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Molecular and functional characterization of *Anopheles gambiae* inward rectifier potassium (Kir1) channels: A novel role in egg production

Rene Raphemot [a, b], Tania Y. Estévez-Lao [c], Matthew F. Rouhier [d], Peter M. Piermarini [d], Jerod S. Denton [a, b, e, f, *], Julián F. Hillyer [c, f, *]

[a] Department of Anesthesiology, Vanderbilt University, Nashville, TN 37232, USA
[b] Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA
[c] Department of Biological Sciences, Vanderbilt University, Nashville, TN 37232, USA
[d] Department of Entomology, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691, USA
[e] Institute of Chemical Biology, Vanderbilt University, Nashville, TN 37232, USA
[f] Institute for Global Health, Vanderbilt University, Nashville, TN 37232, USA

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ABSTRACT

Inward rectifier potassium (Kir) channels play essential roles in regulating diverse physiological processes. Although Kir channels are encoded in mosquito genomes, their functions remain largely unknown. In this study, we identified the members of the *Anopheles gambiae* Kir gene family and began to investigate their function. Notably, we sequenced the *A. gambiae Kir1* (*AgKir1*) gene and showed that it encodes all the canonical features of a Kir channel: an ion pore that is composed of a pore helix and a selectivity filter, two transmembrane domains that flank the ion pore, and the so-called G-loop. Heterologous expression of *AgKir1* in *Xenopus* oocytes revealed that this gene encodes a functional, barium-sensitive Kir channel. Quantitative RT-PCR experiments then showed that relative *AgKir1* mRNA levels are highest in the pupal stage, and that *AgKir1* mRNA is enriched in the adult ovaries. Gene silencing of *AgKir1* by RNA interference did not affect the survival of female mosquitoes following a blood meal, but decreased their egg output. These data provide evidence for a new role of Kir channels in mosquito fecundity, and further validates them as promising molecular targets for the development of a new class of mosquitocides to be used in vector control.

1. Introduction

Inward rectifier potassium (Kir) channels play fundamental roles in excitable and non-excitable cells in mammals, including the regulation of skeletal and cardiomyocyte excitation-contraction coupling, the transport of potassium (K^+) in renal and intestinal epithelia, and the metabolic coupling of blood glucose to insulin secretion in pancreatic beta-cells (Hibino et al., 2010). In humans, there are sixteen Kir channel-encoding genes, which by amino acid homology have been divided into seven subfamilies (Kir1.x – Kir7.x). With the exception of Kir6.x channels, which form hetero-octomeric complexes with regulatory sulfonylurea receptors (SUR), Kir channels are homo- or heterotetramers of membrane-spanning subunits assembled around a water-filled pore through which K^+ moves down its electrochemical gradient (Hibino et al., 2010). Their importance is underscored by the existence of heritable mutations in Kir channel-encoding genes that underlie the human diseases Andersen-Tawil syndrome, Bartter syndrome, neonatal diabetes mellitus, SeSAME/EAST syndrome, and Snowflake vitreoretinal degeneration (Denton and Jacobson, 2012; Hibino et al., 2010; Pattnaik et al., 2012).

In comparison to mammals, relatively little is known about Kir channels in insects, with most of our understanding coming from the model insect, *Drosophila melanogaster*. The *D. melanogaster* genome encodes three members of the Kir gene family, which are named *Kir1*, *Kir2*, and *Kir3* (Döring et al., 2002). Experiments using heterologous expression systems have demonstrated that *Kir1* and *Kir2* encode functional inward rectifier K^+ channels, whereas *Kir3* does not (Döring et al., 2002). In embryos, *Kir2* and *Kir3* are

* Corresponding authors. Department of Biological Sciences (JFH) and Department of Anesthesiology (JSD), Vanderbilt University, Nashville, TN 37232, USA.
E-mail addresses: jerod.s.denton@vanderbilt.edu (J.S. Denton), julian.hillyer@vanderbilt.edu (J.F. Hillyer).

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expressed in the hindgut and the Malpighian (renal) tubules, respectively (Döring et al., 2002), whereas in adult flies all *Kir* encoding genes are expressed in the Malpighian tubules (Evans et al., 2005). Thus, given their spatial expression it has been hypothesized that Kir channels may play a role in osmoregulatory processes (Döring et al., 2002; Evans et al., 2005). Kir channels also appear to be involved in development; a recent study by Dahal et al. (2012) showed that genetic disruption of *Kir2* expression causes wing-patterning defects as a result of dysregulation of bone morphogenetic protein (BMP) signaling.

The genome of the yellow-fever vector mosquito *Aedes aegypti* encodes five members of the Kir channel family, named Kir1, Kir2A, Kir2B, Kir2B′ and Kir3 (Piermarini et al., 2013). Similar to the *Drosophila* Kir family, *A. aegypti Kir1* and *Kir2B*, but not *Kir3*, encode functional channels when heterologously expressed (Piermarini et al., 2013). Also similar to *D. melanogaster*, the expression of *Kir1*, *Kir2B*, and *Kir3* is enriched in Malpighian tubules, consistent with the hypothesis that these genes play important roles in osmoregulation and urine production. Indeed, we recently reported that pharmacologically inhibiting *A. aegypti* Kir1 channels using a small-molecule antagonist reduces urine output, disrupts K^+ homeostasis, and leads to a flightless or dead phenotype within 24 h of treatment. That study showed that Kir channels are essential for proper renal physiology and suggests that inhibiting Kir channels could be a novel insecticidal mechanism for the control of mosquito disease vectors (Raphemot et al., 2013).

The biology of Kir channels in the African malaria vector *Anopheles gambiae* remains unexplored. Here, we identified the members of the *A. gambiae* Kir gene family and began to explore their expression, function, pharmacology, and integrative physiology. Most notably, we found that the expression of *A. gambiae* Kir1 (*AgKir1*) is enriched in the ovaries and that RNAi-mediated knockdown of the channel decreases the number of eggs laid by female mosquitoes.

2. Materials and methods

2.1. Mosquito rearing

A. gambiae Giles *sensu stricto* (G3 strain; Diptera: Culicidae) were reared and maintained in an environmental chamber set to 27 °C and 75% humidity as previously described (Estevez-Lao and Hillyer, 2014). Briefly, eggs were hatched in distilled water and larvae were fed a mixture of koi food and yeast daily. Upon eclosion, adults were fed a 10% sucrose solution *ad libitum*. All experiments were carried out on adult female mosquitoes.

2.2. Sequencing of A. gambiae Kir1 from Malpighian tubule cDNA

As described in previous studies (Piermarini et al., 2010, 2011, 2013), the GeneRacer Kit (Life Technologies, Carlsbad, CA) was used to generate two independent pools of single-stranded cDNA (designated as 5′-cDNA and 3′-cDNA) from Malpighian tubule total RNA (derived from ~50 females). The 5′-cDNA was used as the template for 5′-rapid amplification of cDNA ends (RACE), whereas the 3′-cDNA was used as the template for 3′-RACE.

The 5′- and 3′-RACE reactions were assembled in volumes of 25 μl as recommended by the manufacturer. Each reaction consisted of (1) a GeneRacer Kit primer (5′-Primer or 3′-Primer), (2) a gene-specific primer (designed using the bioinformatic prediction of *AgKir1*), (3) 5′- or 3′ RACE library cDNA, and (4) Platinum PCR Supermix HF (Life Technologies). A "touchdown" thermocycling protocol was used for all RACE reactions as outlined by the GeneRacer Kit. The amplification products of the RACE reactions were visualized by 1% agarose gel electrophoresis, TA-cloned (Life Technologies), and chemically transformed into *Escherichia coli* (Zymo Research, Irvine, CA), as described previously (Piermarini et al., 2010, 2011, 2013). Plasmid DNA from the resulting *E. coli* colonies was sequenced at the Molecular and Cellular Imaging Center of the Ohio State University Ohio Agricultural Research and Development Center (Wooster, OH). A consensus sequence for *AgKir1* was generated after aligning the DNA sequences of the 5′-RACE, 3′-RACE, and full-length PCR products. After assembly, sequences were graphically visualized using Artemis software (Wellcome Trust Sanger Institute, Cambridge, UK). The primers used to determine the full-length sequence of *AgKir1* are presented in Table S1 in Supplementary file 1 and the positions and lengths of *AgKir1* exons and introns in the AgamP3 assembly of the *A. gambiae* genome are shown in Table S2 in Supplementary file 1.

The predicted AgKir1 protein mass was calculated using the Compute pI/Mw tool in the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/), and a search for a signal peptide was done using the SignalP 4.0 server (Petersen et al., 2011). The membrane-associated domains were predicted using the Eukaryotic Linear Motif server (http://www.elm.eu.org), and ExPASy ProtScale (http://web.expasy.org/protscale/) was used to plot hydrophobicity using the Rao and Argos scale (Mohana Rao and Argos, 1986). Prediction of the selectivity filter was done by searching for the canonical T-X-G-Y(F)-G sequence (Hibino et al., 2010), the pore helix was identified by alignment to AeKir1 (Piermarini et al., 2013), and alignment with human Kir sequences was used to predict the G-loop (Nishida et al., 2007).

2.3. Protein alignment and phylogenetic analysis

Protein sequences of Kir channels from *A. aegypti*, *A. gambiae*, *Culex quinquefasciatus*, *D. melanogaster* and *Microcoleus vaginatus* were retrieved from the National Center for Biotechnology Information protein database website (http://www.ncbi.nlm.nih.gov/pubmed/) or VectorBase (https://www.vectorbase.org/), with accession IDs shown in Table S3 in Supplementary file 1. Protein sequences were aligned with the ClustalW algorithm using Geneious Pro v4.8.5 software (http://www.geneious.com; Biomatters, Auckland, New Zealand), and amino acid similarities were calculated using the Blosum62 score matrix (threshold = 0). The LG + I + G + F model of amino acid substitution according to the Akaike information criterion was then identified with ProtTest 2.4 (Abascal et al., 2005), and used to construct a maximum likelihood tree (1000 replicates) in PhyML 3.0 (Guindon et al., 2010), as implemented in the T-REX web server (Boc et al., 2012). The phylogenetic tree was visualized and edited with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The amino acid sequence of Ion transport 2 domain protein (a putative Kir channel) from the cyanobacterium *M. vaginatus* was used as the outgroup.

2.4. Heterologous expression and electrophysiology in Xenopus oocytes

To express *AgKir1* in *Xenopus* oocytes, the open-reading frame of *AgKir1* was sub-cloned into a pGH19 plasmid and the synthesized complimentary RNA (cRNA) was injected into stage IV–V defolliculated *Xenopus laevis* oocytes as previously described (Piermarini et al., 2013). The oocytes were injected with either 7.5 or 15 ng of cRNA to induce *AgKir1* channel expression, and cultured for 3–7 days in OR3 media as described (Piermarini et al., 2010, 2013, 2009). Oocytes that had been injected with nuclease-free H_2O served as controls.

Whole-cell AgKir1 currents were recorded using the two-electrode voltage clamp technique as previously described (Piermarini et al., 2013). Current and voltage commands were