Insect Biochemistry and Molecular Biology 48 (2014) 91–99

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Identification of life-stage and tissue-specific splice variants of an inward rectifying potassium (Kir) channel in the yellow fever mosquito *Aedes aegypti*

Matthew F. Rouhier, Peter M. Piermarini*

Department of Entomology, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691, USA

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form
5 March 2014
Accepted 6 March 2014

Keywords:
Alternative splicing
Molecular cloning
Anopheles gambiae
Drosophila melanogaster

ABSTRACT

Inward-rectifier potassium (Kir) channels play key roles in nerve, muscle, and epithelial cells in mammals, but their physiological roles in insects remain to be determined. The yellow fever mosquito (*Aedes aegypti*) possesses five different genes encoding Kir channel subunits: Kir1, Kir2A, Kir2B, Kir2B′, and Kir3. We have recently cloned and characterized the Kir1, Kir2B, and Kir3 cDNAs in the renal (Malpighian) tubules of adult female *Ae. aegypti*. Here we characterize the expression of the Kir2A gene in *Ae. aegypti*, which was not abundantly expressed in Malpighian tubules. We find that the 1) Kir2A gene is expressed primarily in the midgut and hindgut of adult female mosquitoes, and 2) Kir2A mRNAs are alternatively spliced into three distinct variants (Kir2A-a, -b, and -c). The deduced Kir2A proteins from these splice forms share a completely conserved transmembrane domain (a pore-forming domain flanked by two transmembrane-spanning segments), but possess novel NH_2-terminal and/or COOH-terminal domains. Semi-quantitative RT-PCR analyses indicate that the splice variants exhibit both developmental- and tissue-specific expression. Lastly, we provide insights into the conservation of alternative splicing among the Kir2A genes of dipterans, which may add molecular diversity that compensates for the relatively limited number of Kir channel genes in insects compared to mammals.

1. Introduction

The inward-rectifier potassium (Kir) channels are distinguished from other K^+-channels by their ability to mediate inward K^+ currents at hyperpolarizing membrane voltages more readily than outward K^+ currents at depolarizing membrane voltages. Knowledge concerning the structure and function of Kir channels has been principally derived from vertebrate animals, where Kir channels play important physiological roles in nerve, muscle, endocrine, and epithelial tissues (Hibino et al., 2010). The functional channels are tetrameric, consisting of 4 monomeric Kir subunits. The tetrameric channels can be formed by a single type of monomeric Kir subunit (homomeric) or by two or more different Kir subunits (heteromeric). In mammals, 16 genes encode Kir subunits, thereby resulting in a wide molecular diversity of homomeric and heteromeric Kir channels.

Over more than the past decade, a limited number of studies have begun to provide insights into the Kir channels of insects, namely those of the fruit fly (*Drosophila melanogaster*) (Döring et al., 2002; Evans et al., 2005; Eleftherianos et al., 2011; Dahal et al., 2012), bed bug (*Cimex lectularius*) (Mamidala et al., 2013), and yellow fever mosquito (*Aedes aegypti*) (Piermarini et al., 2013; Raphemot et al., 2013). Insects possess 3 distinct types of genes encoding Kir channel subunits (Kir1, Kir2, and Kir3), and in some cases (e.g., mosquitoes) the Kir2 gene has diversified into further subtypes (e.g., Kir2A, Kir2B). Functional characterization experiments by our laboratory and others have revealed that the insect Kir1 and/or Kir2 genes each encode subunits that form homomeric, barium-sensitive K^+-channels that are constitutively-active when expressed heterologously in *Xenopus* oocytes (Piermarini et al., 2013), HEK-293 cells (Raphemot et al., 2013) and/or S2 cells (Döring et al., 2002). In contrast, the insect Kir3 gene encodes a subunit that does not form functional homomeric channels (Piermarini et al., 2013; Döring et al., 2002).

Our laboratory has focused on elucidating the molecular physiology of Kir channels expressed in the renal (Malpighian) tubules of adult female mosquitoes (*Ae. aegypti*). In particular, we have demonstrated that the Malpighian tubules of *Ae. aegypti* primarily

* Corresponding author. The Ohio State University, Ohio Agricultural Research and Development Center, Department of Entomology, 1680 Madison Avenue, 224 Thorne Hall, Wooster, OH 44691, USA. Tel.: +1 330 263 3641.
E-mail addresses: piermarini.1@osu.edu, pmpierma@yahoo.com (P.M. Piermarini).

http://dx.doi.org/10.1016/j.ibmb.2014.03.003
0965-1748/

express three genes encoding Kir channel subunits (*Ae*Kir1, *Ae*Kir2B, and *Ae*Kir3) (Piermarini et al., 2013). Moreover, we have demonstrated that the injection of a small molecule inhibitor of *Ae*Kir1 (VU573) into mosquitoes incapacitates them within 24 h; the molecule inhibits the production of urine by Malpighian tubules and disrupts hemolymph K^+-homeostasis (Raphemot et al., 2013). Thus, Kir channels appear to play vital physiological roles in mosquitoes and may offer valuable molecular targets for the development of new mosquitocides.

One Kir channel gene that has not received much investigative attention in mosquitoes is Kir2A; this gene is not expressed abundantly in Malpighian tubules of adult females (Piermarini et al., 2013). In *D. melanogaster*, transcripts of the closest ortholog of mosquito Kir2A (i.e., *Dr*Kir2) are alternatively spliced, resulting in predicted proteins with novel NH_2-terminal and/or COOH-terminal domains (Evans et al., 2005; Döring et al., 2002). The goal of the present study is to characterize the molecular expression of the Kir2A gene in the mosquito *Ae. aegypti* and test the hypothesis that transcripts of this gene exhibit splice variation as occurs in *Dr*Kir2. We show that the *Ae*Kir2A gene is expressed primarily in the midgut and hindgut of adult female mosquitoes, and that the *Ae*Kir2A transcripts are alternatively spliced at the 5′ and/or 3′ ends, resulting in three distinct mRNAs (i.e., *Ae*Kir2A-a, -b, and -c). Semi-quantitative RT-PCR analyses indicate that the splice forms exhibit both developmental- and tissue-specific expression. Lastly, we provide insights into the conservation of alternative splicing among the Kir2A genes of dipterans.

2. Methods and materials

2.1. Mosquitoes and tissue isolation

Mosquitoes (*Ae. aegypti*) were raised in the laboratory as described previously (Piermarini et al., 2011). Larvae (4th instar), pupae, and adult females (3–7 days old) were anesthetized on ice, snap frozen in liquid N_2, and stored at −80 °C. For tissue isolations, adult 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 midgut, hindgut, and five Malpighian tubules were carefully separated 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 amounts of tissues were collected, the tube was snap frozen in liquid N_2 and stored at −80 °C. The mosquito Ringer solution contained the following in mM: 150 NaCl, 3.4 KCl, 1.7 $CaCl_2$, 1.8 $NaHCO_3$, 1.0 $MgSO_4$, 5 glucose and 25 HEPES (pH 7.1).

2.2. Cloning of Ae. aegypti Kir2A splice variants

As described in previous studies (Piermarini et al., 2010, 2011, 2013), 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 RNA of each of the following: midgut, hindgut, and whole insect (derived from ~30 females midguts, ~50 female hindguts, and 3 whole females, respectively). 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 *Ae*Kir2A gene (AAEL008928: 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* (Zymo Research) as described previously (Piermarini et al., 2010, 2011, 2013). Plasmid DNA from the resulting *E. coli* colonies was sequenced at the Molecular and Cellular Imaging Center of the Ohio State University Ohio Agricultural Research and Development Center (Wooster, OH).

Once the RACE products were sequenced, primers were designed to the respective 5′- and 3′-ends of the three putative full-length cDNAs identified: *Ae*Kir2A-a, *Ae*Kir2A-b, and *Ae*Kir2A-c (Supplemental Table 1). The full-length cDNAs of the *Ae*Kir2A-a and *Ae*Kir2A-b splice forms were both amplified from hindgut. The full-length cDNA of the *Ae*Kir2A-c splice form was amplified from midgut. All three full-length cDNAs were also amplified from whole insect. A consensus sequence for each cloned *Ae*Kir2A cDNA was generated based on the DNA sequences of the 5′-RACE, 3′-RACE, and full-length PCR products. The consensus sequences of *Ae*KirA-a, *Ae*Kir2A-b, and *Ae*Kir2A-c were based on 19 clones (12 RACE products, 7 full-length), 13 clones (9 RACE products, 4 full-length), and 12 clones (8 RACE products, 4 full-length), respectively. The consensus sequences were submitted to GenBank and assigned the following accession numbers: *Ae*Kir2A-a = KF686744; *Ae*Kir2A-b = KF686745; and *Ae*Kir2A-c = KF686746.

2.3. Qualitative and semi-quantitative RT-PCR

As described in a previous study (Piermarini et al., 2013), qualitative RT-PCR was used to assess the expression of the *Ae. aegypti* Kir channel subunits in the different tissues of adult female mosquitoes. In brief, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) according to manufacturers' instructions. Next, cDNA was synthesized from 1 μg of total RNA using the GoScript Reverse Transcriptase System (Promega, Madison, WI) with random hexamers.

Primer pairs for RT-PCR 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 *Ae*Kir1, *Ae*Kir2A, *Ae*Kir2B′, *Ae*Kir2B, and *Ae*Kir3 cDNAs and a 300 bp region of the cDNA encoding ribosomal protein S7 gene (RPS7; VectorBase accession #AAEL009496) in a single PCR (Supplemental Table 1). 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 Mg^{2+} concentration as described by Marone et al. (2001).

Reactions of 50 μl were assembled for each *Ae*Kir gene that included the following: 1) 0.5 μl of cDNA, 2) 1 μl of an *Ae*Kir gene-specific primer pair (5 μM each primer), 3) 1 μl of the RPS7 primer pair (5 μM each primer), 4) a final concentration of 5 mM $MgCl_2$, 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.

Semi-quantitative RT-PCR was used to compare the expression of *Ae*Kir2A among different mosquito life stages, following the