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Cloning and functional characterization of inward-rectifying potassium (Kir) channels from Malpighian tubules of the mosquito *Aedes aegypti*

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Abstract

Inward-rectifying K⁺ (Kir) channels play critical physiological roles in a variety of vertebrate cells/tissues, including the regulation of membrane potential in nerve and muscle, and the transepithelial transport of ions in osmoregulatory epithelia, such as kidneys and gills. It remains to be determined whether Kir channels play similar physiological roles in insects. In the present study, we sought to 1) clone the cDNAs of Kir channel subunits expressed in the renal (Malpighian) tubules of the mosquito *Aedes aegypti*, and 2) characterize the electrophysiological properties of the cloned Kir subunits when expressed heterologously in oocytes of *Xenopus laevis*. Here, we reveal that three Kir subunits are expressed abundantly in *Aedes* Malpighian tubules (AeKir1, AeKir2B, and AeKir3); each of their full-length cDNAs was cloned. Heterologous expression of the AeKir1 or the AeKir2B subunits in *Xenopus* oocytes elicits inward-rectifying K⁺ currents that are blocked by barium. Relative to the AeKir2B-expressing oocytes, the AeKir1-expressing oocytes 1) produce larger macroscopic currents, and 2) exhibit a modulation of their conductive properties by extracellular Na⁺. Attempts to functionally characterize the AeKir3 subunit in *Xenopus* oocytes were unsuccessful. Lastly, we show that in isolated *Aedes* Malpighian tubules, the cation permeability sequence of the basolateral membrane of principal cells (Tl⁺ > K⁺ > Rb⁺ > NH₄⁺) is consistent with the presence of functional Kir channels. We conclude that in *Aedes* Malpighian tubules, Kir channels contribute to the majority of the barium-sensitive transepithelial transport of K⁺.

Keywords

Kir channels; Mosquito; Malpighian tubule; *Xenopus* oocytes; Electrophysiology; Diuresis

1. Introduction

Together with the ileum and rectum of the hindgut, the Malpighian tubules are the ‘kidneys’ of mosquitoes. The distal, blind-ended Malpighian tubules are responsible for generating the urine, which is isosmotic to the hemolymph (extracellular fluid) and consists primarily of KCl and NaCl (Williams and Beyenbach, 1983). Once formed, the urine can be modified by

downstream reabsorptive and/or secretory processes in the proximal Malpighian tubules and hindgut, before its expulsion as a droplet. Although 'kidney' function is critical to all life stages of the mosquito, the adult females are a particularly interesting case study, because they exhibit a remarkable dynamic range of renal physiology, which can be regulated with switch-like speed, to accommodate the dramatic challenges to salt and water balance associated with gorging on the blood of a vertebrate host (Benoit and Denlinger, 2010; Beyenbach, 2003a,b; Beyenbach and Piermarini, 2011; Coast, 2009).

Although much progress has been made in our understanding of the physiological and molecular mechanisms that mediate and regulate the generation of urine by Malpighian tubules in adult female mosquitoes (Beyenbach and Piermarini, 2011; Coast, 2009), the molecular identities of the K^+ channels on the basolateral membrane of the tubule epithelium have not yet been elucidated. We know that in isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti*, the K^+ channels: 1) account for more than 60% of the basolateral membrane conductance of principal cells (Beyenbach and Masia, 2002); 2) are reversibly blocked by peritubular barium (Beyenbach and Masia, 2002; Masia et al., 2000); 3) are regulated by the metabolic state of the tubule and/or intracellular pH (Wu and Beyenbach, 2003); and 4) are one of two major pathways for the uptake of peritubular K^+ into the epithelium (the other being a bumetanide-sensitive Na,K,2Cl cotransporter) (Scott et al., 2004).

Physiological studies on the Malpighian tubules of other insects (*Formica*, *Rhodnius*, *Drosophila*, *Locusta*, *Tenebrio*) have also demonstrated the presence of barium-sensitive K^+ channels in the basolateral membrane of the epithelium (Haley and Donnell, 1997; Hyde et al., 2001; O'Donnell et al., 1996; Weltens et al., 1992; Wiehart et al., 2003b). Moreover, pharmacological and/or gene-expression studies in Malpighian tubules of *Drosophila* and *Tenebrio* have highlighted the inward-rectifying K^+ (Kir) channels as the most likely candidates for mediating the aforementioned barium-sensitive channel activity in the basolateral membrane (Evans et al., 2005; Wang et al., 2004; Wiehart et al., 2003a). In the present study, we explore the hypothesis that Kir channels mediate the barium-sensitive K^+ conductance of principal cells in *Aedes* Malpighian tubules. On the molecular level, Kir channels are formed by the oligomerization of four monomeric subunits, which are each membrane-bound proteins that contain 1) a central transmembrane domain that includes a pore-forming region; and 2) cytosolic N-terminal and C-terminal domains that regulate the gating and functional properties of the channel (Hibino et al., 2010). Functional Kir channels can be either homomeric (a tetramer of 4 identical subunits) or heteromeric (a tetramer of 2 different subunits).

The results of the present study reveal that the *Aedes* genome contains five genes that encode putative subunits of Kir channels: *AeKir1*, *AeKir2A*, *AeKir2B*, *AeKir2B'*, and *AeKir3*. Three of these genes are expressed abundantly in *Aedes* Malpighian tubules (*AeKir1*, *AeKir2B*, and *AeKir3*) as evidenced by qualitative RT-PCR and the cloning of their full-length cDNAs. Heterologous expression of *AeKir1* or *AeKir2B* subunits in *Xenopus* oocytes elicits inward-rectifying K^+ currents that are blocked by barium, whereas heterologous expression of the *AeKir3* subunit does not. Lastly, we show that in isolated *Aedes* Malpighian tubules, the cation permeability sequence of the basolateral membrane of principal cells is consistent with the presence of functional Kir channels. We conclude that Kir channels contribute to the barium-sensitive K^+ conductance of the basolateral membrane of principal cells.

2. Materials and methods

2.1. Mosquito rearing and isolation of Malpighian tubules

Mosquitoes (*A. aegypti*) were raised in the laboratory as described previously (Piermarini et al., 2011). Only Malpighian tubules from adult females (3–7 days old) were used in the present study. In brief, the females were anesthetized 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 five Malpighian tubules were carefully removed with fine forceps and transferred to a 1.5 ml low-adhesion microcentrifuge tube (USA Scientific, Ocala, FL) with a glass Pasteur pipette. Once the desired number of Malpighian tubules was collected, the tube was snap frozen in liquid N₂ and stored at –80 °C. The mosquito Ringer solution contained the following in mM: 150 NaCl, 3.4 KCl, 1.7 CaCl₂, 1.8 NaHCO₃, 1.0 MgSO₄, 5 glucose and 25 HEPES (pH 7.1).

2.2. Qualitative RT-PCR

Qualitative RT-PCR was used to assess the expression levels of the five *Aedes* Kir (*AeKir*) genes (see Table 2) in the Malpighian tubules of adult female mosquitoes. The assays were performed following a method similar to that of Samra et al. (2012). In brief, total RNA was isolated from 125 Malpighian tubules (25 adult female mosquitoes) and 3 whole adult female mosquitoes using Trizol reagent (Invitrogen, Carlsbad, CA) and a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) according to manufacturers' instructions. Next, cDNA was synthesized from 1 mg of total RNA using the GoScript Reverse Transcriptase System (Promega, Madison, WI) with random hexamers.

Primer pairs for qualitative PCR (see 'Qualitative' in Supplemental Table 1) were designed using Integrated DNA Technologies SciTools-Real-Time PCR application (www.idtdna.com/scitools/applications/primerquest/) to simultaneously amplify a 500 bp region for the *AeKir* cDNAs and a 300 bp region of the cDNA encoding ribosomal protein S7 gene (RPS7; VectorBase accession #AAEL009496) in a single PCR. The expression level of RPS7 served as 1) an internal positive control for each PCR, and 2) a loading control to ensure equal sample loading among the lanes of an agarose gel. Each primer combination was optimized for both temperature and Mg²⁺ concentration as described by Marone et al. (2001).

Reactions of 50 µl were assembled for each *AeKir* gene that included the following: 1) 0.5 µl of cDNA, 2) 1 µl of an *AeKir* genespecific primer pair (5 mM each primer), 3) 1 µl of the RPS7 primer pair (5 µM each primer), 4) a final concentration of 5 mM MgCl₂, and 5) 1.25 units of GoTaq DNA Polymerase (Promega). The thermocycler was programmed to pause at 33 and 53 cycles to remove aliquots of each reaction for analysis. Each PCR was performed in triplicate, and the products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide staining.

2.3. Cloning of *AeKir1*, *AeKir2B*, and *AeKir3* cDNAs from *Aedes* Malpighian tubules

As described in previous studies (Piermarini et al., 2010, 2011), the GeneRacer Kit (Invitrogen, Carlsbad, CA) was used to generate two independent pools of single-stranded cDNA (designated as 5'-cDNA and 3'-cDNA, respectively) from total Malpighian tubule RNA (derived from ~150 Malpighian tubules of ~30 females). The 5'-cDNA was used as a template for the 5'-rapid amplification of cDNA ends (RACE), whereas the 3'-cDNA was used as a template for the 3'-RACE. Gene-specific primers were designed to regions of

predicted exons for the *AeKir1*, *AeKir2B*, and *AeKir3* genes (www.vectorbase.org) and used in the RACE experiments (Supplemental Table 1).

The 5′- and 3′-RACE reactions were assembled in volumes of 25 µl as recommended by the GeneRacer Kit (Invitrogen). Each reaction consisted of 1) a generic GeneRacer primer (GeneRacer 5′-Primer or GeneRacer 3′-Primer, Invitrogen), 2) a gene-specific primer (see ‘5′-RACE’ and ‘3′-RACE’ in Supplemental Table 1), 3) 5′- or 3′-cDNA, and 4) Platinum PCR Supermix HF (Invitrogen). A ‘touchdown’ thermocycling protocol was used for all RACE reactions as outlined by the GeneRacer Kit (Invitrogen). The amplification products of the RACE reactions were visualized by 1% agarose gel electrophoresis, TA-cloned (Invitrogen), and chemically transformed into *Escherichia coli* (Invitrogen) as described previously (Piermarini et al., 2010, 2011). Plasmid DNA from the resulting *E. coli* colonies was sequenced at the DNA Sequencing Center of Cornell University (Ithaca, NY) or the Molecular and Cellular Imaging Center of the Ohio State University Ohio Agricultural Research and Development Center (Wooster, OH).

DNA sequencing of the cloned RACE products allowed for the elucidation of the 5′- and 3′-untranslated regions (UTRs) that flank the predicted open-reading frame of each gene. Thus, for each *AeKir* subunit, gene-specific primer pairs were designed to the flanking UTRs to allow for the amplification of the ‘full-length’ open-reading frame in a single PCR (see ‘Full-length’ in Supplemental Table 1). The one exception was the *AeKir3* gene for which only one genespecific primer was used (primer #21 in Supplemental Table 1). This primer bound to the 5′-UTR of *AeKir3* and was used with the GeneRacer 3′-Primer (Invitrogen) to amplify the full-length open-reading frame. The ‘full-length’ PCR products were TA-cloned and sequenced as described above.

A consensus sequence for each *AeKir* cDNA was generated based on the DNA sequences of the 5′-RACE, 3′-RACE, and full-length PCR products. The consensus sequences were submitted to GenBank and assigned the following accession numbers: *AeKir1*, JQ753065; *AeKir2B*, JQ753067; and *AeKir3*, JQ753066.

2.4. Heterologous expression of *AeKir1*, *AeKir2B*, and *AeKir3* subunits in *Xenopus oocytes*

The open-reading frames of the *AeKir1*, *AeKir2B*, and *AeKir3* cDNAs were each sub-cloned into a pGH19 plasmid (Trudeau et al., 1995) and used as a template to synthesize capped RNA (cRNA; Ambion T7 mMessage mMachine kit, Austin, TX). An RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) was used to purify the generated cRNA, which was then stored in nuclease-free H₂O at –80 °C.

Defolliculated oocytes of *Xenopus laevis* were purchased from a commercial supplier (Ecocyte Bioscience, Austin, TX). To express a particular *AeKir* subunit, oocytes were injected with either 7.5 or 15 ng (in 14 or 28 nl of nuclease-free H₂O, respectively) of the according cRNA and cultured for 3–7 days in OR3 media as described previously (Piermarini et al., 2010, 2009). Control oocytes were injected with 28 nl of nuclease-free H₂O.

2.5. Electrophysiology of *Xenopus oocytes*

All electrophysiological experiments were performed at room temperature. Table 1 describes the compositions of the solutions used in these experiments. When required, barium chloride was dissolved in solution *III* (Table 1) to a final concentration of 1 mM. For the experiments of Fig. 8, 5 or 10 mM of NMDG-Cl in solution *IX* (Table 1) was replaced with NaCl, KCl, TlCl, CsCl, RbCl, or LiCl. All solutions were held in 250 ml glass Erlenmeyer flasks and delivered by gravity to an RC-3Z oocyte chamber (Warner Instruments, Hamden, CT) via polyethylene tubing connected to a teflon 8-way Rotary valve

(Model 5012), Rheodyne, Rohnert Park, CA). The flow rate to the chamber was $\sim 4 \text{ ml min}^{-1}$.

For each experiment, an oocyte was transferred to the chamber under superfusion with solution *I*. The oocyte was then impaled with two conventional glass microelectrodes that were backfilled with 3 M KCl (resistances of 0.5–1.5 M Ω) to measure membrane potential (V_m) and whole-cell membrane current (I_m), respectively. The microelectrodes were bridged to an OC-725 oocyte clamp (Warner Instruments) that was controlled by the Clampex module of the pCLAMP software package (version 10, Molecular Devices, Sunnyvale, CA).

To acquire current–voltage (I – V) relationships, an oocyte was clamped near its spontaneous V_m and a voltage-stepping protocol was initiated by the Clampex module of pCLAMP. The protocol consisted of stepping the membrane potential from -140 mV to $+40 \text{ mV}$ in 20 mV increments for 100 ms each. The resulting V_m and I_m values were recorded digitally by a Digidata 1440A Data Acquisition System (Molecular Devices) and Clampex. The I – V plot was generated using the Clampfit module of the pCLAMP software package (Molecular Devices).

In some oocytes (e.g., Fig. 8), the V_m was clamped continuously at -92 mV or -93 mV using the analog controls on the OC-725 oocyte clamp (Warner Instruments). The resulting I_m values were recorded digitally by the Digidata 1440A Acquisition System (Molecular Devices) and Clampex. All I_m recordings were analyzed using the Clampfit module of the pCLAMP software package (Molecular Devices).

2.6. Diffusion potentials of the principal cell basolateral membrane

Principal cells of isolated Malpighian tubules were impaled with a conventional glass microelectrode to measure the basolateral membrane potential (V_b), as described previously (Piermarini et al., 2011). In brief, the Malpighian tubules were attached to a piece of Parafilm™ (American National Can, Menasha, WI) that was stretched over the bottom of a perfusion bath ($500 \mu\text{l}$ capacity) containing mosquito Ringer solution. A motorized micromanipulator was then used to impale a single principal cell near the distal, blind end of the tubule with a microelectrode (backfilled with 3 M KCl; resistance of 10 – $40 \text{ M}\Omega$). The microelectrode was bridged to a Geneclamp 500 voltage amplifier (Molecular Devices, Sunnyvale, CA). The measurements of V_b were recorded digitally by a Digidata 1440A (Molecular Devices) and the Axoscope module of the pCLAMP software package (Molecular Devices). All V_b recordings were analyzed using the Clampfit module of the pCLAMP software package (Molecular Devices).

In a typical experiment, after recording a stable V_b in the mosquito Ringer solution, the solution through the bath was switched to one in which all of the Cl^- was replaced by gluconate. This replacement was necessary to prevent the precipitation of Ti^+ as its chloride salt. After recording a stable V_b in the gluconate Ringer, the solution through the bath was switched to one in which $30 \text{ mM Na-gluconate}$ was replaced with 30 mM of NH_4Cl , RbCl , KCl , or TiNO_3 .

2.7. Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) or Microsoft Excel. Comparisons between 2 groups were evaluated with an unpaired t -test. To compare means among more than 2 groups, a one-way analysis of variance (ANOVA) was used. Multiple comparisons were performed with a Newman–Keuls posttest or unpaired t tests.

3. Results

3.1. The *Aedes* Kir channel family

Basic local alignment search tool (BLAST) inquiries with the amino-acid sequences of *Drosophila* inward-rectifying potassium (Kir) channel subunits reveal the presence of five genes that encode putative Kir channel subunits in the genome of *A. aegypti* (Table 2). Fig. 1 illustrates the phylogenetic relationships of the *Aedes* Kir channel subunits to those of *Drosophila* and of select human Kir channels. In general, the Kir1 and Kir2 genes of *Aedes* and *Drosophila* appear to 1) have a common ancestry with the Kir channels of humans, and 2) be related closest to the branch of human Kir channels that includes the classical (Kir2.x), ATP-sensitive (Kir6.x), and G protein-gated (Kir3.x) Kir channels vs. the branch that includes the Kir1.1 (ROMK) channel (Fig. 1). On the other hand, the *Aedes* and *Drosophila* Kir3 genes appear to have evolved independently in insects and are related furthest from the human Kir channels (Fig.1).

Within the insect Kir channels, the *AeKir2A* gene is the closest ortholog of *DrKir2*, whereas the *AeKir2B* gene is unique to *Aedes*, which indicates that it may have evolved after the last common ancestor of fruit flies and mosquitoes. Consistent with this notion, the genomes of other mosquitoes (*Anopheles gambiae* and *Culex quinquefasciatus*) each possess at least one ortholog of the *AeKir2A* and *AeKir2B* genes, whereas the available genomes of fruit flies (*Drosophila* sp.) only possess an ortholog of the *AeKir2A* gene (Piermarini, personal observations). Furthermore, the *AeKir2B* gene appears to have duplicated recently within *Aedes*, resulting in the very similar *AeKir2B'*.

3.2. Expression of Kir channel cDNAs in Malpighian tubules vs. the whole mosquito

We next characterized the expression of the five *Aedes* Kir channel genes in isolated Malpighian tubules and adult female mosquitoes using qualitative RT-PCR. As shown in Fig. 2A, Malpighian tubules express detectable levels of all five genes, but the expression levels of *AeKir1*, *AeKir2B*, and *AeKir3* are the most abundant, as indicated by the greater intensity of their bands. Of these three genes, the expression levels of *AeKir2B* and *AeKir3* are stronger than that of *AeKir1*. The comparable intensity of the RPS7 bands among the lanes indicates that the differences in *AeKir* gene expression are not due to unequal sample loading.

Fig. 2B shows that the expression of all five *AeKir* genes is detectable in the whole adult female mosquito, but the expression levels of *AeKir1*, *AeKir2B'*, and *AeKir3* appear to be more robust than those of *AeKir2A* and *AeKir2B*. Again, the comparable intensity of the RPS7 bands among the lanes indicates that these differences in gene expression are not due to unequal sample loading.

3.3. Cloning of *Aedes* Kir channel cDNAs from Malpighian tubules

Given that the *AeKir1*, *AeKir2B*, and *AeKir3* cDNAs are expressed abundantly in Malpighian tubules, we cloned each of their full-length cDNAs using RT-PCR. The lengths of the cloned *AeKir1*, *AeKir2B*, and *AeKir3* cDNAs are 2877 bp, 1731 bp, and 1502 bp, respectively. No evidence of alternative splicing was observed in the open-reading frames of any of the cloned transcripts. Fig. 3 shows an alignment of the amino-acid sequences deduced from the open-reading frames of each cloned cDNA. To our knowledge, these are the first cDNAs encoding putative Kir channel subunits to be cloned from any mosquito and are the first from any insect since those of *Drosophila melanogaster* (Doring et al., 2002).

As expected for any Kir channel subunit, the cloned *Aedes* Kir channel subunits each consist of a transmembrane and cytosolic domain. The transmembrane domain consists of two

predicted transmembrane (TM) segments (TM1 and TM2 in Fig. 3), a pore-forming region ('Pore Helix' in Fig. 3), and the ion selectivity filter ('Selectivity Filter' in Fig. 3) – the latter houses the signature 'GYG' motif of K⁺ channels. The TM2 segment forms the 'inner helix' gate of the channel, and therefore is an important determinant to the activity of the ion permeation pathway (Whorton and MacKinnon, 2011).

The cytosolic domain consists of the N-terminal and C-terminal regions that flank the transmembrane domain. The cytosolic domain primarily provides sites for regulation, ligand binding (e.g., G proteins), and intersubunit interactions (Hibino et al., 2010). However, the cytosolic domain also contributes to the ion permeation pathway of Kir channels by forming a critical cytosolic (G-Loop) gate near the membrane interface (Hibino et al., 2010; Whorton and MacKinnon, 2011).

Overall, the cloned *AeKir* subunits share the highest degree of similarity within the transmembrane domain (Fig. 3), where the *AeKir1* subunit shares 62% and 49% amino-acid identity with that of the *AeKir2B* and *AeKir3* subunits, respectively. The transmembrane domains of the *AeKir2B* and *AeKir3* subunits share 41% amino-acid identity with each other. The bulk of the differences among the cloned *AeKir* subunits occur in the cytosolic domain (Fig. 3), where the *AeKir1* subunit only shares 33% and 29% amino-acid identity with that of the *AeKir2B* and *AeKir3* subunits, respectively. The cytosolic domains of the *AeKir2B* and *AeKir3* subunits share 26% identity with each other.

3.4. Functional characterizations of the homomeric *AeKir* channels in *Xenopus* oocytes

We next sought to characterize the functional properties of the *Aedes* Kir channel subunits that we cloned from Malpighian tubules (*AeKir1*, *AeKir2B*, *AeKir3*) by expressing them heterologously in *Xenopus* oocytes and measuring the corresponding electrophysiological properties of the oocytes. Note that we only investigated the functional properties of the homomeric Kir channels and not the potential heteromeric Kir channels.

3.4.1. Spontaneous membrane potential and current–voltage relationships in standard Ringer solution—When bathed in a standard oocyte Ringer solution (solution I, Table 1), which contains 96 mM Na⁺ and 2 mM K⁺, the mean spontaneous membrane potential (V_m) of the oocytes injected with the *AeKir1* cRNA (*AeKir1* oocytes) or the *AeKir2B* cRNA (*AeKir2B* oocytes) is significantly hyperpolarized compared to that of control (H₂O-injected) oocytes (Table 3). In contrast, the corresponding mean spontaneous V_m of the oocytes injected with the *AeKir3* cRNA (*AeKir3* oocytes) is not significantly different than that of H₂O injected oocytes (Table 3). Given that the expected Nernst potential for K⁺ (E_K) in these oocytes is −102.21 mV, assuming an intracellular [K⁺] of 110 mM; (Ali et al., 2011), the *AeKir1* and *AeKir2B* oocytes appear to have an enhanced membrane permeability (or leak) for K⁺, which is consistent with the presence of constitutively active K⁺ channels. The channels in the *AeKir1* oocyte must be especially active given that the mean spontaneous V_m of these oocytes nearly matches the expected E_K .

Next, we examined the current–voltage ($I-V$) relationships of the *AeKir1*, *AeKir2B*, and *AeKir3* oocytes bathed in solution I. As shown in Fig. 4A, an *AeKir1* oocyte (filled circles, solid line) is characterized by an $I-V$ plot that differs dramatically with that of a H₂O-injected oocyte (open circles, dotted line). In particular, from the V_m values of −140 mV to −80 mV, the inward (negative) and outward (positive) currents of the *AeKir1* oocyte are greater in magnitude than those of the H₂O-injected oocyte and exhibit a steep voltage dependence (Fig. 4A). The $I-V$ relationship then makes a sharp 'break' at −80 mV where the *AeKir1* oocyte exhibits outward currents that are greater in magnitude than those of a H₂O-injected oocyte, but show nominal voltage dependence, from −80 mV to +40 mV (Fig.

4A). The above I - V relationship evinces the presence of inward-rectifying K^+ channels in the *AeKir1* oocyte – i.e., the oocyte favors inward movements of current at hyperpolarizing V_m values vs. outward movements of current at depolarizing V_m values.

As shown in Fig. 4B, an *AeKir2B* oocyte (filled circles, solid line) is characterized by an I - V plot that resembles one of a H_2O -injected oocyte (open circles, dotted line). In contrast to the *AeKir1* oocyte (Fig. 4A), the inward and outward currents elicited by the *AeKir2B* oocyte are only marginally greater in magnitude and voltage dependence than those of the H_2O -injected oocyte (see inset of Fig. 4B). Furthermore, the I - V plot of the *AeKir2B* oocyte does not exhibit any obvious signs of inward rectification (see inset of Fig. 4B). Thus, at least when bathed in solution *I*, which only contains 2 mM K^+ , the *AeKir2B* oocyte appears to harbor K^+ channels that are substantially less active than those in the *AeKir1* oocytes, which is consistent with their respective mean spontaneous V_m values (Table 3).

Fig. 4C shows the I - V relationship of an *AeKir3* oocyte (filled circles, solid line) bathed in solution *I*. The I - V plot of this oocyte is nearly identical to that of a H_2O -injected oocyte (open circles, dotted line) (also see inset of Fig. 4C). Thus, the *AeKir3* oocytes do not appear to possess functional Kir channels, which is consistent with their mean spontaneous V_m values (Table 3).

Fig. 4D–F summarizes the conductive properties of the *AeKir1*, *AeKir2B*, and *AeKir3* oocytes at representative hyperpolarizing and depolarizing V_m values. In particular, we measured the ‘chord conductance’ (g_i) of the oocytes, which according to Helman and Thompson (1982) is defined as the slope of a straight line that connects the I_m value at a given V_m (referred to as the ‘holding voltage’ by Helman and Thompson) to the I_m at the reversal potential, E_{rev} (i.e., zero). Note that in Fig. 4 – and in all of the following figures that report g_i values – the corresponding mean g_i values of H_2O -injected oocytes have been subtracted.

As shown in Fig. 4D, the mean g_i of the *AeKir1* oocytes at a V_m of -120 mV (95.4 ± 9.0 μS) is significantly greater than the mean g_i at a V_m of 0 mV (3.8 ± 1.8 μS). In the *AeKir2B* oocytes (Fig. 4E), the mean g_i at a V_m of -120 mV (0.92 ± 0.15 μS) is also significantly greater than the mean g_i at a V_m of 0 mV (0.41 ± 0.09 μS). However, in the *AeKir3* oocytes (Fig. 4F), the mean g_i at a V_m of -20 mV (0.155 ± 0.03 μS) is not significantly different than the mean g_i at a V_m of 0 mV (0.16 ± 0.03 μS).

The above data indicate that the *AeKir1* and *AeKir2B* oocytes are dramatically and slightly more conductive, respectively, at a hyperpolarizing V_m value that elicits inward currents relative to a depolarizing V_m value that elicits outward currents, which is consistent with the presence of active Kir channels. By extension, there is negligible Kir channel activity in the *AeKir3* oocytes. The remarkably greater magnitudes of the conductances in the *AeKir1* oocytes compared to those in the *AeKir2B* oocytes indicates that the channels expressed in the *AeKir1* oocytes are much more active than those expressed in the *AeKir2B* oocytes.

3.4.2. K^+ vs. Na^+ —We next examined the I - V relationships of the *AeKir1*, *AeKir2B*, and *AeKir3* oocytes in response to increasing extracellular concentrations of K^+ vs. Na^+ . As shown in Fig. 5A, when bathed in a ‘control’ solution containing 0.5 M a^+ and 0.5 mM K^+ (solution *II*, Table 1) the *AeKir1* oocyte exhibits small currents with nominal voltage dependence (open boxes); these currents are similar in magnitude and voltage dependence to those of a H_2O -injected oocyte in the same solution (Fig. 5D). Increasing extracellular K^+ from 0.5 mM to 50 mM (Solution *III*, Table 1) dramatically changes the I - V relationship of the *AeKir1* oocyte and reveals the robust activity of strong inward-rectifying channels (Fig. 5A, filled circles). In contrast, increasing extracellular Na^+ from 0.5 mM to 50 mM

(Solution IV, Table 1) does not noticeably affect the I - V relationship (Fig. 5A, gray circles), relative to that in the control solution. Thus, the currents mediated by the *AeKir1* oocyte are carried by K^+ and not Na^+ .

Fig. 5B shows the same experimental maneuvers performed on an *AeKir2B* oocyte. Similar to the *AeKir1* oocyte, the *AeKir2B* oocyte exhibits small currents with nominal voltage dependence when bathed in the control solution (Fig. 5B, open boxes). Also similar to the *AeKir1* oocyte, elevating extracellular K^+ from 0.5 mM to 50 mM dramatically changes the I - V relationship of the *AeKir2B* oocyte (Fig. 5B, filled circles) and reveals the modest activity (relative to that of the *AeKir1* oocyte) of inward-rectifying channels. Lastly, similar to the *AeKir1* oocyte, increasing extracellular Na^+ from 0.5 mM to 50 mM does not noticeably affect the I - V relationship of the *AeKir2B* oocyte (Fig. 5B, gray circles), which indicates that the currents mediated by the *AeKir2B* oocyte are carried by K^+ and not Na^+ .

Fig. 5C and D shows the same experimental maneuvers performed on an *AeKir3* and H_2O -injected oocyte. In brief, the I - V relationship of the *AeKir3* oocyte (Fig. 5C) exhibits no major differences with that of a H_2O -injected oocyte (Fig. 5D), even in the presence of 50 mM extracellular K^+ , which further indicates that there is no detectable Kir channel activity in *AeKir3* oocytes. As such, we focus on the chord conductances of the *AeKir1* and *AeKir2B* oocytes below.

Fig. 5E and F) summarizes the effects of increasing the extracellular concentrations of K^+ or Na^+ on the conductive properties of the *AeKir1* and *AeKir2B* oocytes. As shown in Fig. 5E, at a V_m of -120 mV, the mean g_i of the *AeKir1* oocytes increases significantly from 0.40 ± 0.75 μS (control) to 49.65 ± 12.17 μS when extracellular K^+ is elevated to 50 mM (Fig. 5E), whereas the mean g_i is not significantly affected (0.43 ± 0.21 μS) when extracellular Na^+ is elevated to 50 mM (Fig. 5E). A similar trend occurs at a V_m of 0 mV, but the conductances are much lower in magnitude, which is consistent with the presence of strong inward rectifier channels. Namely, the mean g_i of the *AeKir1* oocytes increases significantly from 0.19 ± 0.10 μS (control) to 5.40 ± 1.35 S when extracellular K^+ is elevated to 50 mM (Fig. 5E), whereas the mean g_i is not significantly affected (0.17 ± 0.04 μS) when extracellular Na^+ is elevated to 50 mM (Fig. 5E).

In the *AeKir2B* oocytes (Fig. 5F), at a V_m of -120 mV, the mean g_i increases significantly from 0.31 ± 0.62 μS (control) to 10.8 ± 1.2 μS when extracellular K^+ is elevated to 50 mM (Fig. 5F), whereas the mean g_i is not significantly affected (0.46 ± 0.08 μS) when extracellular Na^+ is elevated to 50 mM (Fig. 5F). A similar trend occurs at a V_m of 0 mV, but the conductances are lower in magnitude, which again is consistent with the presence of inward rectifier channels. Namely, the mean g_i of the *AeKir2B* oocytes increases significantly from 0.18 ± 0.04 μS (control) to 1.99 ± 0.29 μS when extracellular K^+ is elevated to 50 mM (Fig. 5F), whereas the mean g_i is not significantly affected (0.23 ± 0.06 μS) when extracellular Na^+ is elevated to 50 mM (Fig. 5F).

Taking the results of Table 3, Figs. 4 and 5 together, we can conclude that the *AeKir1* and *AeKir2B* oocytes express constitutively active Kir channels that are conductive to K^+ and not Na^+ . Furthermore, the data indicate that 1) the *AeKir1* oocytes possess Kir channels that are more robust in activity than those of the *AeKir2B* oocytes, and 2) the *AeKir3* oocytes show no detectable Kir channel activity. Thus, in the remaining experiments we focus on the *AeKir1* and *AeKir2B* oocytes.

3.4.3. Effects of barium on the K^+ currents—We next sought to determine if barium blocks the inward K^+ currents mediated by the *AeKir1* and *AeKir2B* oocytes. Fig. 6A shows a representative I - V plot of an *AeKir1* oocyte. Elevating extracellular K^+ from 0.5 mM in

the control solution (Fig. 6A, open boxes) to 50 mM (Fig. 6A, filled circles) elicits pronounced inward currents in the *AeKir1* oocyte at hyperpolarizing voltages (as was observed in Fig. 5A). However, if barium is added subsequently to the extracellular solution at 1 mM (Fig. 6A, gray circles), then the inward currents are blocked prominently to levels that are similar in magnitude to those of H₂O-injected oocytes in 50 mM K⁺ (e.g., Fig. 5D). After washing the barium out, the inward currents return to levels that are reduced slightly (Fig. 6A, open circles) compared to those in the original exposure to 50 mM K⁺ (Fig. 6A, filled circles).

Fig. 6B shows a representative *I-V* plot of an *AeKir2B* oocyte exposed to similar experimental maneuvers. Elevating extracellular K⁺ from 0.5 mM (Fig. 6B, open boxes) to 50 mM (Fig. 6B, solid circles) elicits enhanced inward currents in the *AeKir2B* oocytes at hyperpolarizing voltages (as was observed in Fig. 5B). Similar to the *AeKir1* oocyte, the addition of 1 mM barium (Fig. 6B, gray circles) inhibits the inward currents to levels that are similar in magnitude to those of H₂O-injected oocytes in 50 mM K⁺ (e.g., Fig. 5D). This block is almost fully reversed after washing out the barium (Fig. 6B, open circles).

Fig. 6C and D) summarizes the effects of barium on the chord conductances of the *AeKir1* and *AeKir2B* oocytes at a V_m of -120 mV. As shown in Fig. 6C, the mean g_i of the *AeKir1* oocytes increases significantly from $1.295 \pm 0.35 \mu\text{S}$ (control) to $30.93 \pm 5.26 \mu\text{S}$ when extracellular K⁺ is elevated to 50 mM (Fig. 6C). The subsequent addition of barium (1 mM) significantly lowers the mean g_i to $0.27 \pm 0.05 \mu\text{S}$, which is statistically similar to the mean g_i in the control solution (Fig. 6C). The wash-out of barium significantly increases the mean g_i to $20.45 \pm 3.31 \mu\text{S}$, which is significantly lower than the original value in 50 mM K⁺ (Fig. 6C).

In the *AeKir2B* oocytes, the mean g_i increases significantly from $0.42 \pm 0.09 \mu\text{S}$ to $9.635 \pm 1.18 \mu\text{S}$ when extracellular K⁺ is elevated to 50 mM (Fig. 6D). The subsequent addition of barium (1 mM) significantly lowers the mean g_i to $0.38 \pm 0.07 \mu\text{S}$, which is statistically similar to the mean g_i in the control solution (Fig. 6D). The wash-out of barium significantly increases the mean g_i to $7.81 \pm 0.94 \mu\text{S}$, which is significantly lower than the original value in 50 mM K⁺ (Fig. 6D).

The above data indicate that the K⁺-conductances of both the *AeKir1* and *AeKir2B* oocytes are reversibly blocked by barium.

3.4.4. Effects of Na⁺ on the K⁺ currents—Fig. 5 demonstrates that an *acute* increase in the extracellular concentration of Na⁺ has no detectable effects on the conductive properties of the *AeKir1* and *AeKir2B* oocytes. We next sought to determine if a *chronic* increase in the extracellular concentration of Na⁺ influences the K⁺ currents mediated by the *AeKir1* and *AeKir2B* oocytes.

As shown in Fig. 7A, when an *AeKir1* oocyte is bathed in a solution containing 0.5 mM Na⁺ and 5 mM K⁺ (solution V, Table 1) it exhibits modest inward currents at hyperpolarizing voltages and nominal outward currents at depolarizing voltages (open boxes, 0 min). Within 5 min of elevating extracellular Na⁺ from 0.5 mM to 93 mM (solution VI, Table 1), the magnitudes of the inward currents are enhanced (Fig. 7A; light gray circles, 5 min). By 10 min and 15 min after elevating Na⁺, the magnitudes of the inward currents are increased even further (Fig. 7A; dark gray and filled circles, 10 min and 15 min). Furthermore, the magnitudes of the outward currents at the initial depolarizing voltages (i.e., -60 mV and -40 mV) show noticeable increases (Fig. 7A). In contrast, the magnitudes of the outward currents at the most depolarizing voltages (i.e., -20 mV to +40 mV) increase marginally over the 15 min period (Fig. 7A). Although not obvious from the *I-V* plot in Fig. 7A, the

E_{rev} of the *AeKir1* oocyte shifts gradually towards the E_K (−79 mV) during the exposure to 93 mM Na^+ from −64.6 mV (0 min) to −71 mV (15 min). The above changes to the $I-V$ relationship of the *AeKir1* oocyte are reversible over a similar time scale (Fig. 7A; light gray boxes, 30 min).

Fig. 7B shows similar experimental maneuvers performed on an *AeKir2B* oocyte. However, in this oocyte, the extracellular concentration of K^+ is 8 mM (solution VII, Table 1) given that the magnitudes of the currents mediated by *AeKir2B* oocytes tend to be lower than those of *AeKir1* oocytes (e.g., Figs. 4 and 5). Accordingly, the extracellular concentration of Na^+ is increased to 90 mM (solution VIII, Table 1) to avoid changing the osmotic pressure of the bath solution. In brief, elevating extracellular Na^+ from 0.5 mM to 90 mM in the presence of 8 mM K^+ does not noticeably affect the $I-V$ relationship of this oocyte over a 15 min time period.

Fig. 7C and D summarizes the effects of elevating extracellular Na^+ on the conductive properties of the *AeKir1* and *AeKir2B* oocytes. For clarity of the figure, we focus on the chord conductances at a V_m of −120 mV, but similar trends also occur at a V_m of 0 mV (data not shown). As shown in Fig. 7C, the mean g_i increases significantly from 14.8 ± 3.4 μS (0 min) to 44.1 ± 8.8 μS within 5 min of increasing extracellular Na^+ from 0.5 mM to 93 mM. After 10 min and 15 min of exposure, the mean g_i continues to increase significantly to 99.9 ± 17.05 μS and 134.2 ± 20.9 μS , respectively (Fig. 7C). The mean g_i decreases significantly to 21.2 ± 3.8 μS (Fig. 7C, 30 min) after returning to 0.5 mM Na^+ for 15 min; this mean g_i value is statistically similar to the one at 0 min (Fig. 7C). In the *AeKir2B* oocytes, the mean g_i does not change significantly when extracellular Na^+ is elevated from 0.5 mM to 90 mM over 15 min of exposure (Fig. 7D; mean $g_i = 2.97 \pm 0.34$ μS at 0 min, 2.92 ± 0.39 μS at 5 min, 2.985 ± 0.39 μS at 10 min, 3.08 ± 0.41 μS at 15 min). After returning to 0.5 mM Na^+ (time 30 min in Fig. 7D), the mean g_i of the *AeKir2B* oocytes (2.83 ± 0.33 μS) is slightly, but significantly, lower than the preceding mean g_i at 15 min (Fig. 7D).

The above data indicate that the conductive properties of the *AeKir1* oocytes are slowly modulated by the presence of extracellular Na^+ , whereas the *AeKir2B* oocytes do not exhibit such modulation.

3.4.5. Monovalent cation selectivity sequence—Lastly, we aimed to characterize the cation selectivity of the *AeKir1* and *AeKir2B* oocytes. A representative experiment for an *AeKir1* oocyte is shown in Fig. 8A; the oocyte is first bathed in the control solution (solution IX, Table 1; 0.5 mM Na^+ and 0.5 mM K^+) and voltage clamped at a V_m of −92 mV. Switching to a solution that contains 5 mM Tl^+ rapidly induces an inward membrane current (I_m) of ~400 nA, which subsequently collapses when the Tl^+ is washed out with the control solution (Fig. 8A). Switching to solutions that contain 5 mM of other monovalent cations show that 1) K^+ elicits an inward I_m comparable in magnitude to that of Tl^+ , 2) Rb^+ produces a weak inward I_m (~70 nA) relative to those of Tl^+ and K^+ , and 3) Na^+ , Cs^+ , and Li^+ each elicit a nominal inward I_m .

Fig. 8B shows similar experimental maneuvers performed on an *AeKir2B* oocyte. However, in this oocyte, the extracellular concentration of a cation is increased to 10 mM given that the currents mediated by *AeKir2B* oocytes tend to be lower in magnitude than those of *AeKir1* oocytes (e.g., Figs. 4 and 5). As shown in Fig. 8B, switching from the control solution to one that contains 10 mM K^+ rapidly induces an inward I_m of ~115 nA; this I_m subsequently collapses when the K^+ is washed out with the control solution (Fig. 8B). Switching to solutions that contain 10 mM of other monovalent cations show that 1) Tl^+ elicits an inward I_m (~125 nA) slightly larger in magnitude than that of K^+ , 2) Rb^+ and Cs^+

each produce a weak inward I_m (~30 nA and 20 nA, respectively) relative to those of K^+ and Tl^+ , and 3) Na^+ and Li^+ each elicit a nominal I_m .

Fig. 8C summarizes the mean change to I_m (ΔI_m) produced by each of the cations tested in the *AeKir1* oocytes after subtracting the corresponding values from H_2O -injected oocytes. Consistent with the representative tracing in Fig. 8A, the mean ΔI_m elicited by K^+ (-353.5 ± 48.5 nA) and Tl^+ (-343.9 ± 46.6 nA) are statistically similar and both are significantly greater than that elicited by Rb^+ (-62.55 ± 9.9 nA). The small mean ΔI_m values produced by Cs^+ , Na^+ , and Li^+ are not significantly different from zero (Fig. 8C). Thus, the monovalent cation selectivity sequence for the *AeKir1* channel is: $K^+ = Tl^+ \gg Rb^+ > Cs^+ = Na^+ = Li^+$; this corresponds to a type IV or V Eisenman sequence (Eisenman and Horn, 1983).

Likewise, Fig. 8D summarizes the ΔI_m produced by each of the cations tested in the *AeKir2B* oocytes. Consistent with the representative tracing in Fig. 8B, the mean ΔI_m elicited by K^+ (-125.4 ± 6.8 nA) and Tl^+ (-129.2 ± 11.35 nA) are statistically similar and both are significantly greater than those elicited by Rb^+ (-25.8 ± 1.4 nA) and Cs^+ (-26.1 ± 2.0 nA); the latter two are statistically similar to one another. The small mean ΔI_m values produced by Na^+ and Li^+ are not significantly different from zero (Fig. 8D). Thus, the monovalent cation selectivity sequence for the *AeKir2B* channel is: $K^+ = Tl^+ \gg Rb^+ = Cs^+ > Na^+ = Li^+$; this corresponds to a type IV Eisenman sequence (Eisenman and Horn, 1983).

3.5. Permeability of the principal cell basolateral membrane to monovalent cations

To further characterize the electrophysiological properties of the principal cell basolateral membrane, we measured the relative ability of various monovalent cations to change the basolateral membrane potential (V_b) of principal cells, as an indicator of membrane permeability. We were particularly curious as to whether the basolateral membrane offers permeability to Tl^+ , given that the *AeKir1* and *AeKir2B* channels conduct this cation to a similar degree as K^+ (Fig. 8).

Fig. 9A shows a representative tracing of principal cell V_b in response to replacing 30 mM of extracellular Na^+ with 30 mM of NH_4^+ , Rb^+ , K^+ , or Tl^+ . In this cell, the spontaneous V_b is approximately -90 mV (arrow) when bathed in the typical mosquito Ringer's solution ('NaCl-Ringer' in Fig. 9A). A replacement of the chloride in the Ringer's solution with gluconate, which is necessary to prevent the precipitation of Tl^+ , leads to a small depolarization of the V_b ('Na-gluconate-Ringer' in Fig. 9A). Subsequently replacing 30 mM of extracellular Na^+ with NH_4^+ causes a small depolarization of V_b that is reversed upon returning the Na^+ . In contrast, replacing 30 mM Na^+ with Rb^+ , K^+ , or Tl^+ each produce a large, reversible depolarization of V_b , with Tl^+ producing the largest depolarization.

Fig. 9B summarizes the relative depolarizations of V_b induced by each of the cations. Note that the absolute magnitudes of the depolarizations triggered by NH_4^+ , Rb^+ , K^+ , and Tl^+ correlated with the spontaneous V_b of principal cells (data not shown). Thus, the membrane depolarizations in Fig. 9B are expressed as a percent change from the spontaneous V_b . As shown in Fig. 9B, the mean depolarizations elicited by the four cations tested, in order of magnitude, are: $6.7 \pm 1.1\%$ for NH_4^+ , $24.4 \pm 3.1\%$ for Rb^+ , $33.5 \pm 3.3\%$ for K^+ , and $60.1 \pm 7.8\%$ for Tl^+ . All of the means are significantly different from one another (Fig. 9B). Accordingly, the permeability sequence of the principal cell basolateral membrane is $Tl^+ \gg K^+ > Rb^+ \gg NH_4^+$; this sequence resembles a type IV or V Eisenman sequence (Eisenman and Horn, 1983).

4. Discussion

4.1. The expression of Kir channel cDNAs in *Aedes* Malpighian tubules

The results of the present study demonstrate that genes encoding three Kir channel subunits (*AeKir1*, *AeKir2B*, *AeKir3*) are expressed abundantly in the Malpighian tubules of adult female *A. aegypti* mosquitoes (Fig. 2). A search of a recently published gene expression atlas for *A. gambiae* mosquitoes (www.tissue-atlas.org) (Baker et al., 2011) reveals a similar pattern of Kir channel subunit expression in the Malpighian tubules of adult females (Piermarini, personal observations). Likewise, in adult *D. melanogaster* fruit flies, the expression of the *DrKir1*, *DrKir2*, and *DrKir3* genes are all enriched in Malpighian tubules relative to that in the whole animal (Chintapalli et al., 2007; Evans et al., 2005; Wang et al., 2004). Thus, in all adult dipterans examined to date, the Malpighian tubules are characterized by the expression of three genes encoding Kir channel subunits. As such, the Malpighian tubules have the potential to express three types of homomeric Kir channels and three types of heteromeric Kir channels (e.g., *AeKir1:AeKir2B*; *AeKir1:AeKir3*; *AeKir2B:AeKir3*).

The most notable difference in Kir channel subunit expression between the Malpighian tubules of mosquitoes and fruit flies appears to lie in the Kir2 genes. That is, mosquitoes express Kir2B—a gene unique to mosquitoes (Fig. 1)—whereas fruit flies express the ortholog of mosquito Kir2A (i.e., *DrKir2*). However, it is unlikely that this subtle difference in gene expression is linked to major functional differences between the Malpighian tubules of blood-feeding female mosquitoes vs. those of frugivorous fruit flies, because male *Anopheles* mosquitoes, which never feed on blood, exhibit the same pattern of Kir channel gene expression in their Malpighian tubules as the hematophagous female *Anopheles* mosquitoes (www.tissue-atlas.org; Piermarini, personal observations).

Although we did not elucidate the cellular localization of gene expression for the Kir channel subunits in *Aedes* Malpighian tubules, a previous study in *Drosophila* has shown that the three *DrKir* genes are expressed exclusively in principal cells (Evans et al., 2005). We assume that the same occurs in *Aedes* Malpighian tubules given the robust barium-sensitive K⁺ conductance detected in the basolateral membrane of *Aedes* principal cells (Beyenbach and Masia, 2002) and the deduced cation permeability of this membrane in the present study (Fig. 9). Subsequent immunolocalization studies in mosquito Malpighian tubules will be required to confirm the expression of the Kir channel subunits in the basolateral membrane of principal cells.

4.2. Functional characterizations of the *Aedes* Kir channels

To our knowledge, the present study provides 1) the first functional characterizations of mosquito Kir channels, and 2) the first successful characterizations of ‘wild-type’ insect Kir channels expressed heterologously in *Xenopus* oocytes. A previous study by Doring et al. (2002) used *Drosophila* S2 cells to functionally characterize the homomeric Kir1 and Kir2 channels of *Drosophila*, but attempts to characterize these wild-type channels in *Xenopus* oocytes were unsuccessful. To uncover the full functional activity of the homomeric *DrKir1* and *DrKir2* channels in *Xenopus* oocytes, it was necessary to generate chimeric constructs that included parts of rat Kir2.1 in their cytosolic N-terminal and/or C-terminal domains (Doring et al., 2002). Furthermore, it was shown that the mutation of a single Val residue (V³⁴) in the cytosolic N-terminal domain of *DrKir1* to a Gln residue was able to uncover some of the functional activity of *DrKir1* when expressed in *Xenopus* oocytes (Doring et al., 2002). Interestingly, the *AeKir1* and *AeKir2B* subunits, which form functional channels in *Xenopus* oocytes, contain a Val (V¹³³) and Ala (A⁹⁶) residue, respectively, at the

corresponding site (Fig. 3). Thus, in mosquito Kir channels, the significance of this residue is questionable as it relates to their functional expression in *Xenopus* oocytes.

4.2.1. The homomeric AeKir1 and AeKir2B channels

4.2.1.1. Similarities: The results of the present study indicate that the homomeric AeKir1 and AeKir2B channels share the following functional features: 1) they are constitutively active (Table 3, Figs. 4 and 5); 2) they mediate inward-rectifying currents that are selective for K⁺ over Na⁺ (Fig. 5); and 3) they are blocked reversibly by barium (Fig. 6). In general, these findings are to be expected, because they are canonical functional features of most Kir channels that have been cloned and characterized from vertebrate and invertebrate animals (Abbas et al., 2011; Doring et al., 2002; Hibino et al., 2010; Tompkins-Macdonald et al., 2009).

To date, the only other insect Kir channels to be functionally characterized are the Kir1 and Kir2 channels of *Drosophila* (*DrKir1* and *DrKir2*). These channels are also constitutively active and mediate barium-sensitive, inward-rectifying K⁺ currents (Doring et al., 2002). Thus, at least the Kir1 and Kir2 genes of dipterans encode subunits that form functional homomeric Kir channels. It remains to be determined whether or not the Kir1 and Kir2 subunits of these insects can also form heteromeric channels with novel functional properties as occurs for some mammalian Kir channels (Hibino et al., 2010).

It should also be noted that, in general, the cation selectivity sequences of the AeKir1 and AeKir2B channels are 1) very similar (Fig. 8) and 2) consistent with those reported for mammalian Kir channels (Chepilko et al., 1995; Zhou et al., 1994). Of particular note is the fact that both the AeKir1 and AeKir2B channels conduct Tl⁺ as well as K⁺ (Fig. 8), which suggests that the mosquito channels may be amenable to high-throughput fluorescence-based functional assays that rely on the flux of Tl⁺ through the channels (Weaver et al., 2004). Such assays have been used to discover novel pharmacological modulators of mammalian Kir channels for development as potential therapeutics (Bhave et al., 2010). Despite the above functional similarities between the AeKir1 and AeKir2B channels, there are also important differences, which are dissected below.

4.2.1.2. Differences in channel activity: Under all of the experimental conditions examined in the present study, the currents mediated by the AeKir1 oocytes are consistently more robust than those of the AeKir2B oocytes (Figs. 4–8). In comparing the amino acid sequences of the AeKir1 and AeKir2B subunits, we find no obvious molecular basis for this functional difference within the predicted transmembrane segments and pore helix (Fig. 3). However, immediately downstream of the second transmembrane segment (TM2) there is a subtle difference in a motif (R²⁶⁵PKK in AeKir1, Fig. 3) that is known to be associated with the activation of mammalian Kir channels by phosphatidylinositol 4,5-bisphosphate, PIP₂ (Hansen et al., 2011). In particular, the positively-charged residues of this motif help coordinate the binding of negatively-charged phosphates of PIP₂ to the channel, which subsequently leads to an opening of the channel's cytosolic gate and thereby greater functional activity (Hansen et al., 2011).

Interestingly, the AeKir1 subunit contains a perfectly conserved PIP₂-binding motif in this region (R²⁶⁵PKK), whereas the AeKir2B subunit contains an uncharged Ser residue in place of the last Lys (R²³²PKS) (see open circle in Fig. 3). Thus, if the absence of one positive charge in this region reduces the ability of PIP₂ to bind to the AeKir2B channel, then a lower functional activity may result. Consistent with this notion, mutagenesis experiments on the mammalian Kir2.1 channel have shown that mutating only a single positively-charged residue in this motif (including the aforementioned 'K') to an uncharged Asn or Cys residue can dramatically reduce both the ability of PIP₂ to bind to the channel and the

functional activity of the channel (Lopes et al., 2002). Similar mutagenesis experiments on the *AeKir1* and *AeKir2B* subunits will be required to test this hypothesis.

Alternatively, it is possible that the gating of the *AeKir2B* channel is more tightly regulated than that of the *AeKir1* channel and requires an agonist to reach its full functional capacity when expressed in *Xenopus* oocytes. Among vertebrate Kir channels, the G protein-gated Kir (KG) channels (also known as GIRK channels), consisting of Kir3.x subunits, can exhibit such functional activity. That is, the expression of KG channels alone results in weak functional activity in *Xenopus* oocytes, but if the KG channels are coexpressed with G proteins, then their functional activity is enhanced (Hedin et al., 1996; Kubo et al., 1993b). In this case, the KG channels are activated by the binding of G protein $\beta\gamma$ subunits to the channel, which opens the helical and cytosolic gates of the channel, thereby leading to a greater flux of K^+ through the channel (Whorton and MacKinnon, 2011).

4.2.1.3. Differences in modulation by Na^+ : As demonstrated in Fig. 7A, elevating extracellular Na^+ slowly activates the *AeKir1* channel, resulting in enhanced inward and outward K^+ currents. Along similar lines, the functional activity of certain mammalian Kir channels, primarily the K_G channels (Kir3.x), are known to be modulated by Na^+ . There is strong molecular and structural evidence that the cytosolic C-terminal domain of most Kir3.x subunits contains a motif (DLR-K/N-SH) that coordinates the binding of intracellular Na^+ ; the Asp and His residues are the most critical determinants of Na^+ binding (Ho and Murrell-Lagnado, 1999a,b; Rosenhouse-Dantsker et al., 2008; Whorton and MacKinnon, 2011). On the atomic level, the binding of Na^+ to the Asp residue has been shown to mimic the effects of G proteins on the Kir3.x channels; i.e., Na^+ binding opens the helical and cytosolic gates of the channel (Whorton and MacKinnon, 2011).

Notably, the proposed intracellular Na^+ -binding motif of mammalian KG channels is well conserved in the *AeKir1* subunit (D²⁹⁶MRKSH; see 'Na⁺-binding Motif' in Fig. 3), including the critical aforementioned Asp and His residues. Furthermore, the slow time course of the modulation (up to 15 min; Fig. 7A) observed in the *AeKir1* oocytes is consistent with Na^+ interacting with an intracellular site of the *AeKir1* channel, because Na^+ needs to first accumulate within the cell before an effect is observed. Likewise, this may explain the slow reversal of the Na^+ effects, because Na^+ needs to first be depleted from the cell. Thus, it is possible that Na^+ imparts its stimulatory effects on the internal currents of the *AeKir1* channel in a manner similar to those of mammalian Kir3.x channels – i.e., the binding of Na^+ relaxes the gating of the channel (Whorton and MacKinnon, 2011).

To our knowledge, the stimulatory effect of Na^+ on the outward K^+ currents (at the initial depolarizing voltages) mediated by the *AeKir1* channel is novel. A mechanism for this effect is unclear, but the finding may indicate that Na^+ alters the channel's conformation in a manner that lowers the ability of intracellular Mg^{2+} and/or polyamines to interact with the channel. The binding of Mg^{2+} and polyamines to Kir channels is known to block the outward movements of K^+ (Hibino et al., 2010; Lu, 2004). Thus, a reduced binding would lower the physical impedance to the outward flux of K^+ , resulting in a channel that mediates larger outward currents, as observed in the *AeKir1* oocytes at the initial depolarizing voltages (Fig. 7A).

In contrast to the *AeKir1* oocytes, the *AeKir2B* channel shows no detectable modulation of its activity by an increase of extracellular Na^+ (Fig. 7B). This is surprising given that the aforementioned Na^+ -binding motif in the *AeKir2B* subunit (D²⁶³LRKSH; see 'Na⁺-binding Motif' in Fig. 3) is identical to that of mammalian Kir3.x channels and is very similar to that of the *AeKir1* subunit, including the essential Asp and His residues. Thus, another region of the mosquito Kir channels must also contribute to their modulation by Na^+ . Elucidating the

location of this region will likely require the generation of chimeric *AeKir* constructs consisting of different domains of the *AeKir1* and *AeKir2B* subunits.

It should be noted that heteromeric, mammalian Kir channels can be formed between Na⁺-sensitive and Na⁺-insensitive subunits. In these cases, the Na⁺-sensitive subunits can confer their Na⁺-sensitivity to the heteromeric channels, as when the Na⁺-sensitive Kir5.1 subunit is coexpressed with the Na⁺-insensitive Kir4.1 subunit (Rosenhouse-Dantsker et al., 2008). It will be interesting to determine if functional heteromeric channels can form between *AeKir1* and *AeKir2B* subunits, and if so, whether *AeKir1* confers its Na⁺-sensitivity to the resulting heteromeric channel.

Lastly, given the pronounced modulatory effects of Na⁺ on the *AeKir1* channel, it is illuminating to return to the robust functional activity that was observed in the *AeKir1* oocytes bathed in solution *I* (Fig. 4A). Solution *I* contains 96 mM Na⁺ and only 2 mM K⁺, but the magnitudes of the inward currents and chord conductances in the *AeKir1* oocytes bathed in solution *I* are comparable to – or even greater than – those observed in *AeKir1* oocytes bathed in solutions containing 25 times more K⁺ (Figs. 5 and 6). Furthermore, the outward currents of the *AeKir1* oocytes are more prominent when bathed in solution *I*. Thus, the large currents and chord conductances measured in the *AeKir1* oocytes bathed in solution *I* are likely made possible by the dramatic activating effects of Na⁺ that were observed in Fig. 7A. As such, in ‘normal’ physiological solutions (i.e., high Na⁺, low K⁺), the *AeKir1* channel is expected to be a highly active channel that can mediate an influx or efflux of K⁺ depending upon the standing membrane potential and chemical gradient for K⁺ across the membrane that it resides in.

4.2.1.4. Differences in cation selectivity: One notable difference in the cation selectivity between the *AeKir1* and *AeKir2B* oocytes is that extracellular Cs⁺ does not produce detectable inward currents in *AeKir1* oocytes, but produces inward currents of similar magnitude as Rb⁺ in the *AeKir2B* oocytes (Fig. 8). This finding is surprising, because Cs⁺ is considered a blocker of wild-type Kir channels and not a conductive cation (Hibino et al., 2010; Kubo et al., 1993a; Zhou et al., 1994). Presumably, the difference in Cs⁺ conductance between the two mosquito channels reflects differences within the ion selectivity filter, but as described below, this may not be the case.

Structure–function studies of mammalian Kir2.1 channels have shown that mutating residues Thr141 and Ser165 – which are near the selectivity filter and in second transmembrane segment, respectively – reduces the blocking effects of Cs⁺ and enhances the permeability of the channels to this cation (Thompson et al., 2000). Likewise, in mammalian Kir1.1 and heteromeric Kir3.1/3.4 channels, mutations of residues associated with the selectivity filter (e.g., Arg¹²⁸ in Kir1.1, Glu¹⁴⁵ in Kir3.1/3.4) can dramatically alter the ion selectivity of the channels and enhance their conductance to Cs⁺ (Dibb et al., 2003; Sackin et al., 2010). However, all of the corresponding residues in the *AeKir1* and *AeKir2B* subunits are identical to one another (see filled circles in Fig. 3). Thus, the molecular basis for the difference in Cs⁺ conductivity between the two mosquito channels is unclear and remains to be determined.

4.2.2. The enigmatic *AeKir3* channel subunit—In the present study, we did not detect functional Kir channel expression in the oocytes injected with cRNA encoding the *AeKir3* subunit, which is consistent with the efforts of Doring et al. who found that the orthologous subunit of *Drosophila* (*DrKir3*) is nonfunctional when expressed heterologously in either *Xenopus* oocytes or *Drosophila* S2 cells (Doring et al., 2002). Thus, to date, the functional characterization of any insect Kir3 channel has been unsuccessful.

Studies on mammalian Kir channel subunits that were once considered ‘non-functional’ provide several possibilities as to why the insect Kir3 subunits may be difficult to characterize. For example, the insect Kir3 subunits may require the expression of an accessory protein, such as a sulphonylurea receptor, to form a functional channel (Inagaki et al., 1996, 1995a,b). Alternatively, insect Kir3 subunits may only be functional when expressed with another Kir subunit to form a heteromeric Kir channel (Pearson et al., 1999; Pessia et al., 1996; Tanemoto et al., 2000; Tucker et al., 2000), or the Kir3 subunits may be expressed in intracellular compartments and regulate the surface expression of other Kir channel subunits in a dominant-negative fashion (Dassau et al., 2011). Clearly, further experimental studies are required to resolve the present enigmas that are insect Kir3 subunits.

4.3. Do Kir channels contribute to the K⁺ conductance on the basolateral membrane of principal cells?

The electrophysiological experiments in isolated Malpighian tubules show that the basolateral membrane of principal cells in *Aedes* Malpighian tubules is highly permeable to Tl⁺ (Fig. 9), which is a cation that is known to substitute for a variety of K⁺ transporters and channels, including Kir channels. In addition, the deduced cation permeability sequence of the basolateral membrane is nearly identical with that described for mammalian Kir channels (Chepilko et al., 1995; Zhou et al., 1994). Taking this result in conjunction with the facts that 1) barium blocks 60% of the basolateral membrane conductance of principal cells in *Aedes* Malpighian tubules (Beyenbach and Masia, 2002), and 2) Malpighian tubules express at least two constitutively active, barium-sensitive Kir channels that are highly conductive to Tl⁺ (Table 3, Figs. 2, 5, 6 and 8), we conclude that Tl⁺-permeable Kir channels dominate the K⁺ conductance of the basolateral membrane of principal cells.

In particular, given the robust conductances mediated by the AeKir1 channel in standard physiological solutions, such as solution I (Fig. 4A), we consider this channel as the best candidate to mediate the barium-sensitive transepithelial transport of K⁺ in *Aedes* Malpighian tubules (Beyenbach and Masia, 2002; Masia et al., 2000; Scott et al., 2004). The AeKir1 channel is an especially good candidate for facilitating the inward movements of K⁺ from the hemolymph to the cytoplasm of principal cells, because of the large inward currents it mediates in the presence of low extracellular K⁺, relative to those of the AeKir2B and AeKir3 channels (Fig. 4). Thus, in isolated *Aedes* Malpighian tubules bathed in a standard mosquito Ringer solution under control conditions, we propose that the barium-sensitive AeKir1 channel operates in parallel with a bumetanide-sensitive Na,K,2Cl cotransporter (NKCC) to provide the dominant mechanisms for the uptake of peritubular K⁺, as originally envisioned by our lab (Scott et al., 2004).

Presumably, both of these K⁺ transport mechanisms would function similarly in isolated *Aedes* Malpighian tubules that are stimulated by kinin diuretic peptides. Kinins, such as leucokinins and aedeskinins, impart their diuretic effects on Malpighian tubules by binding to a G protein-coupled receptor on stellate and principal cells (Lu et al., 2011; Yu and Beyenbach, 2004), which leads to a rise of intracellular Ca²⁺ (Yu and Beyenbach, 2002). Calcium then stimulates the transepithelial secretion of NaCl, KCl, and water by signaling to the paracellular pathway and apical V-type H⁺-ATPase (Beyenbach et al., 2009). Notably, within seconds of kinin application, the paracellular shunt opens (see thick red arrow in Fig. 10A), which effectively short-circuits the epithelium and hyperpolarizes the basolateral membrane of principal cells (Pannabecker et al., 1993). In the case of aedeskinin III, the basolateral membrane hyperpolarizes from approximately -85 mV to -110 mV in less than 30 s (Schepel et al., 2010) (Fig. 10A). Given the effects of hyperpolarizing membrane potentials on the currents mediated by the AeKir1 channel (Fig. 4A), such a kinin-induced hyperpolarization of the principal cell basolateral membrane is expected to enhance the

uptake of peritubular K^+ by the *AeKir1* channel, thereby supporting the diuresis (see thin red arrows in Fig. 10A).

Given that the *AeKir1* channel also mediates outward currents in physiological solutions (Figs. 4 and 7), it is worth speculating on a potential role of this channel in isolated *Aedes* Malpighian tubules that are stimulated by the mosquito natriuretic factor (Petzel et al., 1985), which is now known as the calcitonin-like diuretic peptide (Coast et al., 2005). The calcitonin-like peptide imparts its effects on Malpighian tubules by binding to a receptor on the epithelium, which leads to a rise of intracellular cAMP (Petzel et al., 1987). Cyclic AMP selectively enhances the secretion of NaCl and water (Williams and Beyenbach, 1983) by 1) opening a Na^+ conductance on the basolateral membrane of principal cells (see 'NaC' in Fig. 10B), which depolarizes the basolateral membrane potential from -65 mV to -25 mV (Sawyer and Beyenbach, 1985), and 2) activating the aforementioned bumetanide-sensitive NKCC (Hegarty et al., 1991).

Since a membrane depolarization to -25 mV would at least reduce the inward K^+ current, and likely generate an outward current of K^+ , mediated by the *AeKir1* channel (Fig. 4A), the cAMP-induced depolarization may lead to an efflux of K^+ to the peritubular bath (Fig. 10B). This would result in 1) the recycling of K^+ across the basolateral membrane of principal cells to support the activity of the NKCC (see thin red arrows in Fig. 10B), and 2) a dampening of the amount of K^+ that reaches the apical membrane for transport into the lumen (Fig. 10B). The net effect would be a natriuresis (and not a kaliuresis) that is observed in isolated *Aedes* Malpighian tubules (Williams and Beyenbach, 1983). Thus, cAMP may increase transcellular Na^+ secretion not only by opening a conductive pathway for Na^+ entry across the basolateral membrane (see 'NaC' in Fig. 10B), but also by stimulating a Kir channel-mediated recycling mechanism for K^+ across the basolateral membrane (see 'Kir' in Fig. 10B).

In the mammalian kidney, a similar Kir channel-mediated recycling mechanism for K^+ exists in the thick-ascending limb of the loop of Henle where the apical Kir1.1 channel (ROMK) exports K^+ that enters the cells via an apical NKCC and a basolateral Na^+ , K^+ -ATPase (Hebert et al., 2005; Hibino et al., 2010). In this case, the Kir1.1 channel supports the selective transepithelial *reabsorption* of Na^+ in mammalian renal tubules (Hebert et al., 2005; Hibino et al., 2010). It will be interesting to determine if the *AeKir1* channel supports the selective transepithelial *secretion* of Na^+ in mosquito Malpighian tubules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplemental information

Supplemental Information related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2012.09.009>.

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Fig. 1.

Neighbor-joining tree showing the phylogenetic relationships of *Aedes* (*Ae*), *Drosophila* (*Dr*), and select human (*Ho*) Kir channel subunits (amino-acid sequences). See Section 3.1 for details. The tree was generated by MEGA5 software (Tamura et al., 2011) using Poisson-corrected distance estimates. Bootstrap scores from 1000 replicates are provided for the nodes (filled circles) of the branches. The proportion of amino-acids that differ between two proteins is indicated by the total branch length between them. The scale bar shows a proportional difference (branch length) of 0.1 (i.e., a 10% difference in amino-acids). The tree is rooted to a putative Kir channel of the nematode *C. elegans* (*Celrk3*). GenBank accession numbers are as follows: *AeKir1*, JQ753065; *AeKir2A*, XP_001653530; *AeKir2B*, JQ753067; *AeKir2B'*, XP_001663556; *AeKir3*, JQ753066; *Celrk3*, NP_510395; *DrKir1*, NP_001097884; *DrKir2*, NP_001163700; *DrKir3*, NP_609903; *HoKir1.1*, NP_000211; *HoKir2.1*, NP_000882; *HoKir3.1*, NP_002230; *HoKir6.1*, NP_004973.

Fig. 2.

Expression of genes encoding *Aedes* Kir channel subunits in Malpighian tubules (A) and whole bodies (B) of adult female mosquitoes as determined by RT-PCR. Each column represents the lane of an agarose gel in which the products of a PCR were separated by electrophoresis; both an indicated *AeKir* gene and the *RPS7* gene were amplified simultaneously. The bands in the *AeKir* row are ~ 500 bp and representative of cycle 53, whereas those of the *RPS7* row are ~ 300 bp and representative of cycle 33. Shown are results of one representative experiment each for Malpighian tubules and whole insect; three experiments were run in total.

Fig. 3.

Amino-acid sequence alignment of the *Aedes* Kir channel cDNAs cloned from Malpighian tubules. The filled circles indicate residues that are known to influence the ion permeation properties of mammalian Kir channels, and the open circle indicates a residue involved with binding of PIP₂ to mammalian Kir channels (see Section 4.2.1 for details). Also indicated are the residues predicted to form the so-called 'G-Loop' of the cytosolic domain, which represent the most constricted region of the cytoplasmic pore (Hibino et al., 2010). GenBank accession numbers for *AeKir1*, *AeKir2B*, and *AeKir3* are JQ753065, JQ753067, and JQ753066, respectively. Alignment and shading were performed by the BioEdit Sequence Alignment software, Version 7 (Hall, 1999). Black shading indicates 100% identity among the residues of a position, whereas gray shading indicates 100% similarity among the residues of a position; similarity was determined according to the BLOSUM62 similarity matrix by the BioEdit software.

Fig. 4. Current-voltage (I-V) relationships of AeKir1 (A, filled circles), AeKir2B (B, filled circles), and AeKir3 (C, filled circles) oocytes in comparison to H₂O-injected oocytes (open circles, dashed line). The insets of 'B' and 'C' show the same data plotted on a magnified scale. All oocytes are bathed in solution I (96 mM Na⁺, 2 mM K⁺). V_m , membrane voltage; I_m , membrane current. (D) Mean chord conductances (g_i) $\pm$ SE of AeKir1 oocytes ($N=13$) in solution I at the membrane potentials indicated. Corresponding mean values in H₂O-injected oocytes ($N=10$) are subtracted. (E) Same as in 'D' but for AeKir2B oocytes ($N=19$). Corresponding mean values in H₂O-injected oocytes ($N=9$) are subtracted. (F) Same as in 'D' but for AeKir3 oocytes ($N=8$). Corresponding mean values in H₂O-injected

oocytes ($N=7$) are subtracted. **** indicates significant difference ($P<0.0001$) as determined by an unpaired t -test.

Fig. 5.

Effects of increasing extracellular Na⁺ or K⁺ on the *I-V* relationships of AeKir1, AeKir2B, AeKir3, and H₂O-injected oocytes. Shown are *I-V* plots of representative AeKir1 (A), AeKir2B (B), AeKir3 (C), and H₂O-injected (D) oocytes bathed consecutively (2 min each) in solutions containing 0.5 mM Na⁺ and 0.5 mM K⁺ (Control, open boxes), 50 mM K⁺ (with 0.5 mM Na⁺; filled circles), and 50 mM Na⁺ (with 0.5 mM K⁺; gray circles). V_m, membrane voltage; I_m, membrane current. (E) Mean chord conductances (g_i) ± SE of AeKir1 oocytes (N = 8) in the solutions and at the membrane potentials (V_m) indicated. Corresponding mean values in H₂O-injected oocytes (N = 8) are subtracted. (F) Same as in 'E' but for AeKir2B oocytes (N = 9). Corresponding mean values in H₂O-injected oocytes (N = 5) are subtracted.

Italicized letters indicate statistical categorization of the means within a V_m using a one-way repeated-measures ANOVA with Newman–Keuls posttest; $P < 0.05$).

Fig. 6.

Effects of extracellular barium on the I - V relationships of *AeKir1* oocytes. I - V plots of representative *AeKir1* (A) and *AeKir2B* (B) oocytes bathed consecutively in the following solutions for 2 min each: 1) 0.5 mM K^+ (open boxes), 2) 50 mM K^+ (filled circles, solid line), 3) 50 mM K^+ + 1 mM Ba^{2+} (gray circles, dotted line), and 4) 50 mM K^+ (open circles, dashed line). V_m , membrane voltage; I_m , membrane current. (C) Mean chord conductances (g_i) $\pm$ SE of *AeKir1* oocytes ($N=8$) in the solutions and at the membrane potential (V_m) indicated. Corresponding mean values in H_2O -injected oocytes ($N=6$) are subtracted. (D) Same as in 'C', but for *AeKir2B* oocytes ($N=11$). Corresponding mean values in H_2O -injected oocytes ($N=5$) are subtracted. Italicized letters indicate statistical categorization of the means (one-way repeated-measures ANOVA with Newman-Keuls posttest; $P < 0.05$).

Fig. 7.

Effects of a chronic increase of extracellular Na⁺ on the I - V relationships of *AeKir1* and *AeKir2B* oocytes. Shown are I - V plots of representative *AeKir1* (A) and *AeKir2B* (B) oocytes bathed consecutively in the following solutions: 1) 0.5 mM Na⁺, 5 mM K⁺ (open boxes; 0 min), 2) 93 mM Na⁺, 50 mM K⁺ (light gray, dark gray, and filled circles; 5 min, 10 min, and 15 min, respectively), 3) 0.5 mM Na⁺, 5 mM K⁺ (light gray boxes; 30 min). V_m , membrane voltage; I_m , membrane current. (C) Mean chord conductances $\pm$ SE of *AeKir1* oocytes ($N=6$) at the times and membrane potential (V_m) indicated. Concentrations of extracellular Na⁺ in mM, [Na⁺]_o, are indicated within the shaded bars. Corresponding mean values in H₂O-injected oocytes ($N=8$) are subtracted. (D) Same as in 'C' but for *AeKir2B* oocytes ($N=7$). Corresponding mean values in H₂O-injected oocytes ($N=5$) are subtracted. Italicized letters indicate statistical categorization of the means (one-way repeated-measures ANOVA with Newman-Keuls posttest; $P < 0.05$).

Fig. 8.

Monovalent cation selectivity of the AeKir1 and AeKir2B channels. (A) Representative trace of membrane current (I_m) in an AeKir1 oocyte clamped continuously at -92 mV. The concentration of a particular cation is increased to 5 mM at the times indicated by the horizontal bars; otherwise the oocytes are superfused with the control solution (0.5 mM Na^+ and 0.5 mM K^+). (B) Representative trace of I_m in an AeKir2B oocyte clamped continuously at -93 mV. The concentration of a particular cation is increased to 10 mM at the times indicated by the horizontal bars; otherwise the oocytes are superfused with the control solution. (C) Mean change to I_m (ΔI_m) $\pm$ SE in AeKir1 oocytes ($N=8$) in response to adding each monovalent cation. The respective mean ΔI_m values from H_2O -injected oocytes ($N=4$) are subtracted. (D) Same as in 'C' but for AeKir2B oocytes ($N=6$). The respective mean ΔI_m values from H_2O -injected oocytes ($N=5$) are subtracted. *n.s.*, indicates not significantly different from zero as determined by a one sample *t*-test ($P < 0.05$). Italicized letters indicate statistical categorization of the non-zero ΔI_m means (one-way repeated-measures ANOVA with Newman-Keuls posttest; $P < 0.05$).

Fig. 9.

Cation permeability of the principal cell basolateral membrane. (A) Representative trace of the basolateral membrane potential (V_b) of a principal cell in an isolated *Aedes* Malpighian tubule. The arrow indicates the time of impalement. The gray-shaded periods indicate replacement of 30 mM of the extracellular Na^+ with one of the cations indicated. (B) Mean percent depolarization of $V_b \pm SE$ in response to the cation replacements indicated in 'A'. Number of principal cells (from as many Malpighian tubules) are in parentheses. Lowercase letters indicate statistical categorization of the means as determined by a one-way ANOVA and student *t* tests ($P < 0.01$).

Fig. 10.

Putative roles of Kir channels in Malpighian tubules of adult female mosquitoes stimulated by aedeskinin III (A) or dibutyl- α -cAMP, db-cAMP (B); the latter mimics the effects of the calcitonin-like peptide (Coast et al., 2005). Modified from Piermarini et al. (2011). See Section 4.3 for significance of items highlighted in red. Shown are known mechanisms for the uptake of ions: Cl⁻/HCO₃⁻ anion exchanger, AE (Piermarini et al., 2010); inward-rectifying K⁺ channel, Kir (present study (Beyenbach and Masia, 2002; Scott et al., 2004); Na/H exchanger 3, NHE3 (Pullikuth et al., 2006); Na,K,2Cl cotransporter, NKCC (Hegarty et al., 1991; Scott et al., 2004); Na⁺-channel, NaC (Beyenbach and Masia, 2002; Sawyer and Beyenbach, 1985). Also shown are known and putative mechanisms for the secretion of ions: Cl⁻ channel, ClC (O'Connor and Beyenbach, 2001); Na/H antiporter 1, NHA1 (hypothesized (Xiang et al., 2012); paracellular pathway (Pannabecker et al., 1993); V-type H⁺-ATPase (Weng et al., 2003). The basolateral membrane voltage in 'A' is from Schepel et al. (2010), whereas the apical membrane voltage in 'A' is an estimate based on the assumption that aedeskinin depolarizes the transepithelial potential to a similar degree (i.e., to +6 mV) as leucokinin (Pannabecker et al., 1993). The membrane voltages and transepithelial potential in 'B' are from Sawyer and Beyenbach (1985). The ionic compositions of the luminal fluids in 'A' and 'B' are from Schepel et al. (2010) and Williams and Beyenbach (1983), respectively.

Table 1
Chemical compositions (in mM) of solutions used in *Xenopus* oocyte electrophysiology experiments.

Solution #	I	II	III	IV	V	VI	VII	VIII	IX
NaCl	96	0.5	0.5	50	0.5	93	0.5	90	0.5
NMDG-Cl	0	97.5	48	48	93	0.5	90	0.5	10
NMDG-Gluc	0	0	0	0	0	0	0	0	87.5
KCl	2	0.5	50	0.5	5	5	8	8	0.5
MgCl ₂	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CaCl ₂	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
HEPES	5	5	5	5	5	5	5	5	5

The pH of all solutions was adjusted to 7.5 with NMDG-OH.
The osmolality of each solution was verified to be 190 m Osm kg⁻¹ H₂O (±5 mOsm kg⁻¹ H₂O) by vapor pressure osmometry.
Gluc = gluconate; NMDG =methyl-D-glucammonium.

Table 2Genes encoding putative subunits of Kir channels in *Aedes aegypti*.

VectorBase accession number ^a	Designation in present study	Closest <i>Drosophila</i> ortholog
AAEL008932	<i>AeKir1</i>	<i>DrKir1 (ir)</i>
AAEL008928	<i>AeKir2A</i>	<i>DrKir2 (irk2)</i>
AAEL008931	<i>AeKir2B</i>	<i>DrKir2 (irk2)</i>
AAEL013373	<i>AeKir2B'</i>	<i>DrKir2 (irk2)</i>
AAEL001646	<i>AeKir3</i>	<i>DrKir3 (irk3)</i>

^a www.vectorbase.org.

Table 3Spontaneous V_m values of *Xenopus* oocytes bathed in solution I.

Oocytes (N)	Spontaneous V_m (mV)
AeKir1 (13)	-99.60 ± 0.46 ****
H ₂ O-injected (10)	-39.66 ± 2.17
AeKir2B (19)	-74.19 ± 1.27 ****
H ₂ O-injected (9)	-50.23 ± 2.17
AeKir3 (9)	-54.19 ± 2.49
H ₂ O-injected (7)	-57.64 ± 3.89

Values are means $\pm$ SE. N= number of oocytes.**** indicates significant difference ($P < 0.0001$) from corresponding H₂O-injected oocytes as determined by an unpaired *t*-test.