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Molecular mechanisms of bi-directional ion transport in the Malpighian tubules of a lepidopteran crop pest, *Trichoplusia ni*

Dennis Kolosov[a,*], Maria Tauqir[a], Sabitha Rajaruban[a], Peter M. Piermarini[b], Andrew Donini[c], Michael J O'Donnell[a]

[a] McMaster University, Department of Biology, Hamilton, ON, Canada
[b] The Ohio State University, Ohio Agricultural Research and Development Center, Department of Entomology, Wooster, OH, USA
[c] York University, Department of Biology, Toronto, ON, Canada

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ABSTRACT

Classical studies have described in detail the complex and regionalized morphology of the Malpighian tubule (MT) in larval Lepidoptera. Recent studies revealed unusual aspects of ion transport in the Malpighian tubules of the larva of the cabbage looper, *Trichoplusia ni*. These included: cation reabsorption via secondary cells (SC); coupling of SCs to neighbouring PCs via gap junctions to enable reabsorption; and a reversal from cation secretion to reabsorption by the principal cells in the distal ileac plexus region of the in situ tubule in response to dietary ion loading. The current paper aimed to identify molecular components of ion transport in the MTs of *T. ni* and to describe their role in the recently reported reversal of ion transport in ion-loaded animals. Using a combination of molecular, immunohistochemical and electrophysiological techniques, we assigned roles to Na^+/K^+-ATPase (NKA), V-type H^+-ATPase (VA), $Na^+/K^+/Cl^-$ co-transporter (NKCC), K^+/Cl^- co-transporter (KCC), inward-rectifying K^+ channel (Kir), and Na^+/H^+ exchangers (NHE)-7 and -8 in the transport of Na^+ and K^+ by the distal ileac plexus of *T. ni*. The yellow region of the tubule lacked all of the above except VA, and the white region lacked all of the above transporters but expressed an amiloride-sensitive Na^+ channel (NaC). Overall, the ion transport machinery in the distal ileac plexus of the *T. ni* tubule shows remarkable similarity to that in tubules of other groups of insects, yet this region transports ions very differently. Shutdown of secretory ATPases and utilisation of the same molecular machinery in the face of changing ion gradients may enable ion transport reversal in lepidopteran MTs. We propose that gap junction-based coupling of the two cell types likely aids in toggling between ion secretion and ion reabsorption in this segment.

1. Introduction

The larvae of many lepidopterans, such as the tobacco hornworm *Manduca sexta* and the cabbage looper *Trichoplusia ni* (Gullan and Cranston, 2014), are important agricultural pests. Caterpillars of *T. ni* consume three times their own weight daily, feeding on a wide variety of cultivated plants and weeds (McEwen and Hervey, 1960). The larva faces a number of challenges in regulating levels of Na^+, K^+ and H^+ in the haemolymph. Large quantities of base are transported from the haemolymph into the anterior midgut. The extremely alkaline pH (10–12) which then develops in the larval midgut aids protein assimilation by favouring protein denaturation and dissociation of protein-tannin complexes in the plant diet (Onken and Moffett, 2017). Current models propose that K^+ and base are secreted into the anterior midgut, and then recovered in posterior regions of the gut; K^+ and base are then recycled by anterior movement, either through the haemolymph or by the Malpighian tubules, which are closely applied to the surface of the gut (Moffett, 1994). Additionally, maintenance of adequate Na^+ levels in the rapidly growing larva is complicated by the low levels of Na^+ in a plant-based diet. A further ionoregulatory challenge is the wide range of K^+ and Na^+ content and $[K^+]/[Na^+]$ ratio in plant species consumed by larval lepidopterans. Thus, changing dietary ion content can influence the overall ion homeostasis of the animal.

Excretion in terrestrial insects is accomplished by the combined actions of the Malpighian tubules and hindgut, which together form the functional kidney. Active ion transport from haemolymph into the tubule lumen drives fluid secretion by osmosis. The primary urine is then modified in downstream segments of the tubule or the hindgut; ions and much of the water are reabsorbed and metabolic wastes and toxins are transported into the lumen before the excreta leave the body (Wigglesworth, 1961; Bradley, 1985). In general, the ionomotive enzymes which have evolved to drive fluid secretion employ ions supplied

* Corresponding author.
E-mail address: kolosovd@mcmaster.ca (D. Kolosov).

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in abundance through the insect diet. Current models propose a primary role for a vacuolar-type H^+-ATPase (VA) pump coupled to Na^+/H^+ and/or K^+/H^+ exchangers to drive K^+ and/or Na^+ from cell to tubule lumen (Beyenbach and Wieczorek, 2006). Na^+/H^+ and/or K^+/H^+ exchangers (NHE and KHE) take protons transported into the lumen by apical H^+-ATPase and exchange them for Na^+/K^+, enabling secretion of Na^+/K^+. One advantage of this arrangement is that insects feeding on K^+-rich diets can secrete K^+-rich fluids. The Na^+/K^+ ATPase (NKA) which energizes ion transport in vertebrate epithelia has been assumed to play a less important role. However, studies have suggested that the significance of NKA has been underestimated because organic ion transporters in the tubule limit the effectiveness of the ATPase inhibitor ouabain (Torrie et al., 2004). The tubules are protected by an active ouabain transport system, co-localized with the NKA, which prevents ouabain from reaching inhibitory concentrations. The NKA may thus be as vital in insect tissues as in vertebrates, but can be cryptic to the drugs used to identify its presence.

Inward-rectifying potassium (Kir) channels are so named because the inward flow of K^+ into the cell at any given voltage is greater than the outflow at voltage of the same magnitude but opposing polarity (Nichols and Lopatin, 1997). A role for Kir channels in the movement of K^+ from haemolymph into Malpighian tubule cells has recently emerged in the yellow fever mosquito *Aedes aegypti* and the fruit fly *Drosophila melanogaster* (Evans et al., 2005; Beyenbach et al, 2015). *Aedes* expresses three Kir isoforms: Kir1 and Kir2B localize to the basolateral membranes of secondary and principal cells, respectively, while Kir3 is intracellular in both cell types (Piermarini et al., 2015). Together Kir1 and Kir2B are credited with conducting 66% of total transepithelial K^+ secretion in *Aedes* Malpighian tubules. In the secondary cell-containing distal ileac plexus (DIP, see Supplementary Fig. 2 for a diagram of lepidopteran Malpighian tubule regions) of *T. ni,* principal cells are characterised by secretion of K^+ into Malpighian tubule lumen and reabsorption of Na^+ (O'Donnell and Ruiz-Sanchez, 2015). However, in contrast to *Aedes* tubules, the secondary cells immediately adjacent to the surrounding principal cells reabsorb K^+ in the DIP of *T. ni*.

A recent report describes a reversal in the direction of K^+ transport in the DIP of K^+-fed larvae and demonstrates gap junctional coupling between the secondary cells and surrounding principal cells (Kolosov et al., 2018). An earlier study suggested a role for V-type H^+-ATPase, $Na^+/K^+/Cl^-$ co-transporter (NKCC) and amiloride-sensitive cation-proton exchangers in enabling ion and fluid secretion by the isolated MT of *T. ni* (Ruiz-Sanchez et al., 2015). The current study was designed to characterize the molecular ion transport machinery employed for secretion of Na^+ and K^+ by the DIP of *T. ni* and to describe the molecular basis for the previously reported reversal of Na^+ and K^+ transport in the DIP of ion-loaded animals.

2. Materials and methods

2.1. Experimental animals

Eggs of *Trichoplusia ni* (Hübner, 1800) were obtained from the Great Lakes Forestry Centre (Sault St. Marie, ON). Larvae were kept at 23–25 °C and 40–50% relative humidity and fed on synthetic diet (McMorran, 1965) containing 59 mM [K^+] and 18 mM [Na^+]. Feeding fifth instar larvae were used for all experiments. Larvae were dissected in lepidopteran saline (Maddrell and Gardiner, 1976) adjusted to pH 7.2 and containing (in mM); 15 NaCl, 30 KCl, 2 $CaCl_2$, 30 $MgCl_2$, 10 $KHCO_3$, 5 $KHPO_4$, 10 glucose, 10 maltose, 5 trisodium citrate, 10 glycine, 10 alanine, 10 proline, 10 glutamine, 10 valine, 5 serine, 5 histidine (O'Donnell and Ruiz-Sanchez, 2015).

2.2. K^+-rich and Na^+-rich diets

Diets enriched in K^+ (200 mM, 'High-K^+') or Na^+ (60 mM, 'High-Na^+') were prepared by adding KCl or NaCl, respectively, to the preparation prior to mixing and pouring into diet cups. A survey of crops that *Trichoplusia* feed on confirmed that these ion levels in the diet are physiologically relevant (source: https://ndb.nal.usda.gov/). Diets were thoroughly mixed and set into individual diet cups, allowed to solidify at room temperature, capped with sterilized lids and refrigerated until needed. Larvae were either reared from eggs on ion-rich diets (chronic) or were fed as 5th instars for 24 h (acute). Measurements on larvae fed ion-enriched diet were paired with observations of larvae from the same batch that were fed control diet (see Section 2.1).

2.3. Identification of ion transporters in Trichoplusia

Homologues of *Drosophila melanogaster* β-tubulin, *Aedes aegypti NKA* subunit α, *Aedes aegypti Kir*, *Manduca sexta* VA B subunit (*VAb*), *Manduca sexta nhe-7* and *-8*, *Anopheles gambiae* Na^+/H^+ antiporter 2 (*nha-2*), *Manduca sexta* bumetanide-sensitive *nkcc*, *Aedes aegypti* K^+/Cl^- co-transporter (*kcc*), *Bombyx mori* $Na^+/HCO3^-$ co-transporter (*nbc*) and *Drosophila melanogaster* amiloride-sensitive Na^+ channel (*NaC*) were identified in *T. ni* using NCBI databases. Newly identified sequences were confirmed to be protein-encoding using a BLASTχ search. A reading frame was established using nBLAST alignment and ExPaSy Translate Tool (http://web.expasy.org/translate/). Primers were designed based on the predicted protein-coding regions using Primer3 software (v. 0.4.0). Putative transcript fragments were amplified using reverse transcriptase PCR (RT-PCR) (see below). Amplicon size and identity were verified with agarose gel electrophoresis (see below) and sequencing of purified and isolated PCR samples using a PureLink PCR extraction kit (cat# K220001, ThermoFisher Scientific, Burlington, Canada).

2.4. RNA extraction

Discrete tissues were dissected from fifth instar larvae and total RNA was extracted using Trizol and manufacturer's instructions (e.g., Kolosov and Kelly, 2016; Kolosov et al., 2018). For tissue expression profiles using PCR, distinct portions of the tubule were dissected out from three caterpillars and pooled together so as to avoid missing a transcript based on its absence from a single larva.

2.5. cDNA synthesis

Quality and quantity of isolated RNA was assessed using a NanoDrop ND-1000 spectrophotometer and the A260/A280 ratio. 2 µg of RNA was taken from every sample and topped up to 8 µl with DEPC-treated water. RNA was then treated with DNase I (Amplifications Grade, ThermoFisher Scientific Canada, Inc.) and used for cDNA synthesis. First-strand cDNA was synthesized using SuperScript™ III reverse transcriptase and $Oligo(dT)_{12–18}$ primers (ThermoFisher Scientific, Burlington, Canada).

2.6. RT-PCR/qPCR and gel electrophoresis protocols

Presence of transcripts encoding ion transporters was determined by RT-PCR in the rectal complex, distal ileac plexus, proximal ileac plexus, and yellow and white regions of the Malpighian tubule. Transcript abundance changes in ion-loaded animals were performed using quantitative real-time PCR (qPCR) using EvaGreen 5x qPCR Mastermix (DiaMed Lab Supplies Inc, Mississauga, ON, Canada), a BioRad PCR machine for RT-PCR (PTC-2000; Bio-Rad Laboratories Canada Ltd) and Stratagene MX-3000P qPCR machine (San Diego, California, USA). Primer sets (Table 1) were used for PCR detection and qPCR quantification. The following reaction conditions were used: 1 cycle for denaturation (95 °C, 4 min), followed by 40 cycles of: denaturation (95 °C, 30 s), annealing (see Table 1, 30 s) and extension (72 °C, 30 s), with a final extension step (72 °C, 10 min). To ensure that a single PCR product