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# Inward rectifier potassium (Kir) channels in the soybean aphid *Aphis glycines*: Functional characterization, pharmacology, and toxicology

Peter M. Piermarini[a,*], Edna Alfaro Inocente[a], Nuris Acosta[a], Corey R. Hopkins[b], Jerod S. Denton[c], Andrew P. Michel[a]

[a] Department of Entomology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH, USA
[b] Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE, USA
[c] Departments of Anesthesiology and Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA

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ABSTRACT

Inward rectifier $K^+$ (Kir) channels contribute to a variety of physiological processes in insects and are emerging targets for insecticide development. Previous studies on insect Kir channels have primarily focused on dipteran species (e.g., mosquitoes, fruit flies). Here we identify and functionally characterize Kir channel subunits in a hemipteran insect, the soybean aphid *Aphis glycines*, which is an economically important insect pest and vector of soybeans. From the transcriptome and genome of *Ap. glycines* we identified two cDNAs, ApKir1 and ApKir2, encoding Kir subunits that were orthologs of insect Kir1 and Kir2, respectively. Notably, a gene encoding a Kir3 subunit was absent from the transcriptome and genome of *Ap. glycines*, similar to the pea aphid *Acyrthosiphon pisum*. Heterologous expression of ApKir1 and ApKir2 in *Xenopus laevis* oocytes enhanced $K^+$-currents in the plasma membrane; these currents were inhibited by barium and the small molecule VU041. Compared to ApKir2, ApKir1 mediated currents that were larger in magnitude, more sensitive to barium, and less inhibited by small molecule VU041. Moreover, ApKir1 exhibited stronger inward rectification compared to ApKir2. Topical application of VU041 in adult aphids resulted in dose-dependent mortality within 24 h that was more efficacious than flonicamid, an established insecticide also known to inhibit Kir channels. We conclude that despite the apparent loss of Kir3 genes in aphid evolution, Kir channels are important to aphid survival and represent a promising target for the development of new insecticides.

## 1. Introduction

Inward rectifier $K^+$ (Kir) channels conduct $K^+$-currents into cells at hyperpolarizing membrane potentials more readily than out of cells at depolarizing membrane potentials. The sustained activity of most Kir channels requires the binding of phosphatidylinositol 4,5-bisphosphate ($PIP_2$), and the activities of some Kir channels are modulated by a variety of cellular factors, including adenosine triphosphate, G proteins, and protein kinases (Hibino et al., 2010). Typically, the divalent cation barium blocks Kir channels by occluding the $K^+$-conduction pore (Hibino et al., 2010; Lu, 2004). In mammals, Kir channels contribute to a variety of physiological processes, including maintenance of cell resting-membrane potential, duration of action potential in excitable cells, transepithelial ion transport in renal tubules, and insulin secretion in the pancreas (Hibino et al., 2010). Our group and others have recently shown that Kir channels also play diverse physiological roles in insects, including renal excretion, feeding behavior, immune function, cardiac activity, neuromuscular function, reproduction, and development (Chen and Swale, 2018; Dahal et al., 2012; Eleftherianos et al., 2011; Evans et al., 2005; Kolosov et al., 2018; Piermarini et al., 2015; Raphemot et al., 2014a, 2013; Ren et al., 2018; Rouhier et al., 2014a; Swale et al., 2016, 2017; Wu et al., 2015).

On the molecular level, Kir channels are formed by the oligomerization of monomeric subunits into tetramers. In insects, genes from 3 distinct subfamilies encode Kir subunits: Kir1, Kir2, and Kir3. Most species possess at least one gene from each subfamily; the pea aphid (*Acyrthosiphon pisum*) is a notable exception and lacks a Kir3 gene (Dale et al., 2010). In some taxa, such as mosquitoes, Kir2 genes have diversified, leading to Kir2A and Kir2B subtypes (Piermarini et al., 2013; Raphemot et al., 2014a). Heterologous expression in *Xenopus* oocytes, S2 cells, or HEK-293 cells has revealed that Kir1 and Kir2 each form homomeric, barium-sensitive $K^+$-channels in the plasma membrane

* Corresponding author at: The Ohio State University, Ohio Agricultural Research and Development Center, Department of Entomology, 1680 Madison Avenue, 224 Thorne Hall, Wooster, OH 44691, USA.
*E-mail address:* piermarini.1@osu.edu (P.M. Piermarini).

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(Doring et al., 2002; Piermarini et al., 2013; Raphemot et al., 2014a, 2013; Ren et al., 2018; Swale et al., 2016). Consistent with broad physiological roles of insect Kir channels, expression of Kir1 and Kir2 mRNAs occurs throughout insect life stages and in a wide range of tissues, including the salivary glands, midgut, Malpighian (renal) tubules, and ovaries (Doring et al., 2002; Evans et al., 2005; Mamidala et al., 2013; Piermarini et al., 2013; Raphemot et al., 2014a; Ren et al., 2018; Rouhier and Piermarini, 2014; Swale et al., 2017; Yang et al., 2017). At least in Malpighian tubules of *Aedes aegypti* and *Trichoplusia ni*, immunoreactivities for Kir1 and/or Kir2 localize to the basolateral plasma membrane of epithelial cells involved with transepithelial ion transport (Kolosov et al., 2018; Piermarini et al., 2015).

In contrast to Kir1 and Kir2, the functional properties of insect Kir3 subunits remain enigmatic. Heterologous expression of Kir3 in *Xenopus* oocytes or S2 cells did not induce detectable $K^+$-channel activity (Doring et al., 2002; Piermarini et al., 2013). Kir3 mRNA expression has been shown to be highly enriched in Malpighian tubules (Doring et al., 2002; Evans et al., 2005; Mamidala et al., 2013; Piermarini et al., 2013; Raphemot et al., 2014a; Ren et al., 2018; Yang et al., 2017), and at least in Malpighian tubules of *Ae. aegypti*, Kir3 immunoreactivity occurred within intracellular compartments (e.g., vesicles) of the epithelial cells (Piermarini et al., 2015). However, knockdown of Kir3 mRNA in fruit flies and bed bugs did not produce detectable phenotypes (Mamidala et al., 2013; Wu et al., 2015).

Recently, we initiated the discovery of small-molecule inhibitors of insect Kir channels to facilitate the development of insecticides with novel mechanisms of action (Beyenbach et al., 2015; Raphemot et al., 2013, 2014b; Rouhier et al., 2014b; Swale et al., 2016). Focusing on Kir1 channels of *Ae. aegypti* and *Anopheles gambiae*, we developed high-throughput *in vitro* thallium-flux assays to screen thousands of compounds from the Vanderbilt University (VU) small molecule repository for inhibitors of Kir1 (Raphemot et al., 2013, 2014b; Rouhier et al., 2014b; Swale et al., 2016). Notably, three of the discovered inhibitors (VU573, VU590, VU625) were toxic to adult female *Ae. aegypti* within 24 h of hemolymph injection, and one (VU041) was toxic to adult female *Ae. aegypti* and *An. gambiae* within 24 h of topical application (Raphemot et al., 2013, 2014b; Rouhier et al., 2014b; Swale et al., 2016). Importantly, VU041 was of similar toxic potency against pyrethroid-susceptible and pyrethroid-resistant strains of mosquitoes, suggesting potential for Kir1 inhibitors to by-pass mechanisms of insecticide resistance (e.g., knock-down resistance mutations, enhanced CYP450 activity) (Swale et al., 2016). An independent group recently validated Kir1 as an insecticide target by demonstrating that flonicamid was a potent inhibitor of Kir1 channels from a hemipteran, *Nilaparvata lugens*, and not a chordotonal organ modulator as initially suspected (Ren et al., 2018). Flonicamid was toxic to *Ni. lugens*, and elicited similar toxicological and pathological effects in adult female mosquitoes as did our previously discovered VU small molecule inhibitors of Kir1 (Ren et al., 2018; Taylor-Wells et al., 2018).

With the exception of *Ni. lugens* (Ni) Kir1, functional characterization studies of insect Kir channels and their potential use as insecticide targets have been limited to dipteran species. The goals of the present study were to identify and functionally-characterize Kir channel subunit genes from the soybean aphid (*Aphis glycines*) and determine if small molecule inhibitors of Kir channels were toxic to adult *Ap. glycines*. The soybean aphid is an invasive species of North America and one of the most economically important agricultural pests of soybeans (Koch et al., 2016; Ragsdale et al., 2011). From recently developed transcriptomic and genomic resources for *Ap. glycines* (Bai et al., 2010; Wenger et al., 2017), we identified cDNAs encoding Kir1 and Kir2 subunits (ApKir1, ApKir2), but not Kir3. Heterologous expression in *Xenopus* oocytes revealed that ApKir1 mediated robust barium-inhibitable $K^+$-currents with strong inward rectification, whereas ApKir2 mediated relatively weak barium-inhibitable $K^+$ currents with weak inward rectification. In addition, the $K^+$-currents mediated by ApKir1 and ApKir2 were inhibited by VU041. Topical application of VU041 to adult *Ap. glycines* resulted in dose-dependent mortality within 24 h with better efficacy than the insecticide flonicamid. Our results provide the first functional characterization of aphid Kir channels and further proof of concept that small molecule inhibitors of insect Kir1 channels offer opportunities for the development of insecticides with novel mechanisms of action.

## 2. Materials and methods

### 2.1. Transcriptomic and genomic resources

The current project commenced before the publication of the *Ap. glycines* genome (Wenger et al., 2017). Thus, the cDNAs encoding putative Kir channel subunits were identified in a previously published transcriptome of *Ap. glycines* (Bai et al., 2010) using a basic local alignment search tool (BLAST) with the amino-acid sequences of representative *Ae. aegypti* Kir channel subunits as inquiries: AeKir1 (AAEL008932), AeKir2B (AAEL008931), and AeKir3 (AAEL001646). Following availability of the *Ap. glycines* genome, the two identified cDNA contigs (contig44370, contig42931) were submitted as queries in a BLAST for sequence confirmation and to identify any additional paralogs not present in the transcriptome.

### 2.2. Phylogenetic analysis and sequence alignment

Amino-acid sequences of Kir channel subunits from representative hemipteran and dipteran insects, humans, and a poriferan (*Amphimedon queenslandica*) were acquired from NCBI (Supplemental Table 1) and imported into *MEGA* version 6 for phylogenetic analysis (Tamura et al., 2013). Within *MEGA*, we aligned the sequences using ClustalW and constructed a bootstrap consensus maximum-likelihood phylogenetic tree. The following parameters were used for tree construction: 1000 bootstrap replicates, Jones-Taylor-Thornton model, uniform rates among sites, gaps/missing data deleted completely, nearest-neighbor-interchange ML heuristic method, very strong branch swap filter, and branch cutoff bootstrap score = 70. The amino acid sequence alignment was visualized and shaded with BioEdit Sequence Alignment software version 7 (Hall, 1999). We used a similarity matrix (BLOSUM62) within BioEdit to determine the shading.

### 2.3. Small molecules

VU041 and VU937 were synthesized as described previously (Swale et al., 2016). Flonicamid was purchased from Sigma-Aldrich (St. Louis, MO). For electrophysiology experiments, VU041 and VU937 were first dissolved in dimethylsulfoxide (DMSO) at 100 mM, and then diluted in buffer *III* (Table 1) to 25 μM (0.025% DMSO). For toxicology experiments, VU041 was dissolved in 100% acetone at various concentrations to deliver the doses indicated.

**Table 1**
Chemical compositions (in mM) of buffers used in two-electrode voltage clamping experiments of *Xenopus* oocytes. The pH of all solutions was adjusted to 7.5 with N-methyl-D-glucammonium (NMDG)-OH. A vapor pressure osmometer was used to verify that the osmolality of each buffer was 195 ± 5 mOsm/kg $H_2O$.

| Buffer # | *I* | *II* | *III* |
|---|---|---|---|
| NaCl | 96 | 73 | 73 |
| NMDG-Cl | 0 | 24.5 | 0 |
| KCl | 2 | 0.5 | 25 |
| $MgCl_2$ | 1.0 | 1.0 | 1.0 |
| $CaCl_2$ | 1.8 | 1.8 | 1.8 |
| HEPES | 5 | 5 | 5 |