity. In addition, we present the first evidence, to our knowledge, that the potent kinin peptidomimetic 1728 further triggers female mosquito aversive fly-away or walk-away behaviors upon labellar and tarsal contacts with a sucrose source, overriding sweet taste perception. Electrophysiological recordings from the long labellar sensillum revealed that externally applied kinin analogs inhibited the sucrose-evoked response within milliseconds. Second, the *Aedae*-KR was cloned and sequenced from sensory

Significance

Kinin receptors are known in insects to contribute to osmotic regulation and are expressed in the excretory system, in the Malpighian tubules (renal organs), and in hindgut. We discovered that in *Aedes aegypti* mosquitoes, which are important vectors of human disease, a kinin receptor is also expressed within taste hairs (sensilla) on the legs and mouthparts. A kinin analog engineered to be peptidase resistant activates this kinin receptor with high potency, inhibiting sucrose taste detection directly at the level of the taste organs and eliciting a fast and highly aversive response in females during feeding. This finding suggests that mosquito G protein-coupled receptors could be new targets for discovering compounds to deter mosquitoes and preventing them from feeding.

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The authors declare no conflict of interest.

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appendages, and immunolocalization experiments confirmed its expression in sensory neurons of the tarsi and long labellar sensilla and in accessory cells of the labellum. Moreover, silencing the *Aedae-KR* in female mosquitoes inhibited the aversive behavior resulting from contact with the kinin analog 1728. Taken together, these observations provide new insight into gustatory perception modulated by a canonical GPCR.

Results

No-Choice Feeding Bioassays of Kinin Analogs. To determine the effects of kinin analogs (Fig. S1) on mosquitoes, we exposed females to drops of a sucrose solution mixed with different concentrations of kinin analogs 1728 and 1729. Most often, females touched the diet with their proboscis and prothoracic legs simultaneously (Movies S1 and S2). With analog 1728 at a concentration of 1 mM, females that contacted the diet moved away within a few seconds by exhibiting jump-, fly-, or walk-away behavior (Movie S1). Such an aversive response was rarely observed when females contacted the control sucrose-only solution (Fig. 1 and Movie S2). To quantify these behaviors, we compared the time females spent in contact with diets containing a kinin analog and with the sucrose-only solution (300 mM) during the first hour of exposure (Fig. 1). Analog 1728 at 1 mM significantly reduced the median time spent in contact with the diet (6 s) compared with the other analog concentrations and the sucrose-only control. The maximal time spent in contact with analog 1728 at a concentration of 1 mM was about 2 min and was several fold longer for all other treatments (Fig. 1). The median time spent in contact with analogs 1728 at 600 μ M and 1729 at 1 mM and 600 μ M also was reduced compared with the time spent in contact with the control solution (94.5 s) (Fig. 1). The time females spent in contact with analog

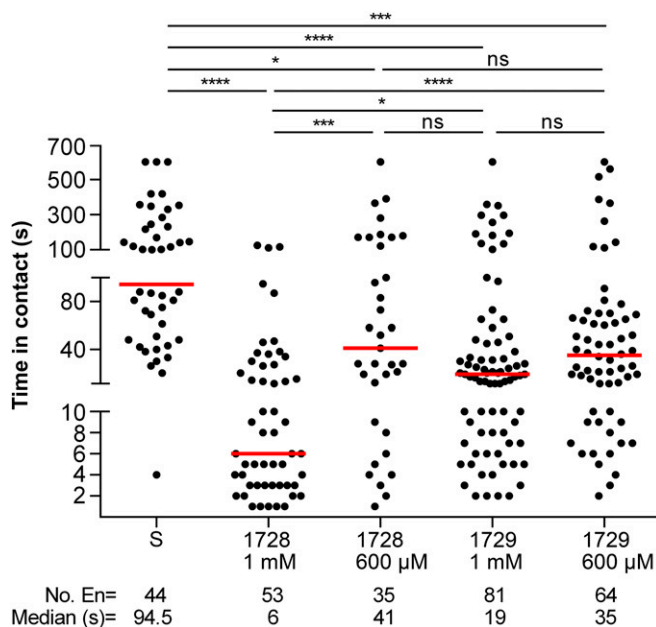


Fig. 1. Median time female mosquitoes spent in contact with kinin analogs 1728 and 1729 (at 1-mM and 600- μ M concentrations in sucrose solution) or with a 10% sucrose solution (S) as control. Females were videorecorded for 1 h (Movies S1 and S2). Each dot represents the duration of a single encounter of a female with the diet, and the median time spent in contact is indicated by a red line. Three independent replicates were performed, for a total of 60 females ($20\text{f} \times 3$) exposed per treatment. The total number of encounters (En) is shown below each column; some females made multiple encounters. The maximal recorded time of an individual encounter was 10 min. Data were analyzed by Kruskal–Wallis nonparametric ANOVA followed by Dunn’s multiple comparisons test. Black lines above the figure define the contrasts in pairs of medians. Asterisks denote significant differences ($*P < 0.05$, $****P < 0.0001$); ns, not significant.

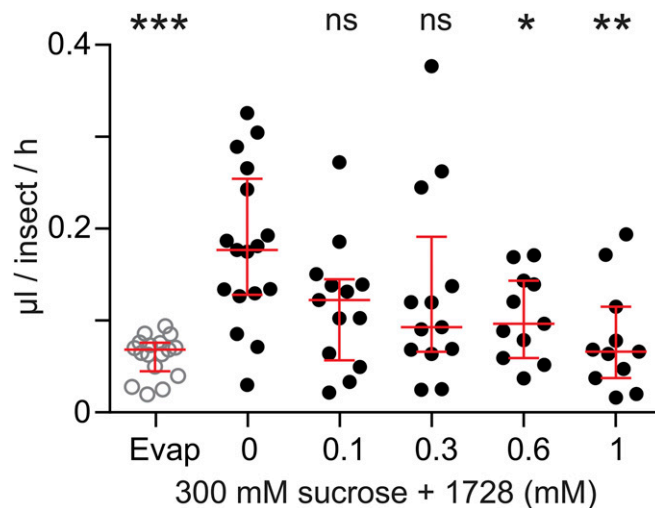


Fig. 2. Consumption of sugar mixed with kinin 1728 ingested by *A. aegypti* females during 2 h in a CAFE assay. Groups of five females were starved for 48 h and then were exposed to a 5- μ L capillary tube containing 300 mM sucrose and kinin 1728. The volume of liquid that disappeared during the experiment was measured. Data are shown as individual measures (dots) and median volume \pm first quartile (red lines) from 11–17 replications. Ordinates: volume expressed in microliters per insect per hour. Abscissa: concentration of analog 1728 (0 to 1 mM) in a 300-mM sucrose solution; Evap, loss of volume of sugar solution from vials without mosquitoes. One-way ANOVA followed by Dunnett’s multiple comparison test was used to compare treatments with the sucrose control ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$); ns, not significant.

1728 at 600 μ M (41 s) did not differ from the time spent in contact with analog 1729 (Fig. 1). To determine if the observed shorter time spent in contact with the two analogs also differentially affected ingestion, capillary feeder (CAFE) assays were performed. Females ingested significantly less of either analog at the 1-mM concentration (Fig. 2 and Fig. S2) and also ingested less analog 1728 at the 600 μ M concentration (Fig. 2), as compared with sucrose. Because kinin analogs may stimulate diuresis during feeding, plate assays were run to determine the number of urine drops (colored blue by the addition of Evans blue to the diets) deposited in the plates and the quantity of Evans blue remaining in females 5 h after diet ingestion when provided sucrose plus one analog at a time (Figs. S3 and S4). Females exposed to analog 1728 at 1 mM and 600 μ M contained less Evans blue than females exposed to analog 1729 at similar concentrations or to the control sucrose solution (Fig. S3A and B). No significant differences in Evans blue content were observed between treatments with analogs 1728 and 1729 (Fig. S3C–F). Importantly, fewer urine drops were observed when females were exposed to analog 1728 at 1 mM and 600 μ M than when females were offered control solution (S) or solution treated with analog 1729 at the same concentrations (Fig. S4A and B). The number of urine drops excreted from females exposed to analog 1728 also was lower at 1 mM than at 600 μ M (Fig. S4B and Inset I). No differences were found between females offered control sucrose-only solution and those treated with analog 1729 (Fig. S4B and Inset II). The results of the median time spent in contact with diets during the first hour of exposure (Fig. 1) and CAFE assays (Fig. 2) indicated that females exposed to analog 1728 at 1 mM or 600 μ M consumed less diet than those exposed to analog 1728 at lower concentrations.

Closer examination of females’ behavior allowed us to determine that the rejection of diets containing analog 1728 at 1 mM and 600 μ M occurred most often after the female contacted the diet with the legs and proboscis simultaneously (Movie S1).

Electrophysiological Recordings on Long Labellar Hair Sensilla. To determine if kinin analogs interfered directly with the detection of sucrose, we performed electrophysiological recordings from long

labellar sensilla on the proboscis (Fig. 3A and Fig. S5A). In this preparation, extracellularly recorded spikes show an amplitude between 0.5 and 2 mV, depending on the insect and on the firing rate of the cells. The responses to 300 mM sucrose mixed with the kinin analogs (10 μ M to 1 mM of 1728, 1729, or 1460) generally showed only one class of action potentials (Fig. 3B), suggesting that these analogs do not activate another taste modality (i.e., salty or bitter), at least in the sensilla tested.

When the analogs were applied at 1 mM, a significant ($*P < 0.05$) decrease in the firing rate—60, 45, and 30% for analogs 1728, 1729, and 1460, respectively—was observed (Fig. 3A) as compared with sucrose only. Analog 1728 was not inhibitory when applied at 10 μ M, but the level of inhibition was intermediate for 1728 and 1729 applied at 100 μ M. Analog 1460 inhibited the sucrose response significantly only when applied at 1 mM and was less inhibitory than the other two at this concentration, with analog 1729 being intermediate in potency (Fig. 3A). The representative traces obtained in response to sucrose in the absence or presence of the three analogs clearly show analog 1728 is the most potent, followed by 1729 and 1460 in that order (Fig. 3B). Kinin analogs depress both the phasic and the tonic portions of the responses to sugar, and this inhibition is concentration dependent (Fig. 3C). Kinin analogs thus exert their inhibition immediately after the female contacts the stimulus solution. The three analogs do not differ in their temporal kinetics, keeping the same rank order of potency, suggesting that they act on the same target. It is clear that the initial response to sucrose is higher than the initial number of spikes per second for analog 1728 at 100 μ M (and 1 mM); this difference is not so obvious for analog 1460. The response is very rapid for analog 1728 at 1 mM and is less rapid for

the other two analogs (Fig. 3C, black traces). These electrophysiological results are in accordance with observations of feeding behavior (Figs. 1 and 2, Figs. S2 and S3, and Movie S1), which indicated that analog 1728 was the most potent molecule.

To determine if analog 1728 had other aversive effects in addition to the inhibition of sucrose perception, we performed no-choice assays in the presence and absence of sucrose (Fig. S6). The time spent in contact with Evans blue only (E) was shorter the time spent in contact with sucrose solution containing Evans blue (SE). Importantly, we showed that Evans blue does not alter the number of spikes per second of the sucrose neuron (Fig. S5C), suggesting that females perceive solution (E) to be similar to water. In addition, there was no difference in the median time females spent in contact with the (E) solution or analog 1728 with Evans blue in the absence of sucrose (1728E). The median time spent in contact did not differ between 1728E and 1728SE, indicating that sucrose is not perceived differentially by these two female groups. However, the median time females spent with 1728SE was shorter than that spent with solution (E) (Fig. S6). These results clearly indicate that analog 1728 at 1 mM interacts strongly with the sucrose perception circuitry.

Aedae-KR Full-Length cDNA Cloning from Female and Male Legs and Female Labellum. The aversive response to analog 1728 appeared to be specific and mediated mainly by labellar and tarsal contact (Fig. 1 and Movie S1). Therefore, to investigate *Aedae-KR* expression in these appendages, full-length cDNAs were cloned from the labellum and legs of females and from the legs of males (Fig. S7). The *Aedae-KR* predicted amino acid sequences obtained were similar to the receptor cloned from the Malpighian tubule (AAT95982) (9), and the region selected for antibody production (residues 328–345; NEKFKREFHKRYPFGRGN) (Fig. S7) also was identical. Thus, transcript expression of *Aedae-KR* was confirmed in appendages, validating the use of the previously reported antibody to localize the receptor (5, 7).

Immunolocalization of *Aedae-KR* and Identification of *Aedae-KR* Sensilla. To verify *Aedae-KR* protein expression, immunohistochemistry was performed in frozen sections of labellum (Fig. 4 and Figs. S8 and S9) and tarsi (Fig. 5 and Fig. S10). The labellum is shown in Fig. 4A and Figs. S8A and S9U. The receptor signal (red) was observed in dendrites of sensory neurons extending to the tip of the long labellar hairs (Fig. 4) and in accessory cells present at the base of long labellar sensilla (Fig. 4 and Figs. S8 and S9), but the signal was detected only in accessory cells of short papillae (Fig. 4 and Fig. S8). Receptor signal was not observed at the base of hairs in the labellar proximal segment (Fig. 4 and Figs. S8 and S9). No signal was observed in tissues incubated with antigen-preabsorbed antibodies (Figs. S8 and S9) or preimmune serum (Fig. S9).

The *Aedae-KR* was immunolocalized in prothoracic tarsal sensory neurons in the second and third tarsomeres (Fig. 5A). Both tarsomeres exhibited a number of immunoreactive neurons along their proximal–distal axis; these neurons have somas that are strongly labeled by the anti-*Aedae-KR* antibody (anti-KR) and are close to the cuticle (Fig. 5B). *Aedae-KR* sensory neurons in tarsi appear to extend their dendrites (Fig. 5B, III-2) into sensilla trichodea (Fig. 5B, III-3). Negative control tarsal tissues, incubated with either antigen preabsorbed antibodies (Fig. S10 A–C) or preimmune serum (Fig. S10 D–F) did not show any receptor signal, as expected.

Effects of *Aedae-KR* Gene Silencing on Feeding Behavior. Gene silencing was performed to confirm that the mosquitoes' aversive behavior to the 1728 analog was mediated by the kinin receptor function in peripheral organs (Figs. 4 and 5). Two days postinjection of dsRNA, *Aedae-KR* expression was significantly reduced by 52% compared with control dsGFP-injected mosquitoes (Fig. 6A). In no-choice feeding assays with kinin analogs, control dsGFP females demonstrated aversive behavior to kinin analog 1728 at 1 mM (Fig. 6B), similar to that observed in naive 1728-challenged females (Fig. 1). In contrast, KR-silenced females no longer displayed the aversive

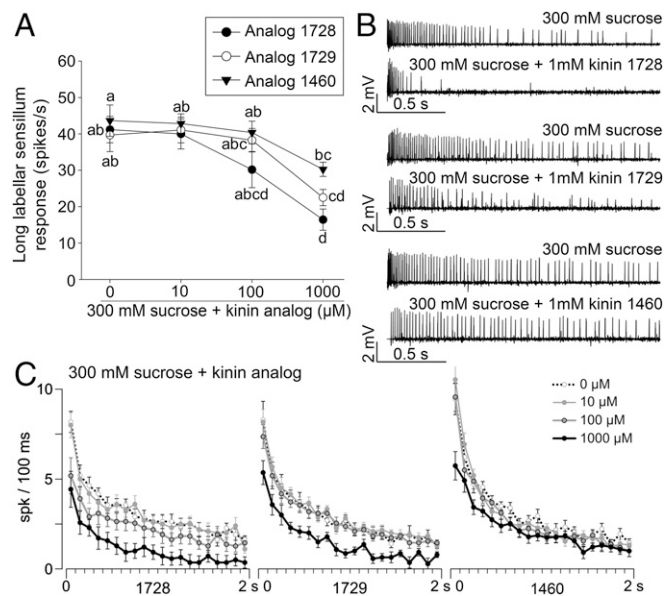


Fig. 3. Electrophysiological responses to kinin analogs recorded from long sensilla in the distal segment of the female labellum. (A) Long labellar sensilla were stimulated with kinin analogs 1728, 1729, and 1460 in 300 mM sucrose. For analogs 1728 and 1729 the maximal inhibition of the sucrose response was observed at 1 mM. Analog 1460 significantly inhibits the sucrose response only when applied at 1 mM, and it was less inhibitory than analogs 1728 and 1729 at the same concentration; analog 1729 was intermediate in potency. Data analysis (the number of spikes during the first second of 2-s recordings) was performed using the SAS command PROC generalized linear mixed model (GLMM) Tukey–Kramer test. Different letters indicate significant differences. Ten females were used to obtain each curve. (B) Consecutive responses to 300 mM sucrose (Upper Traces) and 1 mM of kinin analogs in 300 mM sucrose (Lower Traces) over 2 s. (C) Temporal dynamics of sweet neuron responses to kinin analogs at 1 mM in sucrose solution. In A and C each point represents the mean, and bars represent the SEM.

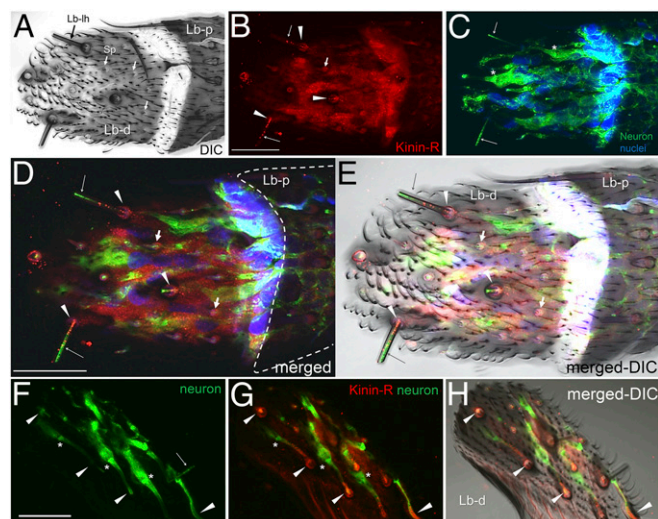


Fig. 4. Confocal analyses of the *Aedae-KR* immunolocalization in long labellar sensilla (Lb-lh, long hair) (black arrow) and short papillae (Sp) (white arrows) in the distal segment of the female labellum. Lb-d, labellar distal segment. (A) Diffused interference contrast (DIC) image. (B–H) The receptor signal (red) is present in dendrites of sensory neurons, indicated by long arrows in B, D, and E, and in accessory cells of both the long labellar sensilla, indicated by arrowheads in B, D, E, G, and H, and short papillae, indicated by short arrows in B, D, and E. Neurons appear green in C–H. Nuclei appear blue in C, D, and E. The root fiber bundles in the ciliary region of sensory neurons are marked by asterisks in C, F, and G. No receptor signal was observed in the labellum proximal segment (the area enclosed by a dashed white line in D). Images were acquired as Z-stacks (Z step: 0.41 μm) using a 100 \times /1.4 oil immersion objective as follows: A and C, 20 sections; D and E, 11 sections; F, 19 sections. B, G, and H show a single optical section (depth, 4.51 μm from the cuticle). (Scale bars, 20 μm .) Two different tissues are shown in A–E and F–H, respectively.

behavior while probing and touching diets containing analog 1728 at a concentration of 1 mM (Fig. 6C). Consequently, the median time those silenced females spent in contact with the diet containing 1-mM analog 1728 (71 s) was similar to the time silenced females spent in contact with the sucrose solution (80 s) (Fig. 6C), suggesting that the KR is directly responsible for the aversion phenotype of the 1728 analog. Similarly, KR-silenced females exposed to analog 1729 at 1 mM did not display significant differences from those exposed to sucrose solution. Although no significant differences were found for the dsGFP females, a trend toward shorter time in contact with analog 1729 was observed for the dsGFP-silenced females (1729: 101.5 s vs. S: 129 s) (Fig. S11), as we had observed previously (Fig. 1).

Discussion

In this work, we clearly show, for the first time to our knowledge, that the gustatory detection of sugars is modulated by a neuropeptide directly at the level of peripheral sensory neurons. This finding suggests that kinin analogs interact with G protein-coupled receptors expressed within gustatory sensilla similar to the way that peripheral olfactory neurons are modulated by tachykinin (16, 17) and neuropeptide F (18–20). These peptides bind to GPCRs in olfactory receptor neurons (ORNs) or in circuits that modulate chemosensory signal-dependent feeding behaviors and food search (21). Here we provide, for the first time to our knowledge, evidence that neuropeptide GPCRs are expressed in taste peripheral neurons and that activating these GPCRs with kinin analogs changes the sensitivity of these neurons to sucrose.

Most mechanistic studies on insect kinins have been conducted with dipterans, *Drosophila melanogaster* and mosquitoes. *Drosophila* kinin is named “drosokinin” or “leucokinin” (*lk*) (22). Three pairs of *lk* subesophageal neurons (SELKs) receive projections from gustatory receptor neurons in the head (23, 24). Peripheral drosokinin

expression was found in sensory cells associated with tarsal sensilla (bristle sensilla) and in labellum by monitoring GFP expression driven by an *lk*-specific GAL4 line (23). Transgenic flies in which drosokinin release was blocked from the brain lateral horn (LHLK) and SELKs had altered olfactory and gustatory responses, respectively (24). Previous reports have questioned the significance of the *lk* system in taste perception in legs and labellum (25), based on the lack of GFP in mouthparts and legs in driver lines. The GFP-expressing cells shown by de Haro et al. (23) did not have the appearance of sensory neurons, and mutant flies did not display defects in the gustatory detection of sucrose (25). Therefore the significance of the kinin signaling system in peripheral taste sensory function in *Drosophila* is still controversial. There is no information about the peripheral functions of kinin neurotransmission in mosquitoes or about behavioral responses to altered kinin signaling in putative “leucokinin” or “leucokinin-responsive” sensory cells in labellum and legs.

Here, we exposed mosquitoes to sucrose solutions containing potent and biostable insect kinin analogs that are lethal to aphids and that are active on the recombinant *Aedae-KR* (12, 26). Floral nectar, which is fed upon by diurnal mosquitoes such as *A. aegypti*, contains ~55% sucrose, and both sexes prefer disaccharides, sucrose and trehalose, over monosaccharides (27). Because

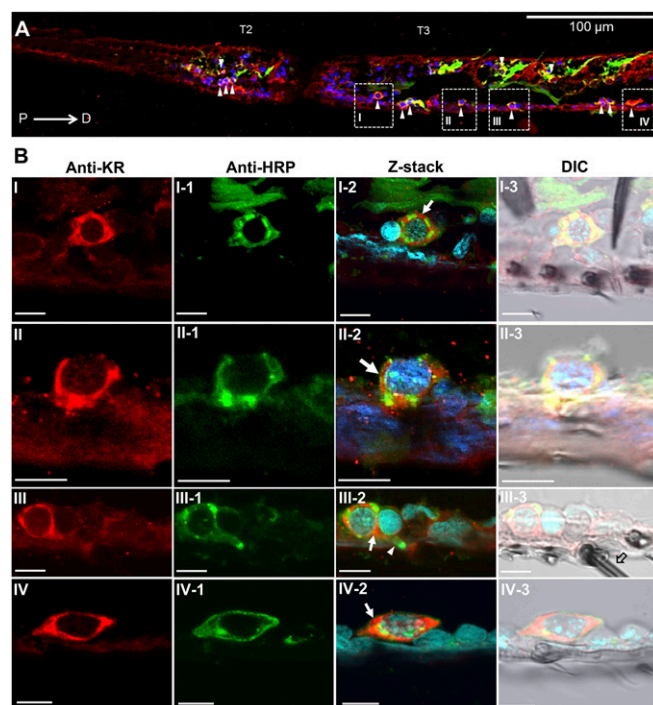


Fig. 5. Confocal analyses of the *Aedae-KR* immunolocalization in prothoracic tarsi. (A) Images of the kinin receptor signal (red, arrowheads) in sensory neurons (green) of the second (T2; four labeled neurons indicated by arrowheads) and third (T3; 10 labeled neurons indicated by arrowheads) tarsomeres. Tarsomeres are oriented from proximal (P) to distal (D) (left to right). The image is an x,y view of a 12.32- μm Z-stack (eight Z-steps, each 1.54 μm). (B) Images from the areas within the areas denoted by dashed boxes I–IV in A, taken using a 100 \times /1.4 oil immersion objective. The first two columns (B, I–IV and B, I-1–IV-1) show the red kinin receptor signal in the plasma membrane (B, I–IV) and cytoplasm of sensory neurons (green in B, I-1–IV-1). The merged images (Z-stack, B, I-2–IV-2) show receptor signal overlapping in neurons (red over green; arrows); nuclei appear blue. The DIC merged images in B, I-3–IV-3 show tarsal sensilla hairs and sockets very close to the receptor-labeled neurons. A receptor-expressing sensory neuron extends its dendrite (green; arrowhead in B, III-2) into a sensillum trichodea (open arrow in B, III-3). The first two columns (Anti-KR, Anti-HRP) show single sections (x,y); the last two columns show respective Z-stacks, as follows: B, I-2, 24 sections, Z-step: 0.25 μm ; B, II-2, 12 sections, Z-step: 0.41 μm ; B, III-2, 14 sections, Z-step: 0.41 μm ; and B, IV-2, 31 sections, Z-step: 0.25 μm . (Scale bars, 5 μm .)

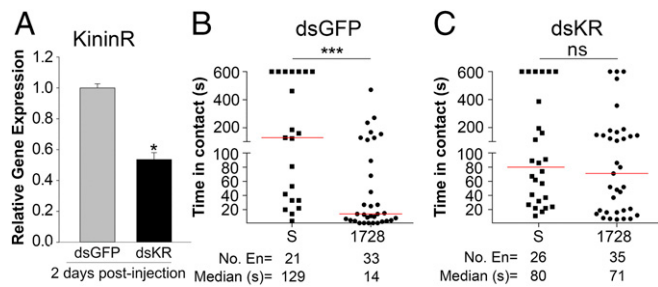


Fig. 6. Silencing of the *Aedae*-KR eliminates the aversive behavior elicited by contact with the 1728 kinin analog. (A) Relative gene expression was measured by quantitative RT-PCR (RT-qPCR) in control (dsGFP) and KR-silenced mosquitoes at 2 d postinjection. *Aedae*-KR expression was reduced significantly (~52%). Data are shown as the mean of three independent experiments \pm SEM. (B) Median time control dsGFP-injected mosquitoes spent in contact with sucrose solution or the kinin analog 1728 at a 1-mM concentration. (C) Median time dsKR-injected mosquitoes spent in contact with sucrose or kinin analog 1728 at a 1-mM concentration. Asterisks denote significant differences ($*P < 0.05$, $***P < 0.001$); ns, not significant. Statistical analyses were performed using GraphPad Prism with an unpaired *t* test to assess the efficiency of knockdown and a Mann-Whitney *u* test to evaluate the time in contact assays (B and C). Three independent RNAi experiments were performed.

most individuals accept a sucrose-only diet, we used a sucrose solution as a driver for providing the kinin analogs (27).

We discovered a previously unidentified function of the kinin-signaling system in mosquitoes in the rapid aversive response to the tasting/feeding of sucrose containing a kinin agonist, 1728, in 300 mM sucrose. The kinin analog 1728 contains pentapeptide kinin core residues (Phe-X¹-X²-Trp-Gly-NH₂) identical to those of drosokinin (22), further supporting the role of insect kinins in chemosensory responses for taste perception of sugars, similar to that reported for trehalose in adult *Drosophila* (24). Before feeding, females normally touched diets with their labellum and tarsi but quickly avoided the agonist 1728 at 1 mM by flying, jumping, or walking away (Fig. 1 and Movie S1). Results from CAFE assays corroborated the observations that females significantly rejected this diet and also ate less diet with analog 1728 at 600 μ M (Fig. 2). This aversion was also reflected in plate assays showing that at these concentrations females retained less Evans blue (Fig. S3 A and B) and deposited fewer urine drops (Fig. S4). Similar results were obtained for analog 1729 at 1 mM in CAFE assays, but in plate assays the differences among analog concentrations in remaining Evans blue (Fig. S3 A and Inset II) and in the number of urine drops deposited (Fig. S4 B and Inset II) were not detected after 5 h. We then investigated if the aversive response to 1728 was correlated to the expression of the *Aedae*-KR in chemosensory appendages. The *Aedae*-KR immunostaining observed in the distal labellum (Fig. 4 and Figs. S8 and S9) coincides with dendrites of sensory neurons in the long labellar sensilla. These *A. aegypti* labellar hairs, from which recordings were also obtained (Fig. 3A and Figs. S5A, S8A, and S9A), are identical to the long labellar sensilla described in *A. aegypti* (28, 29) and are similar to the long labellar sensilla (trichoid) of *Anopheles gambiae* (30) and *Culiseta inornata* (31, 32). Accessory cells surrounding sensory neurons immunostained for the *Aedae*-KR could be the trichogen or tormogen (30, 33), because these cells are believed to secrete the dendrite bathing fluid (34, 35). The *Aedae*-KR staining at the sensilla base is reminiscent of accessory cells associated with sensory neurons in the proboscis of *Anopheles stephensi*, a mosquito vector of malaria (36).

We show that the *Aedae*-KR is expressed in sensory neurons associated with sensilla (Fig. 3 B, III-3) in the tarsi, where it was associated with tarsal sensilla trichodea (Fig. 3 B, III-2 and III-3). However, we cannot exclude the possibility that the receptor is present in neurons housed by other types of sensilla (37, 38). The *Aedae*-KR transcript expression and receptor localization in tarsi and in the distal segment of the labellum strongly linked the observation of the shorter time females spent in contact with

diet containing analog 1728 in feeding assays to the specific action of the kinin analog on the *Aedae*-KR. We performed single-sensilla recordings of long labellar sensilla in which the receptor was immunolocalized (Fig. 3A). Because *A. aegypti* prefer sucrose solutions of 100 mM or higher, a 300-mM sucrose solution was chosen for assays (27). Electrophysiological recordings on the long labellar sensillum of female *A. gambiae* showed that responses of the sucrose receptor cell reached a plateau at sucrose concentrations of 25–292 mM (10% sucrose) (30). We found that kinin analogs of diverse chemical structure inhibited the firing of neurons in response to sucrose in the female labellum (Fig. 3). The number of spikes per second decreased significantly in response to all three analogs at 1-mM concentrations, although 1728 appears to be the most potent (Fig. 3B). These results are consistent with the observation of reduced feeding of sucrose plus analog 1728 (Fig. 2). Moreover, using RNAi-mediated gene knockdown, we verified the fundamental role of *Aedae*-KR in mediating the aversive behavior resulting from contact with the 1728 analog. Females with silenced *Aedae*-KR no longer display aversive behavior in response to the 1728 analog at 1 mM (Fig. 6). This result strongly supports our conclusion that *Aedae*-KR expressed in sensory peripheral organs plays an important role in the control of feeding behavior. The kinin analogs tested have been characterized extensively in vitro and in vivo for their activity on insect kinin receptors. Two types of kinin analog structures were tested, the first containing α -amino isobutyric acid (Aib) (analog 1728 and 1729) and the second containing β^3 Pro (analog 1460) (Fig. S1). All activate recombinant mosquito and tick kinin receptors in CHO-K1 cells, with 1728 being the most potent on the *Aedae*-KR (EC₅₀, 76 nM), followed by 1460 (EC₅₀, 367 nM) and 1729 (EC₅₀, 625 nM) (12, 26). Analog 1728 and 1460 (12, 26) also have potent diuretic activity in the Malpighian tubules of *A. aegypti* in vitro (8). Although both the Aib-containing analogs 1728 and 1729 elicit hindgut contractions in *Rhodnius*; 1728 is more potent (39). Structural modifications of insect kinins, such as the incorporation of Aib and β -amino acids with an additional methylene group (-CH₂-), render these peptides biostable, because they are protease resistant (8, 10–12, 26). Our hypothesis is that the potent analog 1728 enters the labellar and tarsal sensilla (30) and diffuses through the aqueous sensillum lymph to activate the *Aedae*-KR expressed in sucrose taste neurons, thereby decreasing sucrose taste perception. We are not certain why analog 1728 elicits fast walk-, fly-, or jump-away behaviors (Movie S1) that are not observed for either analog 1729 or 1460, which also inhibit the sucrose response, although with less potency (Fig. 3C). It is possible that analog 1728 may stabilize the *Aedae*-KR in a specific conformation by homologous functional selectivity (biased agonism) (8). It is known that aedeskinins hyperpolarize the basolateral membrane voltage by increasing the chloride conductance of Malpighian tubule cells in *A. aegypti* (8, 40). A simple speculative explanation is that, acting via the *Aedae*-KR, analog 1728 changes the chloride concentration in the sensillum lymph, thus affecting the chloride channels involved in the repolarization of gustatory neurons and making the sucrose receptor cells insensitive (41). There is a lack of knowledge regarding chemosensory reception in mosquito leg sensilla. Our finding of the *Aedae*-KR in sensilla trichodea is supported by earlier studies showing that tarsal sensilla trichodea are involved in mosquito gustatory behaviors associated with sugar (42). In *Drosophila* the mapping of taste sensilla in tarsomeres revealed specific sensilla in tarsomeres 5–2 that detect sugars (43). Such a detailed map does not exist for any mosquito species. Our work contributes to the understanding of taste in mosquitoes by providing a receptor marker for a subpopulation of sensory cells in tarsi that can be pharmacologically manipulated and now can be explored further.

Our results pertaining to *Aedae*-KR peripheral function may extend the current knowledge about the modulation of ORNs by GPCRs in olfactory systems (discussed above) to those of chemosensory neural networks in taste organs such as labellum and legs, for which less information is available. Our pharmacological manipulation of the *Aedae*-KR by an externally applied

synthetic molecule provides proof of principle for the search of environmental deterrents (or repellents) modulating GPCRs in peripheral sensory neurons. Further, it demonstrates that these analogs are valuable tools for investigating how peripheral chemosensory systems define insect behavior. In mosquitoes the leucokinin system in peripheral organs appears to be involved in a behavioral avoidance mechanism in the context of sucrose feeding, in addition to its known role in the hormonal control of water and ion homeostasis. This avoidance mechanism could be present in other pests and might be exploited for their control.

Materials and Methods

Mosquito rearing was as described in ref. 5. Details of mosquito rearing, kinin analogs, no-choice feeding assays with kinin analogs, frozen-section immunohistochemistry of *Aedae-KR* in labellum and prothoracic tarsi, electrophysiological recordings on labellar long hair sensilla, the effects of gene

silencing on feeding behavior, dsRNA synthesis and the efficiency of knock-down, and statistical analyses are provided in *SI Materials and Methods*. The structures and synthesis of analogs are shown in Fig. S1. Primers are listed in Table S1.

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