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Dynamic expression of genes encoding subunits of inward rectifier potassium (Kir) channels in the yellow fever mosquito *Aedes aegypti*

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ABSTRACT

Inward rectifier potassium (Kir) channels play fundamental roles in neuromuscular, epithelial, and endocrine function in mammals. Recent research in insects suggests that Kir channels play critical roles in the development, immune function, and excretory physiology of fruit flies and/or mosquitoes. Moreover, our group has demonstrated that mosquito Kir channels may serve as valuable targets for the development of novel insecticides. Here we characterize the molecular expression of 5 mRNAs encoding Kir channel subunits in the yellow fever mosquito, *Aedes aegypti*: *Kir1*, *Kir2A-c*, *Kir2B*, *Kir2B'*, and *Kir3*. We demonstrate that 1) Kir mRNA expression is dynamic in whole mosquitoes, Malpighian tubules, and the midgut during development from 4th instar larvae to adult females, 2) *Kir2B* and *Kir3* mRNA levels are reduced in 4th instar larvae when reared in water containing an elevated concentration (50 mM) of KCl, but not NaCl, and 3) Kir mRNAs are differentially expressed in the Malpighian tubules, midgut, and ovaries within 24 h after blood feeding. Furthermore, we provide the first characterization of Kir mRNA expression in the anal papillae of 4th instar larval mosquitoes, which indicates that *Kir2A-c* is the most abundant. Altogether, the data provide the first comprehensive characterization of Kir mRNA expression in *Ae. aegypti* and offer insights into the putative physiological roles of Kir subunits in this important disease vector.

1. Introduction

Inward rectifier K^+ (Kir) channels belong to a large 'superfamily' that includes the voltage-gated, two-pore, calcium-gated, and ATP sensitive K^+ channels (Miller, 2000; Prole and Marrion, 2012). The evolutionary history of Kir channels is ancient; homologs are present in prokaryotes, protozoans, and nearly all metazoan phyla (Cheng et al., 2009; Prole and Marrion, 2012; Tompkins-Macdonald et al., 2009). On the molecular level, Kir channels are tetrameric proteins composed of either identical subunits (homomeric) or different subunits (heteromeric). Insects possess 2–6 genes encoding Kir channel subunits, which fall into 3 general clades: *Kir1*, *Kir2*, and *Kir3* (Doring et al., 2002; Mamidala et al., 2013; Piermarini et al., 2013; Raphemot et al., 2014a). In mosquitoes, the *Kir2* clade has expanded into two sub-clades: *Kir2A* and *Kir2B* (Piermarini et al., 2013; Raphemot et al., 2014a); *Ae. aegypti* possesses a duplicate copy of the *Kir2B* gene (i.e., *Kir2B'*) (Piermarini et al., 2013), whereas *Anopheles gambiae* possesses a duplicate copy of the *Kir2A* gene (i.e., *Kir2A'*) (Raphemot et al., 2014a). The *Kir1* and *Kir2* clades of insects appear to have a common ancestry with vertebrate Kir subunits, whereas the *Kir3* clade appears to have evolved independently in insects. Consistent with this apparent ancestry, heterologous expression of representative Kir subunits from *Drosophila melanogaster*, *Aedes aegypti*, and *Anopheles gambiae* in *Xenopus* oocytes, mammalian cell lines, or insect cell lines suggest that members of the *Kir1* and *Kir2* clades encode bonafide barium-sensitive K^+ channels that mediate inward-rectifying currents, whereas members of the *Kir3* clade do not encode functional K^+ channels (Doring et al., 2002; Piermarini et al., 2013; Raphemot et al., 2013, 2014a, 2014b; Rouhier et al., 2014b).

The functional roles of Kir channels are best understood in mammals where they perform key physiological functions in nerve, muscle, endocrine, and epithelial tissues (Hibino et al., 2010). Insect Kir channels have not been as extensively studied, but recent studies in dipteran insects suggest putative roles of Kir channels in fundamental physiological processes. In *Drosophila melanogaster*, the systemic

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depletion of *Kir1 (ir)*, *Kir2 (irk2)*, or *Kir3* (*irk3*) mRNAs leads to embryonic mortality or severe developmental defects to the wings (Dahal et al., 2012). Moreover, in *D. melanogaster*, knocking down *ir* and *irk2* mRNAs inhibits immune responses against cardiotropic viruses (Eleftherianos et al., 2011) and K^+ secretion in isolated Malpighian tubules (Wu et al., 2015). Furthermore, molecular expression and pharmacological studies in *D. melanogaster* implicate Kir channels as important contributors to Malpighian tubule function (Doring et al., 2002; Evans et al., 2005; Wang et al., 2004).

Our group has sought to elucidate the molecular physiology of Kir channels in mosquitoes. In adult female *Ae. aegypti* and/or *An. gambiae*, we have shown that Kir mRNAs are widely expressed throughout various body compartments, including epithelial tissues (e.g., gut and Malpighian tubules) and the ovaries (Piermarini et al., 2013; Raphemot et al., 2014a; Rouhier and Piermarini, 2014). In *Ae aegypti*, *Kir2A* mRNAs are expressed as splice variants (i.e., *Kir2A-a*, *-b*, and *-c*), of which *Kir2A-c* is the most abundant (Rouhier and Piermarini, 2014). In addition, we have demonstrated that at least *Kir1* in *An. gambiae* and *Kir2A* in *Ae. aegypti* exhibit differential mRNA expression during development from larvae to adults (Raphemot et al., 2014a; Rouhier and Piermarini, 2014). Moreover, in adult female *An. gambiae*, silencing *Kir1* mRNA expression via RNA interference does not affect the survival of mosquitoes, but causes a decrease in fecundity, suggesting a role of *Kir1* in reproduction (Raphemot et al., 2014a).

In adult female *Ae. aegypti*, we have demonstrated that Kir1 and Kir2B channels play especially important roles in the physiology of Malpighian tubules. Immunoreactivities for Kir1 and Kir2B subunits localize to the basolateral membranes of stellate and principal cells, respectively (Piermarini et al., 2015). In isolated Malpighian tubules, inhibiting Kir1 and Kir2B with small molecule VU625 reduces 1) the secretion of fluid and K^+ and 2) the electrical conductance of principal cells, indicating that Kir channels are major entry points for K^+ into the epithelium from the hemolymph (Piermarini et al., 2015). Consistent with an important role of Kir channels in renal function, injecting mosquitoes with small molecule inhibitors of Kir1 impairs their capacity for 1) -mediating a diuresis and 2) surviving a load of K^+ to their hemolymph (Piermarini et al., 2013; Raphemot et al., 2013, 2014b; Rouhier et al., 2014a, 2014b). Thus, Kir channels appear to play central roles in the regulation of extracellular fluid volume and K^+ homeostasis, offering potentially valuable molecular targets for disrupting the mosquito life cycle (Beyenbach et al., 2015).

Despite the apparent physiological importance of Kir channels in adult female mosquitoes, it is not known how the mRNA expression of each Kir gene changes during development (e.g., from larvae to adults). Moreover, Kir mRNA expression in tissues of larval or pupal mosquitoes has not previously been characterized. The goal of the present study was to use quantitative real-time PCR (qPCR) to compare the molecular expression of Kir mRNAs in *Ae. aegypti* during development, with a focus on two major tissues of the alimentary canal—the midgut and Malpighian tubules. In addition, since Kir channels have been implicated in mosquito osmoregulation, excretion, and reproduction, we sought to characterize the expression of Kir channel mRNAs 1) in larval mosquitoes reared in environments with elevated concentrations of K^+ or Na^+, 2) in the anal papillae of larval mosquitoes, and 3) after a blood meal in adult female mosquitoes. The data provide the first comprehensive characterization of Kir mRNA expression in *Ae. aegypti* mosquitoes and offer valuable insights into the putative functional roles of Kir subunits in mosquitoes.

2. Methods and materials

2.1. Mosquitoes and tissue isolation

All mosquitoes (*Ae. aegypti*) used in this study were raised in an environmental chamber set at 28 °C and 80% relative humidity with a 12 h:12 h light:dark cycle, as described previously (Piermarini et al., 2011). For whole mosquito samples, 10 intact larvae (4th instar), pupae, or adult females (3–7 days old) were immobilized on ice, submerged in TRIzol reagent (Invitrogen, Carlsbad, CA), and stored at −80 °C until RNA isolation. For isolating tissues, 30–50 mosquitoes were immobilized on ice and decapitated with fine forceps (Dumont #5; Fine Science Tools, Inc., Foster City, CA). The remaining carcass (thorax and abdomen) was submerged in a mosquito Ringer solution and the alimentary canal was isolated by pulling on the last abdominal segment with fine forceps. From the alimentary canal, the midgut and Malpighian tubules were carefully separated with fine forceps and transferred to separate 1.5 ml microcentrifuge tubes (USA Scientific, Ocala, FL) containing Ringer solution. For larvae, the anal papillae were also removed from the carcass with fine forceps. Once the desired amounts of tissues were pooled, they were transferred out of the Ringer solution to a 1.5 ml tube containing TRIzol reagent and then frozen at −80 °C. The mosquito Ringer solution contained the following in mM: 150 NaCl, 3.4 KCl, 1.7 $CaCl_2$, 1.8 $NaHCO_3$, 1.0 $MgCl_2$, 5 glucose and 25 HEPES (pH 7.1).

2.2. Rearing of larvae in elevated K^+ or Na^+

Approximately 100–200 eggs were hatched in distilled water under vacuum for 2 h and then ~30 first instar larvae each were transferred to small dishes containing ~200 ml of 50 mM KCl, 50 mM NaCl, or distilled water (control). Larvae were fed daily with finely ground TetraMin flakes (Melle, Germany) for 7 days, and then 10 whole larvae (4th instar) were transferred to TRIzol reagent and frozen at −80 °C, as described above.

In a separate set of experiments, 30–50 4th instar larvae each (6 days post hatching, reared in dH_2O) were transferred to one of three containers with ~200 ml of 50 mM KCl, 50 mM NaCl, or dH_2O (control) without food. Twenty-four hours later, the larvae were transferred directly to TRIzol reagent and frozen at −80 °C, as described above. In some experiments, the anal papillae were isolated from 30 larvae, transferred to TRIzol reagent, and frozen at −80, as described above.

2.3. Blood feeding

A total of 120–160 adult female mosquitoes (4–10 days old) were placed into two separate cages (~60–80 per cage) without access to 10% sucrose. Twenty-four hours later, one cage of mosquitoes was offered heparinized rabbit blood (HemoStat Laboratories, Dixon, CA) containing 0.01 g/ml of ATP (adenosine 5′-triphosphoric acid disodium salt) using a membrane feeder (Hemotek, Blackburn, UK). The other cage was offered a cotton wick soaked with 10% sucrose as a non-blood fed control. Mosquitoes were allowed to feed for 30–60 min before the blood or sucrose was removed from the cage. At 3 h or 24 h after removing the feeders, the Malpighian tubules, midgut, and ovaries were isolated in Ringer solution from 30 blood fed (confirmed by visual inspection) and 30 non-blood fed control mosquitoes, transferred to TRIzol reagent, and stored at −80 °C. An additional 3 intact female mosquitoes from each group were immersed in TRIzol reagent and stored at −80 °C for each time point.

2.4. RNA isolation, cDNA preparation and quantitative real-time PCR

Total RNA was isolated from whole animals or isolated tissues using TRIzol reagent, as described in previous studies (Calkins et al., 2015; Piermarini et al., 2013; Rouhier and Piermarini, 2014). First-strand cDNA was synthesized from total RNA using the GoScript Reverse Transcription System (Promega) according to manufacturer instructions. For a specific sample, qPCR was performed in three replicates of 10 μl reactions, each consisting of 5 μl of GoTaq® qPCR Master Mix (Promega), 40 ng cDNA, 400 nM forward and reverse primers, and nuclease free H_2O. The reactions took place in 96-well unskirted, low profile plates (Bio-Rad Laboratories, Hercules, CA) sealed with

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