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Role of inward rectifier potassium channels in salivary gland function and sugar feeding of the fruit fly, *Drosophila melanogaster*

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ABSTRACT

The arthropod salivary gland is of critical importance for horizontal transmission of pathogens, yet a detailed understanding of the ion conductance pathways responsible for saliva production and excretion is lacking. A superfamily of potassium ion channels, known as inward rectifying potassium (Kir) channels, is overexpressed in the *Drosophila* salivary gland by 32-fold when compared to the whole body mRNA transcripts. Therefore, we aimed to test the hypothesis that pharmacological and genetic depletion of salivary gland specific Kir channels alters the efficiency of the gland and reduced feeding capabilities using the fruit fly *Drosophila melanogaster* as a model organism that could predict similar effects in arthropod disease vectors. Exposure to VU041, a selective Kir channel blocker, reduced the volume of sucrose consumption by up to 3.2-fold and was found to be concentration-dependent with an EC_{50} of 68 μM. Importantly, the inactive analog, VU937, was shown to not influence feeding, suggesting the reduction in feeding observed with VU041 is due to Kir channel inhibition. Next, we performed a salivary gland specific knockdown of Kir1 to assess the role of these channels specifically in the salivary gland. The genetically depleted fruit flies had a reduction in total volume ingested and an increase in the time spent feeding, both suggestive of a reduction in salivary gland function. Furthermore, a compensatory mechanism appears to be present at day 1 of RNAi-treated fruit flies, and is likely to be the Na^+-K^+-$2Cl^-$ cotransporter and/or Na^+-K^+-ATPase pumps that serve to supplement the inward flow of K^+ ions, which highlights the functional redundancy in control of ion flux in the salivary glands. These findings suggest that Kir channels likely provide, at least in part, a principal potassium conductance pathway in the *Drosophila* salivary gland that is required for sucrose feeding.

1. Introduction

The molecular architecture of arthropod salivary glands have been analyzed in detail for a variety of model organisms, like *Drosophila melanogaster* [1,2], and hematophagous arthropods, including mosquitoes [3–5], ticks [6–8], fleas [9], and blackflies [10]. In the case of hematophagous arthropods, this work yielded a general understanding of saliva constituents that contribute to blood feeding through regulation of blood haemostasis by vasodilation, inhibitors of blood clotting, anesthetics, and anti-immune factors [11,12]. Yet, an understanding of the molecular machinery and physiological systems within arthropod insect salivary glands to enable salivation is limited.

Currently, tick salivary glands are the most commonly studied of all arthropods. Park and colleagues [13,14] have reported the presence of two dopamine receptors in the salivary glands of the blacklegged tick (*Ixodes scapularis*) that are expressed at two distinct locations of the gland and separately control the inward fluid transport and release of fluid to coordinate salivary secretion in ticks. Although considerable pharmacological evidence suggests the dopaminergic system is a major physiological pathway required for arthropod salivation, additional physiological pathways are present that likely contribute to salivary gland function as well. For example, mammalian salivary glands have been shown to rely on chloride (Cl^-)- and potassium (K^+)- ion gradients for proper salivary gland function [15], yet little to no literature exists on the K^+ or Cl^- ion channels responsible for maintaining these gradients.

K^+ ion transport within the mammalian salivary glands is critical for generating saliva and inwardly rectifying potassium (Kir) channels have been shown to be essential to mammalian salivary gland function [16–19]. These channels function as biological diodes due to the unique biophysical property that facilitates the flow of potassium ions in the inward direction more easily than the outward direction [20]. All Kir channels share a similar molecular structure and are tetramers assembled around an aqueous membrane-spanning pore that are gated by

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polyvalent cations that occlude the pore at cell potentials more positive than the K^+ equilibrium potential (E_k) [21,22]. The numbers of genes encoding Kir channel constructs vary depending on species with humans possessing 16 Kir channel encoding genes [20], *Aedes aegypti* mosquito possessing 5 [23], and *D. melanogaster* with 3 [24]. The *Drosophila* Kir genes are termed *Ir*, *Irk*2, and *Irk*3 and encode Kir1, Kir2, and Kir3, respectively [24]. Tissue expression patterns of *Drosophila* Kir channels are highly variable and are described in Zhuo and Hong-Sheng [24] with the expression patterns in excretory systems summarized in Table 1.

Several recent lines of genetic and pharmacological evidence suggest Kir channels play important physiological roles in exocrine systems of dipteran insects as well. In *D. melanogaster*, embryonic depletion of Kir1 and Kir2 mRNA in Malpighian tubules significantly reduces transepithelial secretion of fluid and K^+ transport [25]. In *Aedes aegypti* and *Anopheles gambiae* mosquitoes, researchers have shown that pharmacological inhibition of *Ae*Kir1 with structurally distinct small-molecules (i.e. VU573, VU041) disrupts the secretion of fluid and K^+ in isolated Malpighian tubules, as well diuretic capacity, and K^+ homeostasis in adult females [26–29]. Furthermore, the *Ir* gene encoding *Drosophila* Kir1 is enriched in exocrine tissues and gene expression is increased by 37-fold in the salivary glands of larval and adult life stages (Table 1) [24,30]. The *Drosophila* salivary gland mainly consists of secretory cells that synthesize and secrete proteins required for feeding [31], and the high expression of Ir in the salivary gland may suggest a role in promoting secretion of salivary constituents.

Considering 1) the overexpression of *Ir* in *Drosophila* salivary, 2) the critical role Kir channels serve in Malpighian tubules and 3) that salivary gland cells are also an exocrine tissue that rely on ionic gradients and water transport to generate saliva, we hypothesized that Kir channels are essential to proper salivary gland function and are critical in the highly intricate physiological processes of arthropod feeding. Therefore, the goals of the present study were to use pharmacological inhibition and salivary gland-specific genetic depletion of Kir channels in the model organism *D. melanogaster* to determine the physiological importance of Kir channels in fly salivary gland function as measured through sucrose feeding efficiency.

2. Methods

2.1. Drosophila stocks and rearing conditions

Four strains of *D. melanogaster* were used in this study. The wildtype Oregon-R (OR) strain was provided by Dr. Jeffrey Bloomquist at the University of Florida and was originally donated by Doug Knipple, Cornell University, Ithaca NY, USA. All GAL4-UAS fly strains were purchased from Bloomington Drosophila Stock Center (Bloomington, IN, USA). The GAL4-UAS strain 6870 expresses the promoter in the larval and adult salivary glands, the strain 42644 expresses dsRNA for RNAi of Kir1 (*Ir*) under UAS control, and the strain 41554 expresses hairpin RNA (hpRNA) under the control of UAS for RNAi of GFP and was used as a negative knockdown control. The genotypes of each strain are as follows: 6870, w[1118]; P{w[+mC] = Sgs3-GAL4.PD}TP1; 42644, y [1] sc[*] v [1]; P{y[+t7.7] v[+t1.8] = TRiP·HMS02480}attP2; 41554, y [1] sc[*] v [1]; P{y[+t7.7] v[+t1.8] = VALIUM20-EGFP.shRNA.2}attP2.

All fly strains have been maintained in culture at the Louisiana State University since April 2015. All fly strains were reared on standard medium in *Drosophila* tubes at 25 °C, 12·hour-12·hour photoperiod and 55% relative humidity. For dissection, flies were anaesthetized by chilling on ice and decapitated before dissecting out salivary glands in Schneider's medium (Invitrogen, Paisley, Scotland, UK).

2.1.1. Chemicals

The Kir channel inhibitor VU041 and the inactive analog VU937 were originally discovered in a high-throughput screen against the *Anopheles gambiae* Kir1 channel [29]. Both compounds were synthesized by Dr. Corey Hopkins at the Vanderbilt Center for Neuroscience Drug Discovery using methods described in Swale et al. [29]. Dopamine and the D_1/D_2 antagonist fluphenazine dihydrochloride were purchased from Sigma-Aldrich. Structures of VU041 and VU937 are shown in Fig. 1.

2.2. Feeding assay

The capillary feeding assay (CAFE) was used to quantify the volume of sucrose solution consumed over a period of time and was performed essentially as described in Ja and colleagues [32]. A schematic representation and micrograph image of the assay design is shown in Fig. 2A and 2B, respectively. Briefly, both sexes were used in this assay due to the absence of any literature suggesting differential expression between the genders. One adult fly was placed into a 2 mL glass vial with a screw lid that was pierced with a glass microcapillary tube via a truncated 200-μL pipette tip. The microcapillary tubes contained 5% (wt/vol) sucrose solution that had a 5 μL mineral oil overlay to minimize evaporation during the time course of the experiment. Each experiment included an identical CAFE chamber without flies to determine evaporative losses (typically 5–10% of ingested volumes), which were subtracted from experimental readings. A concentration of 100 μM dopamine and 100 μM fluphenazine were used in Fig. 3A and 200 μM VU041 and 700 μM VU937 (solubility limits) was used in Fig. 3B to determine the influence pharmacological agents have on fly feeding. Exposure to VU041 yielded approximately 30% mortality whereas <10% mortality was observed in control and VU937 treated animals. All dead flies were excluded from all time points of the study regardless of the time point of death. Total consumption volume was calculated by measuring the change in meniscus changes from time zero with 1 cm = 1 μL. Mean ($n > 25$) values are shown as points for all figure panels that measure total consumption.

To determine the effect of increased potassium ions on the VU041-mediated reduction in sugar consumption, 500 μM potassium chloride was added to the 5% sucrose solution and ingested volume was

Table 1
Anatomical expression of Kir channels expressed in excretory systems of *Drosophila* [24].

mRNA Signals							
Localizations	Ir			Irk2		Irk3	
	Larval	Adult	Enrichment	Larval	Adult	Larval	Adult
Salivary gland	725	7480	37.2	67	235	3	8
Crop	–	2871	1.4	N/A	1722	–	6
Midgut	782	506	2.5	36	117	7	4
Hindgut	302	83	0.4	3856	4564	4	48
M. tubules	844	1099	5.5	N/A	805	2898	4932

Note: Data was originally obtained from http://flyatlas.org and values shown were extracted from Luan and Li [24]. Values indicate intensities of RNA signal. N/A: no informative data. Enrichment is defined as the mRNA expression of the tissue/whole body mRNA expression.

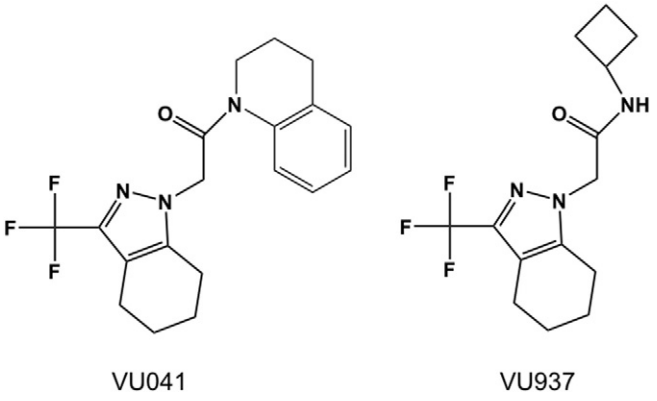

Fig. 1. Chemical structures of Kir channel inhibitors used in this study.