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

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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 RIP 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 in mosquito fecundity, and further validates them as promising molecular targets for the meal, but decreased their egg output. These data provide evidence for a new role of Kir channels development of a new class of mosquitocides to be used in vector control.

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Keywords

Inward rectifier potassium channel; Kir; *Anopheles gambiae*; mosquito; ovary; fecundity; oviposition

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 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 hours 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.

Materials and Methods

Mosquito rearing

Anopheles 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.

Sequencing of *Anopheles gambiae* Kir1 from Malpighian tubule cDNA

As described in previous studies (Piermarini et al., 2010; Piermarini et al., 2011; Piermarini et al., 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; Piermarini et al., 2011; Piermarini et al., 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 analyzed using 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).

Protein alignment and phylogenetic analysis

Protein sequences of Kir channels from *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Drosophila 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.

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 complementary RNA (cRNA) was injected into stage IV-V defollicular *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; Piermarini et al., 2013; Piermarini et al., 2009). Oocytes that had been injected with nuclease-free H₂O 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 generated with a Digidata 1440A Data Acquisition System (Molecular Devices, Sunnyvale, CA) and the Clampex module of pCLAMP software (version 10, Molecular Devices). For current recordings, an oocyte was transferred to the holding chamber under superfusion with a control solution and was impaled with two conventional-glass microelectrodes backfilled

with 3M KCl (resistances of 0.5-1.5 MW). The control solution (ND96) contained (in mM): 96 NaCl, 0 NMDG-Cl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES, with pH adjusted to 7.5 with NMDG-OH. For some experiments, a low-K⁺ solution was used, which contained (in mM): 0.5 NaCl, 97.5 NMDG-Cl, 0.5 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES. Alternately, a high-K⁺ solution was used, which contained (in mM): 0.5 NaCl, 48 NMDG-Cl, 50 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES. AgKir1 currents were inhibited with Ba²⁺ (1 mM), a non-specific K⁺ channel blocker (Ho et al., 1993; Kubo et al., 1993; Newman, 1993). For current-voltage (I-V) recordings, oocytes were voltage clamped near their spontaneous membrane potential (V_m) and stepped from -140 mV to +40 mV in 20 mV increments for 100 ms each. All recordings were performed at room temperature, and the I-V plots were generated using the Clampfit module of pCLAMP.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from whole adult female mosquitoes as previously described (Hillyer and Estevez-Lao, 2010). Briefly, RNA extracted from 10 to 20 mosquitoes was isolated using TRIzol® Reagent (Life Technologies, Carlsbad, CA) and purified using the RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from poly(A)+RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies) according to manufacturer instructions. Real-time quantitative PCR (qRT-PCR) was then done using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7300 Real-Time PCR System. Relative quantification was carried out using the 2^{-CT} method (Livak and Schmittgen, 2001), and the ribosomal protein S7 (*rps7*) was used as the reference gene (Coggins et al., 2012; Estevez-Lao et al., 2013).

AgKir1 mRNA levels, and the mRNA levels of other *A. gambiae* Kir genes, were measured by real-time quantitative PCR (qRT-PCR) essentially as described (Estevez-Lao and Hillyer, 2014). To quantify *AgKir1* mRNA levels in different developmental stages, cDNA was synthesized from RNA purified from ~200 eggs, 50 second instar larvae, 40 third instar larvae, R30 fourth instar larvae, 20 pupae (callow or black) or 15 adults (24 h, 5 days or 10 days old). Two biological replicates were conducted and each was analyzed in duplicate. The graphed output displays the average fold-change in mRNA levels relative to eggs.

To quantify *A. gambiae* Kir gene expression in different body segments, cDNA was synthesized from RNA purified from 10 whole bodies, 20 heads, 20 thoraces or 20 abdomens from adult mosquitoes at 4 days post-eclosion. To quantify *A. gambiae* Kir gene expression in dissected tissues, cDNA was synthesized from RNA purified from midguts, Malpighian tubules, ovaries and fat bodies. Tissues were collected from 25 mosquitoes as previously described (Estevez-Lao and Hillyer, 2014), except that in this study the Malpighian tubules were separated from the midgut and processed separately. These are standard methods of tissue dissection, although the procedure for fat body collection suffers from the fact that other cell types are collected as well, including the dorsal vessel, other muscle, the ventral nerve cord, pericardial cells, and sessile hemocytes (Andereck et al., 2010; King and Hillyer, 2013). The body segment and tissue experiments were conducted in parallel, with three independent trials being performed and each being analyzed in duplicate. The graphed output of body segment and tissue analyses displays the average

fold-difference in mRNA levels relative to whole bodies. The primers used for qRT-PCR are presented in Table S4 in Supplementary file 1.

RNA interference (RNAi)-based gene silencing

An RNAi-based strategy was used to silence gene expression. A 492 bp double stranded RNA (dsRNA) construct specific to *A. gambiae Kir1* was synthesized using the MEGAscript® kit (Life Technologies, Carlsbad, CA) as described earlier (Estevez-Lao et al., 2013). As a control, a 214 bp dsRNA construct specific to *bla(Ap^R)*, the ampicillin-resistant gene that is encoded in Novagen's pET-46 Ek/LIC vector (EMD Chemicals, Gibbstown, NJ), was synthesized from DNA purified from BL21(DE3) *Escherichia coli* cells containing the pET-46 plasmid.

To knockdown mRNA levels, approximately 500 ng of *AgKir1* dsRNA or *bla(Ap^R)* dsRNA was intrathoracically injected into 3-day-old female mosquitoes. To verify knockdown efficiencies, 10 mosquitoes were collected at days 4, 8, and 11 post-injection, total RNA was purified, cDNA was synthesized, and relative gene expression was quantified by qRT-PCR. For each timepoint, 2-3 biological replicates were conducted, and each was assayed in duplicate. Gene silencing data are presented as mRNA levels relative to the *bla(Ap^R)* dsRNA-injected control groups. The primers used for RNAi and gene expression are presented in Table S4 in Supplementary file 1.

Antibody production

An affinity purified, polyclonal rabbit antibody raised against a synthetic peptide (SRRIRKRVIFKQGDC) corresponding to a putative fragment of the cytosolic NH₂-terminal domain of *AgKir1* (S128-C142) was developed by 21st Century Biochemicals (Marlboro, MA).

Western blotting

To prepare protein lysates, whole mosquitoes were homogenized in a 10% Ringer solution. After determining the protein concentration using the Bradford reagent, the lysates were diluted by addition of one volume of high-urea buffer that was composed of the following: 6 M Urea, 15 mM TrisHCl, 0.3% SDS and 0.25 mM NaCl at pH 7.4 (Piermarini et al., 2010). The high-urea lysates were mixed with an appropriate volume of a 5X Laemmli gel loading buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 0.5% β-mecaptoethanol, 0.1% bromophenol blue) and incubated at 100°C for 5 minutes. Approximately 30 µg of protein was separated by SDS-PAGE on a denaturing 12% polyacrylamide resolving gel (with a 4% stacking gel; ProtoGel®Quick-Cast, National diagnostics, Atlanta, GA) using an XCell SureLock Mini Cell electrophoresis unit (Life Technologies). After electrophoresis, the stacking gel was discarded and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, Rockford, IL) using an XCell II blot module (Life Technologies) according to the manufacturer's protocol.

To detect *AgKir1* immunoreactivity, the PVDF membrane was washed three times with Tris-buffered saline containing 0.01% Tween 20 (TBST; 10 mM TrisHCl, 150 mM NaCl, and 0.01% Tween 20, pH 7.4), blocked for 30 min with 5% nonfat dry milk dissolved in

TBST (blocking buffer), and incubated overnight at 4°C with the anti-AgKir1 antibody (1:1000 dilution) in blocking buffer. The following day, the PVDF membrane was washed three times (5 min each) with TBST, incubated for 1 h with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:20,000 dilution; Pierce Biotechnology, Thermo Scientific, Rockford, IL) in blocking buffer, and washed three times (5 min each) with TBST. To visualize AgKir1 immunoreactivity, a luminescent substrate of HRP (DuraWest Chemiluminescent, Pierce Biotechnology) was applied to the PVDF membrane, and the signal was detected with ChemiDocVersatile Document Imager (Bio-Rad Laboratories, Hercules, CA).

Survival assays

For survival assays, 3-day-old adult female mosquitoes were intrathoracically injected with *bla*(*Ap^R*) dsRNA or *AgKir1* dsRNA and then returned to the environmental chamber and provided with 10% sucrose. At day 8 following the dsRNA injections, the mosquitoes were allowed to blood-feed on mice anesthetized with a mixture of ketamine and xylazine for approximately 30 min, and subsequently maintained on 10% sucrose. Beginning with the time of blood feeding, mosquito survival was recorded daily for 19 days, and the graphed output represents the mean percent survival from the three group biological replicates (24-27 mosquitoes per in each trial).

Fecundity assays

For the fecundity assays, 3-day-old adult female mosquitoes were intrathoracically injected with *bla*(*Ap^R*) dsRNA or *AgKir1* dsRNA and allowed to blood feed 8 days later. Forty-eight hours after blood-feeding, fully engorged mosquitoes were individually transferred to *Drosophila* vials (Fisher Scientific, Pittsburgh, PA) containing 2-3 ml of distilled water and capped with a cotton ball. Mosquitoes were monitored for the next 3 days, and eggs were counted once oviposition had taken place. A total of 5 biological replicates were performed.

Results

Anopheles gambiae Kir1 gene structure

Reciprocal tblastn searches of the AgamP3 assembly of the *A. gambiae* genomic sequence and the AaegL2 assembly of the *A. aegypti* genomic sequence in www.vectorbase.org revealed that *A. gambiae* AGAP001280 and *A. aegypti* AAEL008932 are 1:1 orthologs (reciprocal E-values = 0.0). Thus, because AAEL008932 is named *AeKir1* (Piermarini et al., 2013), we named AGAP001280 *AgKir1*.

To determine the complete mRNA sequence of *AgKir1*, the entire transcript was sequenced by 5' and 3' RACE. Assembly of the RACE sequences revealed that the *AgKir1* mRNA is 3,452 nucleotides (base pairs; bp) in length, and is composed of a 1,085 bp 5' untranslated region (UTR), a 1,668 bp open reading frame (ORF) and a 669 bp 3' UTR (Fig. 1; GenBank ID: Pending). Alignment of the mRNA sequence and the chromosome 2R genomic sequence revealed that the *AgKir1* gene is composed of 11 exons that span 68,906 bp of genomic sequence. Although initially predicted as AGAP001280, the mRNA sequence is significantly larger than the prediction. Starting at the 5' end, *AgKir1* spans the predictions

of AGAP013159 and AGAP001280. Thus, these two VectorBase gene IDs should be merged into a single entry.

Conceptual translation of the full-length mRNA revealed that *AgKir1* encodes a 555 amino acid (aa) protein with a predicted mass of 62.1 kDa. As expected of an ion channel, the ORF does not encode a signal peptide. The ORF, however, does encode all of the characteristic features of inward rectifier potassium channels (Kirs). Specifically, ELM analysis predicts three membrane-associated domains, located in aa positions 171-193, 213-235, and 248-270. The first and third predictions are the classical transmembrane domains seen in Kir channels (Hibino et al., 2010). The second prediction is the ion pore, which includes the pore helix (aa 219-230; 100% identity with the predicted pore helix in AeKir1; (Piermarini et al., 2013)) and the selectivity filter TIGYG (aa 232-236; (Hibino et al., 2010)). A hydrophobicity plot of the conceptual translation of *AgKir1* supports the prediction of the two transmembrane domains and the ion pore (Fig. S1 in Supplementary File 1). Finally, alignment of *AgKir1* with human Kirs (Nishida et al., 2007) predicts that aa 390-400 form the so-called G-loop.

Phylogenetic analysis of *AgKir* channels

A tblastn search of the AgamP3 assembly of thA. *gambiae* genomic sequence and thCpipJ1 assembly of the *C. quinquefasciatus* genomic sequence in www.vectorbase.org using *AgKir1* as the query sequence, followed by the identification of characteristic features of Kir channels, identified five additional members of the *A. gambiae* Kir gene family (for a total of six Kir genes), and five members of the *C. quinquefasciatus* Kir gene family. Based on sequence alignment and a phylogenetic analysis (see below), the *A. gambiae* genes were named *AgKir2A*, *AgKir2A'*, *AgKir2B*, *AgKir3A* and *AgKir3B*, and the *C. quinquefasciatus* genes were named *CqKir1*, *CqKir2A*, *CqKir2B*, *CqKir3A* and *CqKir3B*.

For the *A. gambiae* Kir genes, alignment of the conceptual translation of *AgKir1* with the putative amino acid sequences of *AgKir2A*, *AgKir2A'*, *AgKir2B*, *AgKir3A* and *AgKir3B* revealed several characteristic features of Kir channels, including a pore-forming domain containing the signature “TIGYG” K⁺ selectivity filter (STGYG in the case of the two *AgKir3* genes), two transmembrane-spanning domains (TM1 and TM2), and the G-loop (Figs. 1-2) (Hibino et al., 2010; Nishida et al., 2007). The core region (between filled circles in Fig. 2; (Döring et al., 2002)) of *AgKir1* shares approximately 60% amino acid sequence identity with *AgKir2* subtypes, but only about 40% identity with *AgKir3* subtypes. Finally, it is interesting that all mosquito (*Anopheles*, *Aedes* and *Culex*) Kir3 genes encode a non-canonical form of the selectivity filter, whereas *DmKir3* contains the canonical form. Given that *AeKir3* and *DmKir3* do not encode a functional channel (Döring et al., 2002; Piermarini et al., 2013), it is possible that the *A. gambiae* and *C. quinquefasciatus* Kir3 genes do not encode functional channels as well (for an alignment of the selectivity filters of dipteran and human Kir channels see Fig. S2 in Supplementary File 1).

A maximum likelihood tree of dipteran Kir channels using Ion transport 2 domain protein (a putative Kir channel) of *M. vaginatus* as the outgroup shows that Kir genes can be divided into two major clades: Kir1/Kir2 and Kir3 (Fig. 3). Consistent with the neighbor-joining analysis of Piermarini et al. (Piermarini et al., 2013), the Kir1/Kir2 clade can be subdivided

into Kir1 and Kir2 groups, and the Kir2 group can be further subdivided into Kir2A and Kir2B subgroups. Interestingly, whereas *D. melanogaster* only encodes three Kir genes (Kir1, Kir2 and Kir3), the Kir gene family has expanded in the mosquito lineage. That is, the genomes of all three mosquito species encode at least two Kir2 genes, and the genomes of *A. gambiae* and *C. quinquefasciatus* encode two Kir3 genes.

Functional characterization of AgKir1 channels in *Xenopus* oocytes

We have shown that a small molecule inhibitor of AeKir1 (VU573) (1) incapacitates adult females of several mosquito species (*A. aegypti*, *A. albopictus*, *A. gambiae* and *C. pipiens*) and (2) disrupts the excretory physiology of at least *A. aegypti* (Raphemot et al., 2013). Thus, because of the important role that AeKir1 plays in renal physiology, our first assessment of Kir gene function in *A. gambiae* focused on *AgKir1*. As shown in Figure 4A, when bathed in a control (ND96) solution, heterologous expression of AgKir1 channels in *Xenopus* oocytes gave rise to functional channels exhibiting spontaneous large inward and small outward currents between -140 mV and -80 mV. Moreover, the spontaneous resting membrane potential of the AgKir1-expressing oocytes (-96.9 ± 1.3 mV) was (1) hyperpolarized compared to the H₂O- injected oocytes (-42.0 ± 2.5 mV), and (2) close to the estimated Nernst potential for K⁺ (-102 mV). As shown in Figure 4B, the inward AgKir1 channel currents increase with increased extracellular K⁺ concentrations and are inhibited by barium. These functional characteristics are canonical of most animal Kir channels, and very similar to those of AeKir1 (Raphemot et al., 2013). Thus, AgKir1 functions as an inward rectifier K⁺ channel.

Developmental and tissue distribution of *Kir1* in *Anopheles gambiae*

To gain insight into the possible in vivo functions of AgKir1, we analyzed the expression patterns of *AgKir1* at different life stages. Quantitative RT-PCR analyses revealed that *AgKir1* is ubiquitously expressed in all mosquito life stages, with transcript levels being highest in the pupal stage and lowest in third instar larval stage (Fig. 5; ANOVA $P = 0.0003$). Specifically, relative to eggs, there is a trend for *AgKir1* mRNA levels to decrease by half during the larval stages and then increase between 2- and 3-fold in the early (callow) and late (black) pupal stage. After eclosion, *AgKir1* mRNA levels return to levels similar to those observed in eggs and remain unchanged through the 10th day following adult emergence (the last timepoint assayed).

To determine the locations of *AgKir1* transcription, we analyzed the expression pattern of *AgKir1* in different body segments and tissues of 4-day-old adult female mosquitoes. Quantitative RT-PCR analyses revealed that *AgKir1* mRNA levels are detectable in all body segments and in all tissues assayed (Fig. 6). Specifically, mRNA levels in the head, thorax, abdomen and Malpighian tubules are comparable to the levels detected in the mosquito whole body. *AgKir1* mRNA levels trended to be lower in the midgut and fat body tissues when compared to the whole body, a finding that is in agreement with the *Kir1* gene expression profile reported in MozAtlas (Baker et al., 2011). However, of all the tissues examined, we observed the significant enrichment of *AgKir1* mRNA in the ovaries: levels were almost 2.5-fold higher than those found in the whole body (ANOVA $P = 0.0008$). Furthermore, when compared to other *Anopheles* Kir genes, enrichment of Kir mRNA in the

ovaries was only observed for *AgKir1* (Fig. S3 in Supplementary File 1). This finding suggested a potentially novel role of Kir channels in mosquito fecundity.

***AgKir1* knockdown does not affect survival**

We have shown that injection of a small-molecule inhibitor of AeKir1 (VU573) into the hemocoel of adult female mosquitoes (*A. aegypti*, *A. albopictus*, *A. gambiae*, *C. pipens*) induces incapacitation and/or death (Raphemot et al., 2013). Moreover, VU573 disrupts renal excretory physiology and hemolymph K^+ homeostasis in at least *A. aegypti* (Raphemot et al., 2013). These findings suggest that Kir channels are essential regulators of physiological events affecting osmoregulation. Thus, we sought to determine whether *AgKir1* is important for mosquito survival following a blood meal, which is a period when mosquitoes face extreme stresses to hemolymph Na^+ , K^+ , Cl^- , and water homeostasis (Beyenbach, 2003; Williams et al., 1983)

Toward this goal, we developed methods for knocking down *AgKir1* mRNA levels by RNA interference. Relative to the *bla(Ap^R)* dsRNA controls, injection of *AgKir1* dsRNA significantly reduced *AgKir1* mRNA levels by 24% ($P = 0.3487$) and 58% ($P = 0.0108$) at 4 and 8 days post-treatment, respectively (Fig. 7A). To determine if *AgKir1* knockdown affects survival following blood feeding, we blood fed mosquitoes 8 days after injecting *AgKir1* dsRNA or *bla(Ap^R)* dsRNA, discarded the mosquitoes that were not fully engorged, and monitored their survival over the next 19 days. Surprisingly, in three independent trials, knockdown of *AgKir1* had no effect on mosquito survival as compared to the control *bla(Ap^R)* group (Logrank P values = 0.6500, 0.8523 and 0.9288; Fig. 8). In fact, the slopes and R-square values of the survival curves of the *AgKir1* dsRNA ($y = -3.43$; $R^2 = 0.985$) and *bla(Ap^R)* dsRNA ($y = -3.17$; $R^2 = 0.941$) groups were nearly identical.

Engorged mosquitoes usually take 2-3 days to digest their meal. To ensure that *AgKir1* remained suppressed at that time, we measured *AgKir1* mRNA and protein levels at 72 hrs post-blood feeding, which also represented 11 days post-injection of dsRNA. As shown in figure 7, *AgKir1* mRNA levels were decreased by >95% ($P = 0.0014$) relative to the *bla(Ap^R)* dsRNA control group, and *AgKir1* protein was reduced considerably, but not completely. These findings suggest that a partial knockdown of *AgKir1* (1) might not be sufficient to affect mosquito survival following a single naïve blood meal, (2) that the function of *AgKir1* may be redundant with other Kir channels, or (3) that the functional role(s) of Kir1 may differ between thanopheline and culicine lineages.

***AgKir1* knockdown decreases fecundity**

Because *AgKir1* is enriched involved in the ovaries (Fig. 6), we hypothesized that it could be involved in fecundity. Therefore, we investigated the effect of *AgKir1* RNAi-based knockdown on egg production using a similar protocol to that used for survival experiments, except that the mosquitoes were placed in an oviposition environment at 48 hrs post-blood feeding, and egg laying was monitored for up to three days thereafter. As shown in Figure 9, mosquitoes that were treated with *AgKir1* dsRNA oviposited an average of 21.41 eggs whereas the mosquitoes that were treated with *bla(Ap^R)* dsRNA oviposited an average of 30.11 eggs ($n = 151$ for both groups; Mann-Whitney $P = 0.0485$). Furthermore, the

percentage of mosquitoes that laid any eggs in the *AgKir1* dsRNA and *bla(Ap^R)* dsRNA was 32% and 42%, respectively. Together, these data indicate that silencing of *AgKir1* significantly impairs a mosquito's ability to produce and oviposit eggs.

Discussion

The large volume of blood that is commonly ingested by mosquitoes represents a major physiological challenge, thereby necessitating an efficient excretory system that rapidly off-loads excessive weight, water, and solutes (Beyenbach, 2003; Williams et al., 1983). In a previous study, we showed that pharmacological inhibition of Kir1 (1) incapacitates adult female mosquitoes of several species (*A. aegypti*, *A. albopictus*, *A. gambiae*, *C. pipiens*) and (2) reduces urine production/excretion and disrupts the maintenance of hemolymph K⁺ homeostasis in at least *A. aegypti* (Raphemot et al., 2013). That study, besides showing that Kir channels are essential for proper renal excretory physiology in mosquitoes, suggested that inhibiting Kir channels could be used to control mosquito populations, and by extension, mosquito-borne diseases. However, the two major mosquito lineages, the Culicinae (e.g., *Aedes* sp., *Culex* sp.) and the Anophelinae (e.g., *Anopheles* sp.), diverged approximately 200 million years ago (Reidenbach et al., 2009), and many physiological differences are known to exist between these two groups (Bartholomay et al., 2010; Beaty and Marquardt, 1996; Becker et al., 2010; Coggins et al., 2012). In the present study, we began the characterization of the Kir gene family in the African malaria mosquito, *Anopheles gambiae*. Among other things, we identify the members of the *A. gambiae* Kir gene family, describe their developmental and tissue expression, and show that silencing *AgKir1* reduces mosquito fecundity.

Whereas the genome of *Drosophila melanogaster* encodes three Kir genes, the genomes of *A. aegypti*, *A. gambiae* and *C. quinquefasciatus* encode between five and six Kir genes. This gene expansion in mosquitoes may reflect an increase in the molecular complexity of homomeric or heteromeric Kir channel complexes that are required to regulate unique physiological processes. Indeed, the importance of functional complexity was demonstrated in *Drosophila*, where disruption of Kir channel heteromerization results in the dysregulation of BMP signaling, which causes major developmental defects (Dahal et al., 2012). Thus, by encoding six Kir genes, *A. gambiae* have potentially increased the diversity of heteromeric and homomeric complexes available to carry out a multitude of diverse physiological processes.

Using heterologous expression in *Xenopus* oocytes, we show that *AgKir1* encodes a canonical inward-rectifying K⁺ channel with similar properties as *AeKir1* (Piermarini et al., 2013). That is, the channel mediates strong inward K⁺ currents that are blocked by barium. Moreover, the electrophysiological properties of *AgKir1*-expressing oocytes in ND96 solution (i.e., resting membrane potentials and I-V relationships) closely resemble those of *AeKir1*-expressing oocytes (Piermarini et al., 2013).

AgKir1 is transcribed in all developmental stages. Peak expression of *AgKir1* occurs in pupae, which suggests a role for *AgKir1* during post-embryonic development, and more specifically, metamorphosis. Interestingly, a reciprocal pattern of expression for at least

Kir2A occurs in the development of *A. aegypti*, where *Kir2A* abundance is elevated in larvae and adults but is decreased in pupae (Rouhier and Piermarini, 2014). The reorganizations associated with metamorphosis are known to result in changes in membrane excitability. As pertains to Kirs, earlier studies have implicated a role for K⁺ channels in regulating membrane potential during post-embryonic development of motor neurons in the fruit fly, *Drosophila melanogaster* (Duch et al., 2008), and the tobacco hornworm, *Manduca sexta* (Duch and Levine, 2000; Hayashi and Levine, 1992). Further supporting a role for Kirs in development, a recent study showed that *D. melanogaster* DmKir2 is required for normal wing development, and that Kir channel involvement occurs through the regulation of BMP signaling (Dahal et al., 2012).

AgKir1 is expressed in all adult tissues and body segments. Interestingly, *AgKir1* expression was enriched in the ovaries. This enrichment suggested that Kir channels could be involved in fecundity. Previous studies have demonstrated a role for ionic currents in the maturation of oocytes. For example, ionic currents such as inward rectifying K⁺ currents undergo dynamic modulation (i.e., K⁺ current amplitude decreases with oocyte maturation) upon in vitro hormone-stimulation in maturing starfish oocytes (Moody and Lansman, 1983). Moreover, ion channel currents (e.g. calcium currents) are involved in oocyte maturation and fertilization in vertebrate and invertebrate animals (Tosti, 2006; Tosti and Boni, 2004). Therefore, given that *AgKir1* expression is enriched in the ovaries and that *AgKir1* gene silencing reduces fecundity, it is possible that *AgKir1* may participate in the regulation of mosquito oocyte maturation by modulating membrane excitability in these tissues. In insects, oocyte maturation is generally arrested during meiotic cell division and resumes in a fertilization independent manner that requires a mechanical stimulus, such as oviduct passage or rehydration (Von Stetina and Orr-Weaver, 2011; Yamamoto et al., 2013). Thus, future studies should investigate whether *AgKir1* plays a role in oogenesis.

Although we detected a role for *AgKir1* in fecundity, we originally anticipated that, based on our previous findings in *A. aegypti* and *A. gambiae* (Raphemot et al., 2013), gene silencing of *AgKir1* would result in a dead or flightless phenotype following blood feeding. However, no significant increase in post-blood feeding mortality was observed between *AgKir1* dsRNA and *bla(Ap^R)* dsRNA-treated groups, and the flightless phenotype was not observed either. The fact that we did not observe an obvious phenotype following gene silencing may reflect the difference in efficiency between pharmacologic and reverse-genetic approaches. For example, acute pharmacological inhibition of protein function is expected to yield an extreme phenotype (e.g., death or bloating), whereas partial gene silencing by RNAi may not sufficiently reduce protein levels to produce an obvious effect. Alternatively, it is possible that (1) other Kir channels expressed in the Malpighian tubules of *A. gambiae* can compensate for the reduced levels of *AgKir1*, (2) Kir1 performs different functions in the Malpighian tubules of *A. gambiae* and *A. aegypti*, or (3) RNAi efficiency is markedly different between tissues (Boisson et al., 2006). Nevertheless, our survival experiment is consistent with studies in *D. melanogaster*, where there is no apparent lethal effect following Kir1 knockdown, but rather a sub-lethal effect on immune function (Eleftherianos et al., 2011).

In conclusion, here we present the first evidence that Kir channels play a role in mosquito fecundity, which together with their important roles in mosquito renal physiology (Raphemot et al., 2013), further validates them as promising molecular targets for the development of a new class of mosquitocides to be used in vector control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Anopheles gambiae* encodes six putative Kir channels, named AgKir1, AgKir2A, AgKir2A', AgKir2B, AgKir3A and AgKir3B.
- *AgKir1* encodes a functional, barium-sensitive Kir channel.
- *AgKir1* expression peaks during the pupal stage and is enriched in adult ovaries.
- RNAi-mediated gene silencing of *AgKir1* does not impact mosquito survival after a blood meal, but decreases mosquito fecundity.

Figure 1. Gene structure of *Anopheles gambiae* AgKir1
The open reading frame (ORF) is the gray boxes, the untranslated regions (UTRs) are the white boxes, and the introns are the horizontal lines. The locations that encode the transmembrane domains (TM1 and TM2), pore helix, selectivity filter and G-loop are marked, and the sizes (in bp) of the UTRs, ORF, exons and introns are provided.

Figure 2. Amino acid sequence alignment of *Anopheles gambiae* Kir channels

ClustalW alignment of the conceptual translations of the RACE sequenced *AgKir1* and the other predicted members of the *A. gambiae* Kir gene family. The transmembrane domains (TM1 and TM2), the pore-helix, the selectivity filter (SF) and the G-Loop are indicated. The core region is flanked by filled circles. Shadings denote the amino acid similarity at a given position, with black highlight, dark grey highlight, light grey highlight, and no highlight indicating 100%, 80-99%, 60-79% and <60% similarity, respectively.

Figure 3. Phylogenetic analysis of the *Anopheles gambiae*, *Aedes aegypti*, *Culex quinquefasciatus*, and *Drosophila melanogaster* Kir channel families

Protein sequences were aligned by ClustalW and the maximum likelihood tree computed with PhyML 3.0. A putative Kir channel from the cyanobacterium *M. vaginatus* was used as the outgroup, and bootstrap support for the clades are shown for each node. The proportion of amino acid changes among the proteins is indicated by the branch length. The gene IDs are presented in Table S3 in supplementary file 1.

Figure 4. Current-voltage (I-V) relationships of AgKir1 channels in *Xenopus* oocytes
 (A) The I-V relationship of AgKir1 channel-expressing oocytes (filled circles; N = 6) reveals robust inward rectifying currents when bathed in the control (ND96) solution, whereas those of H₂O-injected oocytes (open circles; N = 8) exhibit nominal currents. Data are mean $\pm$ SEM. (B) Barium (1 mM) blocks inward-rectifying K⁺ currents in AgKir1 channel-expressing oocytes. Oocytes were bathed consecutively in the following solutions: (1) low-K⁺ solution (open squares), (2) high-K⁺ solution (filled circles), (3) high-K⁺

solution with Ba^{2+} (open circles). Shown is a representative I-V plot from an oocyte. V_m , membrane voltage; I_m , membrane current.

Figure 5. Quantitative RT-PCR analysis of the expression of *A. gambiae* AgKir1 in different developmental stages

Transcript levels were measured in eggs, developing first instar larvae, second through fourth instar larvae, callow (early) and black (late) pupae, and adults at 24,h, 5 days and 10,days after eclosion. Data are mean $\pm$ SEM fold-difference (two biological replicates) in mRNA levels relative to eggs, using *RPS7* as the reference gene. ANOVA $P = 0.0003$, and the asterisk indicates $P < 0.05$ when compared to eggs (Tukey's test).

Figure 6. Quantitative RT-PCR analysis of the expression of *A. gambiae* *AgKir1* in different body segments and tissues

Transcript levels were measured in the whole body, head, thorax, abdomen, Malpighian tubules, midgut, fat body and ovaries. Data are mean $\pm$ SEM fold-difference (three biological replicates) in mRNA levels relative to the whole body, using *RPS7* as the reference gene. ANOVA $P = 0.0008$, and the asterisk indicates $P < 0.05$ when compared to whole body (Tukey's test).

Figure 7. Quantitative RT-PCR and western blot analyses showing *AgKir1* RNAi-based knockdown efficiencies

(A) Quantitative RT-PCR analysis demonstrating the efficiency of *AgKir1* RNAi-based knockdown at 4, 8 and 11 days post-dsRNA injection. Data are mean $\pm$ SEM fold-difference (two or three biological replicates) in mRNA levels relative to the *bla(Ap^R)* dsRNA control group. ANOVA $P = 0.0016$, and asterisks (*, **) indicate <0.05 or $P < 0.01$ respectively when compared to the *bla(Ap^R)* dsRNA group (Tukey's test). (B) Western blot analysis demonstrating the partial knockdown of AgKir1 protein levels in *A. gambiae* mosquitoes at 11 days post *bla(Ap^R)* dsRNA or *AgKir1* dsRNA injection.

Figure 8. *AgKir1* knockdown does not affect mosquito survival after a blood meal

Paired cohorts composed of 24-27 *A. gambiae* each were injected with *bla(Ap^R)* dsRNA or *AgKir1* dsRNA, provided a blood meal 8 days later, and the survival was tracked for the next 19 days. Data are mean $\pm$ SEM of 3 biological replicates.

Figure 9. *AgKir1* knockdown decreases mosquito fecundity

Paired cohorts of *A. gambiae* were injected with *bla(Ap^R)* dsRNA or *AgKir1* dsRNA, provided a blood meal 8 days later, and allowed to oviposit. The circles represent the number of eggs laid by each female ($n = 151/\text{group}$), and the horizontal lines mark the means. The two groups were statistically different (*, Mann–Whitney $P = 0.0485$).