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RESEARCH ARTICLE

Malpighian tubules of *Trichoplusia ni*: recycling ions via gap junctions and switching between secretion and reabsorption of Na^+ and K^+ in the distal ileac plexus

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ABSTRACT

The functional kidney in insects consists of the Malpighian tubules and hindgut. Malpighian tubules secrete ions and fluid aiding in hydromineral homeostasis, acid–base balance and metabolic waste excretion. In many insects, including lepidopterans, the Malpighian tubule epithelium consists of principal cells (PCs) and secondary cells (SCs). The SCs in the Malpighian tubules of larvae of the lepidopteran *Trichoplusia ni* have been shown to reabsorb K^+, transporting it in a direction opposite to that in the neighbouring PCs that secrete K^+. One of the mechanisms that could enable such an arrangement is a gap junction (GJ)-based coupling of the two cell types. In the current study, we have immunolocalized GJ protein Innexin-2 to the PC–PC and SC–PC cell–cell borders. We have demonstrated that GJs in the SC-containing region of the Malpighian tubules enable Na^+ and K^+ reabsorption by the SCs. We also demonstrated that in ion-loaded animals, PCs switch from Na^+/K^+ secretion to reabsorption, resulting in an ion-transporting phenotype similar to that of tubules with pharmacologically blocked GJs. Concomitantly, mRNA abundance encoding GJ proteins was downregulated. Finally, we observed that such PC-based reabsorption was only present in the distal ileac plexus connected to the rectal complex. We propose that this plasticity in the PC function in the distal ileac plexus is likely to be aimed at providing an ion supply for the SC function in this segment of the tubule.

KEY WORDS: Malpighian tubule, Secondary cell, Ion transport, Gap junction

INTRODUCTION

Lepidopterans are a holometabolous group of plant-feeding insects, where larval and adult life stages undergo trophic partitioning, with separate feeding habits. Larval caterpillars of lepidopterans are voracious eaters and can consume up to four times their own weight in food every day (McEwen and Hervey, 1960). Increased dietary intake leads to an increased need for excretion of rapidly accumulating metabolic waste. In the Malpighian tubules (MT) of insects, metabolic waste excretion is coupled to fluid secretion (Wigglesworth, 1961; Bradley, 1985). In plant-feeding insects that ingest K^+-rich diets, fluid secretion relies primarily on the transport of K^+ by the epithelial cells in the secretory portion of the MT with a primary role for a vacuolar-type H^+-ATPase proton pump coupled to a K^+/H^+ exchanger to drive K^+ from the cell to the tubule lumen (Beyenbach and Wieczorek, 2006).

As shown in Fig. 1, larval lepidopterans demonstrate a so-called cryptonephric condition where the distal ends of the MTs are embedded into the rectal complex, juxtaposed to the rectal epithelium and bathed in fluid contained within the perinephric space which is separated from haemolymph by the enveloping perinephric membrane (Irvine, 1969; Ramsay, 1976; O'Donnell and Ruiz-Sanchez, 2015). Classic studies suggest that the primary function of the rectal complex in developing lepidopterans is to regulate the Na^+/K^+ content of their haemolymph when presented with rapid changes due to the start and cessation of feeding (Ramsay, 1976). Recently, the rate and direction of K^+ transport in the MTs of *Trichoplusia ni*, an important agricultural lepidopteran pest, have been shown to differ in larvae with full versus empty guts (O'Donnell and Ruiz-Sanchez, 2015). In this study, we set out to examine further how the ion content of the gut affects MT function by feeding and rearing larvae on ion-rich diets. Examination of the Na^+ and K^+ content of common crops that lepidopterans feed on indicates $[K^+]/[Na^+]$ ratios ranging from ∼7 to ∼70 (source: https://ndb.nal.usda.gov/). Thus lepidopteran pests like *T. ni* may face variations in Na^+ and K^+ intake depending on the plant they feed on.

The MT epithelium of insects consists of two secretory epithelial cell types. The principal cells (PCs) comprise the majority of the tubule and in dipterans (e.g. mosquitoes and flies) enable cation secretion, while the secondary cells (SCs) (also termed 'stellate' in dipterans because of their shape) secrete anions into the tubule lumen (O'Donnell et al., 1996). Although MT morphology and physiology is quite distinct in different orders of insects, PC–SC dichotomy in the MT epithelium is suggested to have evolved early on in insect evolution prior to the divergence of Endopterygota and Exopterygota (Halberg et al., 2015).

Previous studies using isolated tubule assays have implicated the distal ileac plexus (DIP) portion of lepidopteran MT in ion and fluid secretion (Irvine, 1969; Ramsay, 1976; Ruiz-Sanchez et al., 2015). However, PCs and SCs in the DIP were described to transport K^+ bidirectionally, i.e. K^+ is secreted by the PCs and reabsorbed by the neighbouring SCs (O'Donnell and Ruiz-Sanchez, 2015). This pattern is paradoxical as SCs of insects studied to date contribute to ion secretion (Dow, 2012). In mosquitoes, in particular, SCs play an important role in K^+ secretion (Piermarini et al., 2015). Presumably, reabsorption of ions by SCs would reduce total ion and fluid secretion by the DIP. The function and molecular mechanisms of ion reabsorption by SCs remain unknown. However, one of the mechanisms that could enable reabsorption would be coupling of the neighbouring PCs and SCs by gap junctions (GJs).

From *Hydra* to humans, GJs are intercellular channels that allow the passage of ions and small molecules between adjacent cells

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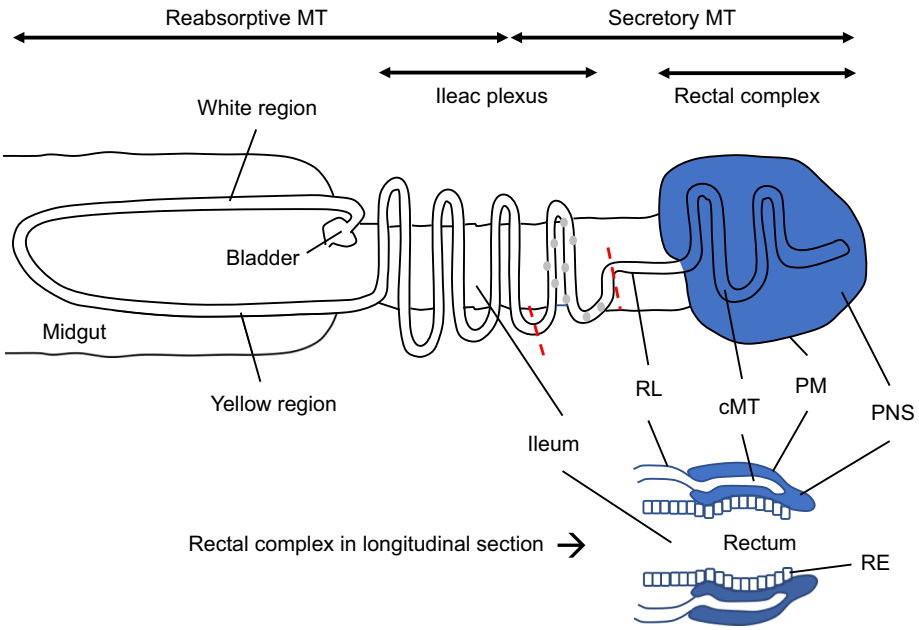

Fig. 1. Schematic representation of the cryptonephric condition in larval *Trichoplusia ni*. The distal end of each of the six Malpighian tubules, termed cryptonephridial tubule (cMT), is embedded into perinephric space (PNS), juxtaposed onto the underlying rectal epithelium (RE) and covered by the perinephric membrane (PM). The rectal lead (RL) connects each cMT to the downstream tubule region, which is applied to the ileum and termed the ileac plexus. The distal portion of the ileac plexus (delimited by dashed red lines) contains most secondary cells and comprises ~19% of the total length of the tubule. The proximal ileac plexus then terminates in the downstream yellow and white regions that are closely applied to the posterior midgut, prior to terminating into the urinary bladder at the midgut–hindgut juncture. Studies to date indicate that the cMT and DIP form the secretory portion of the MTs, while the rest of the ileac plexus together with yellow and white regions forms reabsorptive MT in *T. ni*. Grey circles in the DIP are used to denote the secondary cells. Dashed red lines are used to indicate the region used in isolated preparations.

(Phelan et al., 2008; Skerrett and Williams, 2016). The GJs of invertebrates are members of the Innexin family of proteins that are evolutionary homologues of vertebrate pannexins (Phelan et al., 2008). Coupling via GJ channels has been described in many animal tissues. In excitable tissues (e.g. muscle, neurons), such coupling allows for the transmission of signals from one cell to another within the tissues (Skerrett and Williams, 2016). However, the role of GJ coupling in ion-transporting epithelial tissues remains largely unexplored.

Innexin-based GJ channels are involved in many processes in invertebrates, including communication between cells, small molecule transfer, coordination of apoptotic events, and signal transduction synchronization in neurons (Luo and Turnbull, 2011). The role of GJ channels in the MTs of insects has emerged recently with emphasis on the MTs of dipterans. Weng and colleagues demonstrated that MTs of *Aedes aegypti* express *inx-1*, *inx-2*, *inx-3* and *inx-7* and that PCs exhibit significant coupling via GJs (Weng et al., 2008). Calkins and Piermarini later demonstrated that the GJ blocker carbenoxolone reduces the volume of excreted fluid in volume-loaded mosquitoes (Calkins and Piermarini, 2015). In addition, the same study demonstrated that transcriptional knockdown of *inx*s results in increased mortality of blood-feeding adults (Calkins and Piermarini, 2015). However, detailed examinations of the effects of GJ channel blockers on the ion transport physiology of insect MTs are lacking. Interestingly, carbenoxolone has been demonstrated to block GJ channels in a lepidopteran cell culture model, which means that it may be able to effectively block GJ channels in the MTs of *Trichoplusia* (Luo and Turnbull, 2011).

In this study, we present evidence for plasticity of rates and direction of ion transport in the DIP of *T. ni* in response to variations in dietary ion content. We also present molecular and physiological evidence supporting a role for PC–SC gap junctions in maintenance of Na^+ and K^+ secretion and reabsorption by the DIP region of the MT.

MATERIALS AND METHODS

Experimental animals

Eggs of *Trichoplusia ni* (Hübner 1800) were purchased biweekly from the Great Lakes Forestry Centre (Sault St Marie, Ontario, Canada). Larvae were maintained at ambient temperature and humidity on synthetic McMorran diet (McMorran, 1965) containing 59 mmol l^{-1} K^+ and 18 mmol l^{-1} Na^+. All experiments were performed in feeding fifth instar larvae that were dissected in physiological lepidopteran saline (originally described in Maddrell and Gardiner, 1976) adjusted to pH 7.2. The saline contained (mmol l^{-1}): 15 NaCl, 30 KCl, 2 $CaCl_2$, 30 $MgCl_2$, 10 $KHCO_3$, 5 $KHPO_4$, 10 glucose, 10 maltose, 5 sodium citrate, 10 glycine, 10 alanine, 10 proline, 10 glutamine, 10 valine, 5 serine and 5 histidine (O'Donnell and Ruiz-Sanchez, 2015).

K^+- and Na^+-rich diets

A diet enriched in K^+ (200 mmol l^{-1}) or Na^+ (60 mmol l^{-1}) was 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-enriched diets (chronic exposure) or were reared on a control diet and then fed as fifth instars for 24 h on ion-enriched diets (acute exposure). Measurements on larvae fed an ion-enriched diet were paired with observations of larvae from the same batch fed a control diet.

Scanning ion-selective electrode technique

Hardware, software and methodology for acquiring scanning ion-selective electrode technique (SIET) data and calculating ion fluxes have been described in detail in previous publications (Donini and O'Donnell, 2005; O'Donnell and Ruiz-Sanchez, 2015). SIET was used to measure ion fluxes for isolated DIPs and also for the DIP *in situ* (i.e. with intact connections upstream to the rectal lead and rectal complex, and downstream through the yellow and white segments and ureter to the gut). For measurements on isolated DIPs, feeding fifth instar larvae were dissected. Dissection for *in situ* measurements was carried out according to O'Donnell and Ruiz-Sanchez (2015). Briefly, the larva was pinned in a Sylgard dish and a longitudinal incision was made to expose the gut and overlying

MT. Tracheal connections of the gut to the larval exoskeleton were severed and the exoskeleton with underlying fat body removed. The isolated DIP was placed into Petri dishes pre-coated with poly-L-lysine to facilitate tissue adherence and filled with saline (for detailed procedure, see Naikkhwah and O'Donnell, 2011). The DIP was isolated by severing its connection to the rectal lead and the middle ileac plexus before mounting. Sampling rules were as follows: each PC and SC was sampled across its surface at 25 µm intervals to identify the location of maximal flux. Three values, the maximum and two values 25 µm either side of the maximum, were averaged for each cell. Five PCs and five SCs were scanned in each DIP and the values for DIPs from five larvae were averaged for each treatment.

Ramsay assay and measurement of [K^+]

The Ramsay assay on isolated DIP and [K^+] content of the secreted fluid was carried out as described previously (Ruiz-Sanchez et al., 2015) with several modifications. Briefly, the DIP was separated from tracheae and mounted into a 70 µl droplet of lepidopteran saline. Distal and proximal ends of the tubule were affixed to steel pins. Droplets that formed at the proximal end were collected at 15 min intervals; the first droplet was discarded. Collected droplets were placed at the bottom of a separate Petri dish filled with oil and diameters (d) were measured by an ocular micrometer. Fluid secretion rate (nl min^{-1}) was then calculated from droplet volume ($\pi d^3/6$) divided by 15. Droplet [K^+] was measured using K^+-selective microelectrodes prepared as described in Donini et al. (2008).

Gap junction channel blockers

Previously reported GJ channel blockers octanol (Pappas et al., 1996; Adler and Woodruff, 2000) and carbenoxolone (Luo and Turnbull, 2011; Spéder and Brand, 2014; Calkins and Piermarini, 2015) were applied at concentrations of 1 mmol l^{-1} and 100 µmol l^{-1}, respectively, to isolated DIP preparations. Octanol was dissolved directly in saline, while carbenoxolone was first dissolved in 100% ethanol and then added to saline to a final concentration of ethanol of 0.1%. Each DIP was first sampled with SIET (see above), the selected GJ blocker was added and the measurements were repeated after 10 min. Thus, the effect of each GJ blocker was based on paired observations. Preliminary experiments showed that ion fluxes were stable for >10 min and were unaffected by addition of saline or 0.1% ethanol. GJ blockers at concentrations used in this study did not affect the slopes or response time of Na^+- and K^+-selective electrodes.

Identification of innexins in *T. ni*

Homologues of *Drosophila* innexins (*inx-1*, *inx-2*, *inx-3* and *inx-7*) 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 *inx*-encoding regions using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). Putative *inx* 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 was carried out using a PureLink PCR extraction kit (catalogue number K220001, Thermo Fisher Scientific, Burlington, Canada).

RNA extraction

Total RNA was extracted using previously published protocols (e.g. Kolosov and Kelly, 2016). Briefly, DIP was separated from tracheae and excised from every MT of the fifth instar (control or dietary condition) and kept on ice immersed in RNAlater (Thermo Fisher Scientific). Six DIP samples originating from the same caterpillar were pooled together to constitute an N=1 for molecular analysis. Five caterpillars were dissected for all dietary conditions. Samples were immersed in RNAlater and kept refrigerated as per the manufacturer's instructions. The following morning, RNAlater was removed by aspiration and tissues were immersed in 0.5 ml Trizol (Thermo Fisher Scientific) per six tubules. Tissues were homogenized in Trizol using a 26G syringe needle and allowed to remain at room temperature for 5 min to dissociate nucleoprotein complexes. Molecular grade chloroform (100 µl) was then added to every sample. Tubes were vigorously shaken by hand and left at room temperature for 3 min. Samples were then spun at 12,000 ***g*** for 15 min at 4°C to force phase separation, after which the clear aqueous phase containing total RNA was collected into a set of new, sterile 1.5 ml Eppendorf tubes and kept on ice. Molecular grade isopropanol (250 µl) was added to every sample to precipitate RNA. Tubes were agitated gently and left at room temperature for 10 min, after which they were spun at 12,000 ***g*** for 10 min at 4°C to concentrate the precipitate into the pellet. Supernatant was removed from every tube by aspiration and pellets were washed in 75% molecular grade ethanol solution in diethylpyrocarbonate (DEPC)-treated water, and centrifuged at 7500 ***g*** for 5 min to immobilize pellets. Ethanol was then removed by aspiration and pellets were left to air-dry for 1 min at room temperature, after which they were dissolved in 12 µl of DEPC-treated water.

cDNA synthesis

Quality and quantity of isolated RNA was assessed using a NanoDrop ND-1000 spectrophotometer and the A_{260}/A_{280} ratio. Two micrograms of RNA was taken from every sample and topped up to 8 µl with DEPC-treated water. RNA was then treated with DNase I (Amplification Grade, Thermo Fisher Scientific) and used for cDNA synthesis. First-strand cDNA was synthesized using SuperScript III reverse transcriptase and Oligo$(dT)_{12–18}$ primers (Thermo Fisher Scientific).

RT-PCR/qPCR and gel electrophoresis protocols

Presence and abundance of transcripts encoding *inx-2*, *inx-3* and *inx-7* (*inx-1* could not be detected in the DIP) were determined by RT-PCR and quantitative real-time PCR (qPCR) using EvaGreen 5X qPCR master mix (DiaMed Lab Supplies, Mississauga, Ontario, Canada), a Bio-Rad PCR machine for RT-PCR (PTC-2000; Bio-Rad Laboratories, Canada) and Stratagene MX-3000P qPCR machine (Stratagene, San Diego, CA, USA). Primer sets (Table 1) were used

Table 1. Primer sets used for PCR and qPCR analysis of innexin mRNA expression and abundance

Transcript amplified	Forward primer	Reverse primer	PCR amplicon size (bp)	NCBI accession number
Tubulin (*tub*)	AGAAGTGGAGACGGGGGAAC	CGAAAACACAGATGAAACCTACTG	218	MG000943
Innexin-2 (*inx-2*)	CAGTGGGTGTGTTTTGTGTTATTC	GCGTAGAAGTTTTGGGTATGC	203	KC018473.1
Innexin-3 (*inx-3*)	ATGAACCAGGAACAACGAACAG	GATAAACCAGAACCACAGGAAGA	175	MG000944
Innexin-7 (*inx-7*)	ATGGCGTCTTTCTACTTTTACCG	CCCTACTCTACAACTCCTGTTTCC	359	MG000945

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for PCR detection and qPCR quantification. The following reaction conditions were used: one cycle for denaturation (95°C, 4 min), followed by 40 cycles of: denaturation (95°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 30 s), with a final extension step (72°C, 10 min). To ensure that a single PCR product was synthesized during reactions, a dissociation curve analysis was carried out after each qPCR run. Transcript abundance was normalized to that of *Trichoplusia* tubulin (*tub*). The use of *tub* for gene of interest normalization in dietary ion-loading studies was validated by statistically comparing *tub* threshold cycle values between tissues to confirm that no statistically significant changes occurred (P=0.842, one-way ANOVA).

Whole-mount immunohistochemistry procedures

Whole-mount immunohistochemistry (IHC) procedures have been described in detail by D'Silva et al. (2017) and Patrick et al. (2006). Briefly, larvae were dissected in lepidopteran saline and the DIP was removed and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C. Tissues were then rinsed in PBS and dehydrated (20% v/v, stepwise to 100%) and rehydrated in methanol/PBS series (100%→PBS). Tissues were then permeabilized and blocked in 0.1% Triton X-100 PBS solution (PBT), containing 2% bovine serum albumin w/v (BSA). After blocking, tissues were incubated with primary rabbit anti-BombyxINX-2 antibody (84% sequence identity between the epitope and Inx-2 sequence from *T. ni*) (Fushiki et al., 2010). The antibody was a generous gift from Dr Ryoichi Yoshimura, Kyoto Institute of Technology. The incubation took place overnight at 4°C at 1:100 dilution in PBT/1% BSA. An additional preparation was included with primary antibody omitted to act as negative control. Tissues were washed the following morning in PBT/1% BSA supplemented with normal goat serum three times for 15 min each with constant agitation to remove unbound primary antibody. Following washes, tissues were incubated with 1:1000 of secondary goat anti-rabbit TRITC-conjugated secondary antibody (Cedarlane, Burlington, Ontario, Canada) in the dark at room temperature for 2 h. Following incubation with secondary antibody, PBT/1% BSA wash was repeated three times for 15 min. Tubules were then mounted on slides using a transfer pipette. Slides were then blotted dry using KimWipes and preparations were mounted using ProLong Antifade with DAPI nuclear stain (Thermo Fisher Scientific) reagent under coverslips and left to cure in the dark. Images were obtained using a laser-scanning confocal Leica LSM700 microscope at York University imaging facilities.

Statistical analysis

Significant differences due to experimental treatment were determined using a Student's *t*-test in SigmaPlot (version 11; Systat, Inc., San Jose, CA, USA) statistical software. Statistical significance was based on the observation of a fiduciary P<0.05 limit.

RESULTS

Pharmacological evidence of GJ-based coupling between PCs and SCs

The addition of GJ blocker carbenoxolone to control saline affected transport of both K^+ and Na^+ via the principal and secondary cells. Firstly, carbenoxolone reduced reabsorption of K^+ and Na^+ via SCs in isolated MTs (see Fig. 2A,B; positive values indicate ion reabsorption from lumen into haemolymph). Carbenoxolone treatment simultaneously reversed secretion of K^+ and Na^+ via PCs (negative values in Fig. 2B,D) into reabsorption. Similar results were obtained with a second GJ blocker octanol (see Fig. S1). These results were obtained in isolated DIP preparations in order to avoid confounding effects of GJ blockage on the muscle and nervous tissues of the rectal complex. This ensured that if the tidal flow and expelling of the fluid from the rectal complex was affected, it would not contribute to the changes in ion transport observed in the downstream DIP.

Innexins in the DIP of MTs in *T. ni*

Transcripts encoding for *inx-2*, *inx-3* and *inx-7* were identified in the transcriptome of *Trichoplusia ni* (see Fig. S2 for phylogenetic

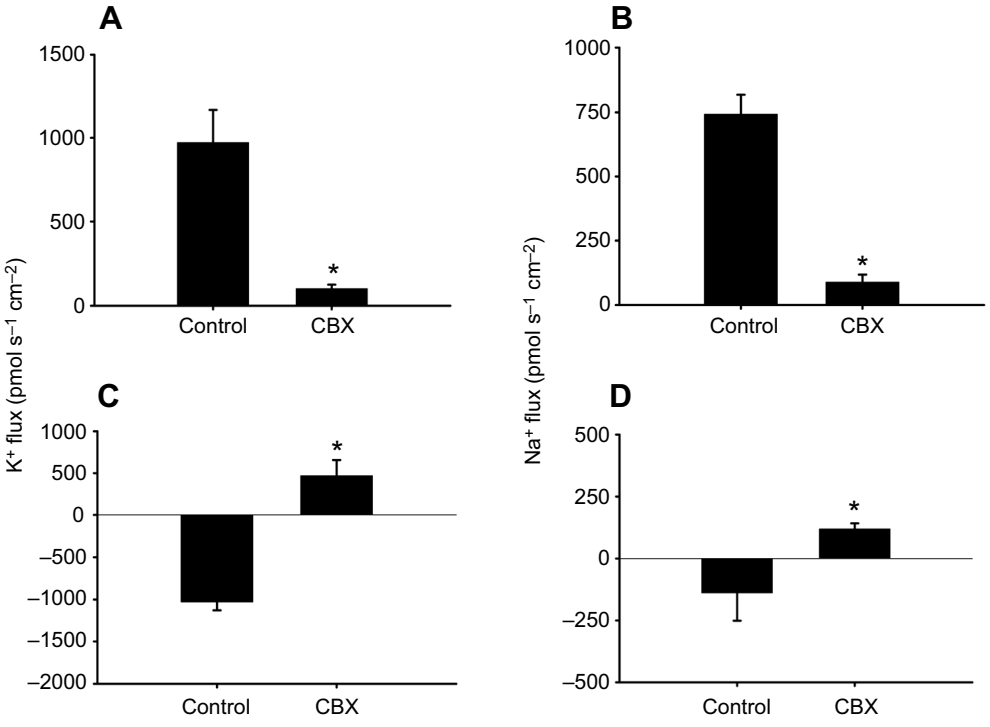

Fig. 2. The effect of GJ blocker carbenoxolone on transport of Na^+ and K^+ by the principal cells and secondary cells of the DIP in the MTs of *Trichoplusia ni*. Addition of 100 µmol l^{-1} of carbenoxolone (CBX) to the bathing saline decreased reabsorption of (A) K^+ and (B) Na^+ by the secondary cells and reversed secretion of (C) K^+ and (D) Na^+ by the principal cells into reabsorption. All data are means±s.e.m. (N=5). Asterisks denote significant difference from control treatment as determined by a Student's *t*-test (*P<0.05). Negative values indicate ion secretion and positive values indicate ion reabsorption.

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relationship with insect innexins). Transcript expression of *inx-2*, *inx-3* and *inx-7* were detected in the DIP of the larval cabbage looper *T. ni* (Fig. 3A). In order to investigate how mature (fifth instar) larvae deal with excess ions in the diet, they were exposed to ion-rich diets for 24 h ('acute exposure'). Alternatively, to determine how rearing on ion-rich diets has an impact on *inx* abundance in the MTs, larvae were raised from egg to fifth instar on the same diets ('chronic exposure'). For larvae acutely exposed to ion-rich diets, transcript abundance of *inx-2* and *inx-7* decreased under K^+-enriched diet conditions, and *inx-3* and *inx-7* mRNA abundance decreased with acute exposure to Na^+-enriched diet (Fig. 3B). For larvae chronically exposed to ion-rich diets during rearing (Fig. 3C), transcript abundance of *inx-3* decreased in the DIP of animals reared on K^+-enriched diet, while mRNA abundance encoding all three innexins decreased in animals reared on Na^+-rich diet.

Immunolocalization of Inx-2 was investigated because an antibody against lepidopteran Inx-2 was previously developed by the Yoshimura laboratory using an antigenic peptide with high sequence identity to that of *Trichoplusia ni* (Fushiki et al., 2010). Protein immunolocalization of Inx-2 was detected in the SCs and between neighbouring PCs and PCs bordering SCs (Fig. 4A). Neighbouring epithelial cells express Inx-2 at the cell–cell junction (Fig. 4C,D).

Ion transport direction switch in intact versus excised DIP

PCs switched from K^+ secretion to reabsorption in the intact preparations of larvae chronically exposed to K^+-rich diet (chronic; Fig. 5A). Intact DIP preparations retained both their connection through the rectal lead to the rectal complex upstream, and their connection through the proximal ileac plexus to the downstream yellow and white segments. However, PCs of DIP that were isolated from the rectal complex (by excising them as shown in Fig. 1) reverted back to K^+ secretion (Fig. 5A). In addition, whereas Na^+ reabsorption was evident in both PCs and SCs of *in situ* preparations

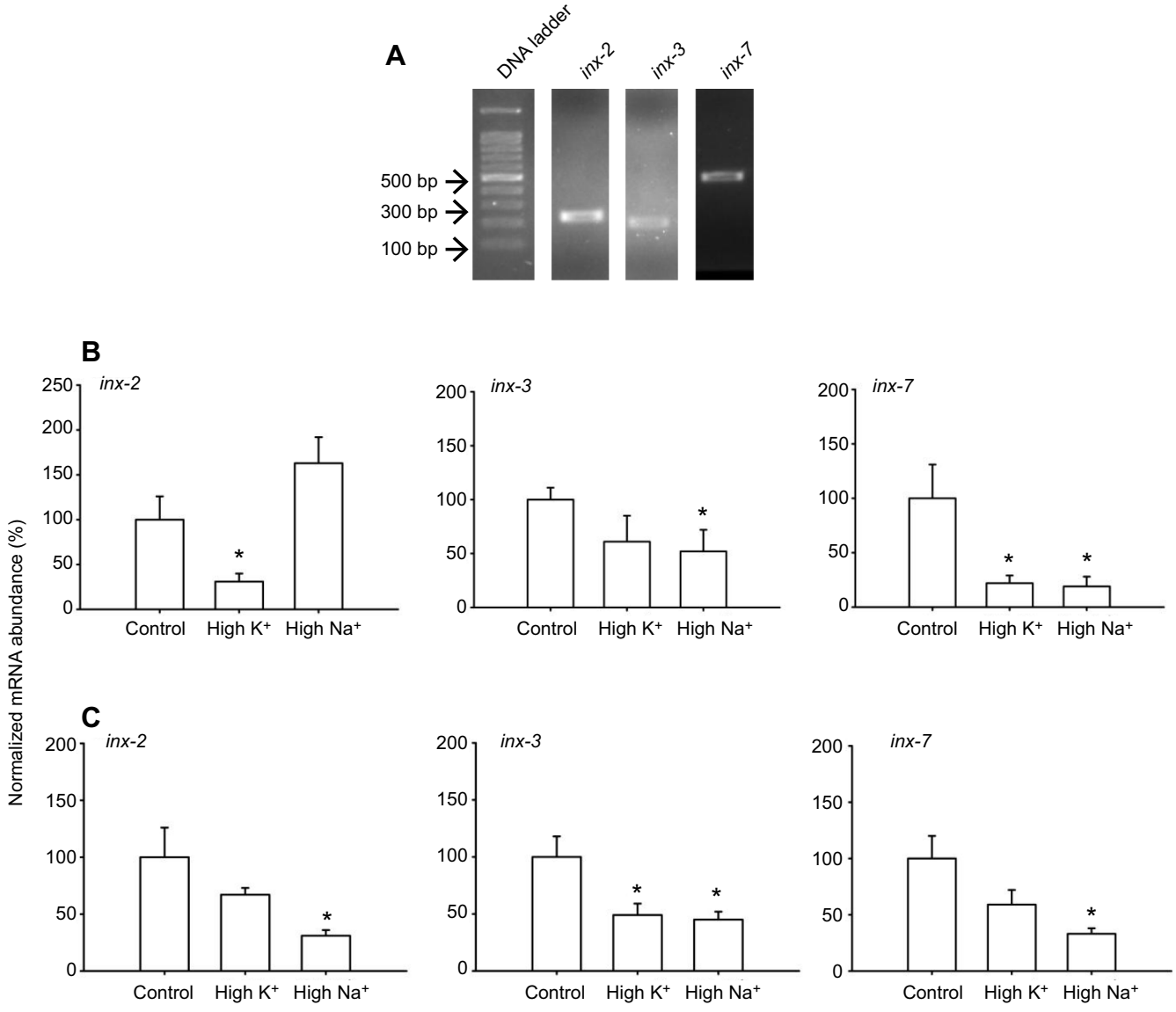

Fig. 3. The distal ileac plexus of *Trichoplusia ni* expresses transcripts for *inx-2*, *inx-3* and *inx-7*. Expression of *inx-1* was not detected. (A) RT-PCR detection of transcripts encoding *inx-2*, *inx-3* and *inx-7* in the DIP of *Trichoplusia ni*. DNA ladder is shown to denote the molecular weight of PCR amplicons, with weight indicated in base pairs (bp). (B) Transcript abundance changes for *inx-2*, *inx-3* and *inx-7* in response to acute exposure of fifth instar larvae to Na^+- or K^+-rich diet. (C) Transcript abundance changes for *inx-2*, *inx-3* and *inx-7* in response to chronic exposure of larvae (from egg to fifth instar) to Na^+- or K^+-rich diet. All data are means±s.e.m. (*N*=5). Asterisks denote significant differences from control treatment as determined by a Student's *t*-test (**P*<0.05).

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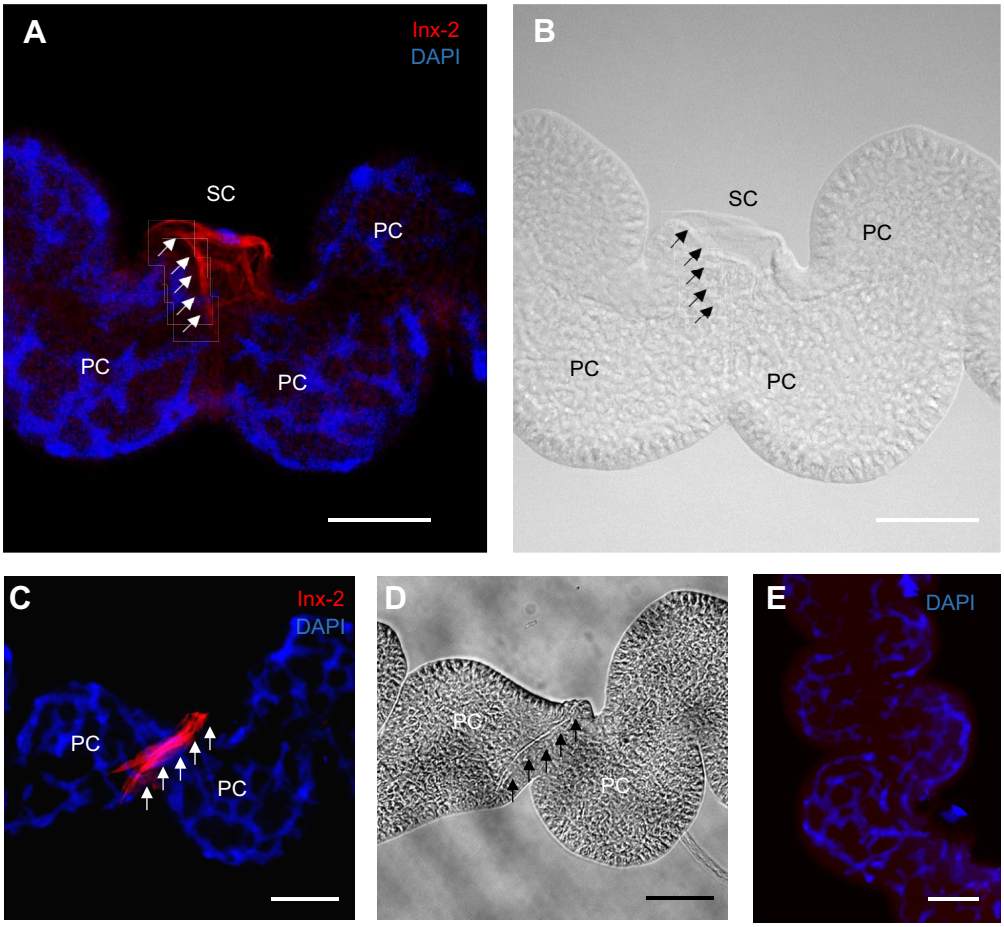

Fig. 4. Whole-mount immunohistochemistry showing expression of protein Inx-2 in the principal cells and secondary cells of the DIP of in the MTs of *Trichoplusia ni*. (A) Localization of Inx-2 in the area of cell–cell contact between adjacent principal and secondary cells (PC and SC, respectively). (C) Immunoreactivity between adjacent PCs, clearly demonstrating expression of Inx-2 in membranes of two adjacent PCs. Arrows in A–D denote cell–cell junctions. B and D show brightfield images of the areas shown in A and C, respectively. (E) Negative control preparation lacking primary antibody. Nuclear DAPI staining can be seen in blue in A, C and E. Scale bars, 100 µm.

and increased significantly with chronic enrichment in dietary Na^+, isolated tubules on control diet demonstrated Na^+ secretion by the PCs (Fig. 5B). As the reversal of Na^+ flux from secretion to reabsorption did not occur (compared with control-fed animals) for the PCs *in situ* in response to Na^+-rich diet, isolated preparation data were collected only from control-fed animals to demonstrate the reversal upon isolation. Interestingly, animals chronically exposed to diets enriched in Na^+ or K^+ demonstrated increased reabsorption of these ions through the SCs (Fig. 5A,B). Lastly, the *in situ* preparations of caterpillars fed K^+-enriched diet, which demonstrated K^+ reabsorption in the PCs, not only reverted back to secretion (SIET data; Fig. 5A), but when mounted for a Ramsay assay, K^+ content (Fig. 6A) in the secreted fluid as well as the fluid secretion rate (Fig. 6B) remained comparable to the corresponding values for preparations isolated from larvae reared on the control diet. Similar to above, as no reversal of Na^+ transport was noted in Na^+-fed animals, Ramsay assay data were not collected as isolated DIP has been reported to secrete Na^+ in a previous Ramsay assay study (see Ruiz-Sanchez et al., 2015) even though Na^+ was reabsorbed by both cell types of the DIP *in situ* using SIET (O'Donnell and Ruiz-Sanchez, 2015).

DISCUSSION

Overview and significance

Our data provide novel insights into three important aspects of MT ion transport in lepidopterans. Firstly, we have demonstrated that *in situ* the DIP reabsorbs Na^+ and K^+ under ion-rich conditions. Secondly, by combining Ramsay assay and SIET data, we have directly demonstrated that reabsorption of Na^+ and K^+ across PCs in ion-loaded animals takes place *in situ*, but not in isolated tubules (i.e. *in vitro*). Lastly, this study implicates GJs in the mechanism that enables SC-based ion reabsorption and promotes local ion recycling in the DIP. Interestingly, ion reabsorption by the SCs was not affected by any of the treatments (including excision of the DIP), except by gap junction blockers. We propose that the plasticity of the DIP in response to variations in dietary ion content enable SC function in response to changing hydromineral status. Our evidence that isolated and *in situ* segments of the MTs do not behave similarly must be taken into account in future studies.

Gap junctions facilitate ion reabsorption by secondary cells

The DIP of *T. ni* was found to express *inx*s and functionally rely on the GJs to supply ions for SC-based ion reabsorption (Fig. 2A,C). We used two previously reported GJ channel blockers: octanol (Pappas et al., 1996; Adler and Woodruff, 2000) and carbenoxolone (Luo and Turnbull, 2011; Spéder and Brand, 2014; Calkins and Piermarini, 2015) to pharmacologically impair GJ channels, which led to reduced Na^+/K^+ reabsorption through the SCs. Interestingly, PCs switch from Na^+/K^+ secretion to reabsorption when GJs are blocked. Artificial GJ blockage may lead to a shutdown of ion secretion by the PCs, thus avoiding intracellular accumulation of ions when ions cannot be transferred to the SCs. GJs in the MTs of *Aedes* have been reported to close naturally when ATP synthesis is interrupted with application of dinitrophenol, concomitant with basolateral K^+ channel closure, presumably protecting the intracellular milieu against metabolic stress (Weng et al., 2008). In addition, a GJ blocker has been reported to disrupt diuresis in mosquitoes (Calkins and Piermarini, 2015), consistent with the

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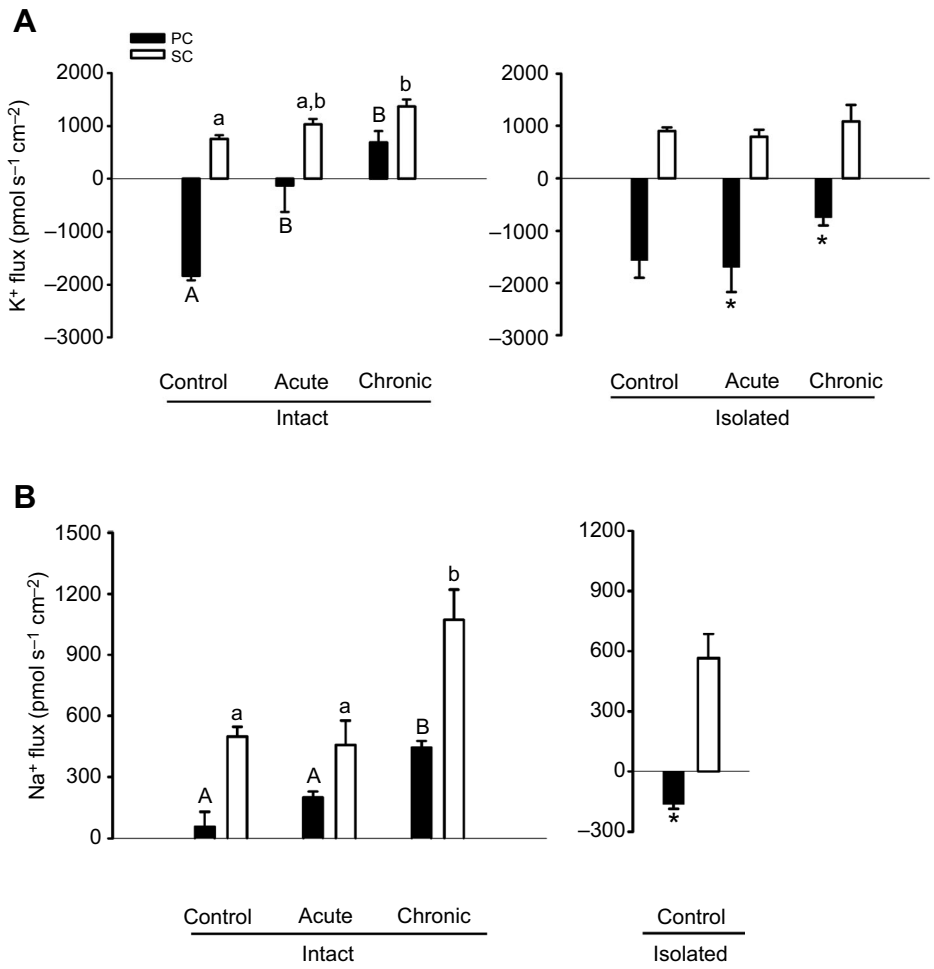

Fig. 5. The effects of ion-rich diet on the transport of Na^+ and K^+ in the DIP of MTs of larval *Trichoplusia ni*. Larvae were either exposed to (A) K^+-enriched and (B) Na^+-enriched diet for 24 h at the fifth instar stage (acute), or raised on these diets from eggs through to the fifth instar (chronic). *N*=5, data are means±s.e.m. Uppercase letters denote significant differences in ion flux by the PCs between dietary treatment groups. Lowercase letters denote significant differences in ion flux by the SCs as determined by a one-way ANOVA coupled with a Holm–Šídák *post hoc* test. Asterisks denote significant differences in ion flux owing to isolation of the tubule (compared with the *in situ* preparation on the left) as determined by Student's *t*-test (*P<0.05).

findings in the current study demonstrating that blocking GJs switches off PC-based secretion of Na^+ and K^+ (Fig. 2B,D). As in an isolated DIP there is no source of ions from the upstream cryptonephridial MT of the rectal complex, PCs switching off ion secretion resulted in decreased reabsorption by the SCs.

This GJ-based coupling between the neighbouring PCs and SCs may be aimed at local ion recycling, so that in an intact ileac plexus, ions reabsorbed by the SCs could be used in PC-based secretion again (proposed in Fig. 7A,B). An instance of K^+ recycling by neighbouring cell types using GJs has been documented in mammalian cochlea, where GJ channels between cochlear supporting and root cells allow for K^+ picked up from endolymph by the hair cells to be rerouted back into the endolymph (Kikuchi et al., 2000).

Ion-rich diet downplays GJ coupling and switches direction of ion transport

A salt-enriched diet has been shown to alter fluid and ion secretion (Naikkhwah and O'Donnell, 2011, 2012), and activate gene expression of ion transporters (Stergiopoulos et al., 2009) in MTs of insects. GJs clearly play a role in recycling of ions transported by PCs and SCs under regular dietary conditions, where recycling may be of benefit (see above). However, under K^+-rich diet conditions, where the *in situ* DIP reabsorbs K^+ through *both* PCs and SCs (Fig. 5A), our data show downregulation of *inx* mRNA abundance (Fig. 3C,D). Expression of Inx-2, Inx-3 and Inx-7 has previously been reported in epithelial tissues of invertebrates (Bauer et al., 2003; Phelan, 2005; Ostrowski et al., 2008; Weng et al., 2008; Beyenbach and Piermarini, 2011; Calkins and Piermarini, 2015). Inx-2 and Inx-3 are required for epithelial morphogenesis and function in *Drosophila* and *Caenorhabditis elegans*, respectively (Phelan, 2005). Inx-2 and Inx-3 have been studied in lepidopterans in relation to apoptosis (Liu et al., 2013) and development (Hong et al., 2009). Inx-2 is particularly important for the epithelial function of developing *Drosophila* embryos (Bauer et al., 2003). Inx-7 has been associated with the epithelial tissues of *Drosophila* and *Aedes* (Ostrowski et al., 2008; Calkins and Piermarini, 2015). Therefore, the importance of these Innexins in epithelial tissues of insects has been established to date. We suggest that the observed downregulation limits the supply of cations from the PCs into the SCs under the conditions of decreased necessity for PC-based ion supply in ion-loaded animals (see Fig. 7C).

In addition, there were observed differences in which *inx* mRNA was downregulated with acute and chronic exposure to an ion-rich diet. This is probably indicative of a regulatory network capable of adjusting GJ coupling between epithelial cells in the MT in response to both short-term variation in the dietary ion content (acute exposure), as well as prolonged differences in dietary ion content during rearing (chronic exposure). In mature larvae, acute exposure is likely to disturb ion transport of primary electrolytes and co-transport of coupled solutes. In contrast, in chronically exposed larvae, increased dietary ion content may influence positive ion and water balance needed for constant growth and haemolymph expansion (Jungreis et al., 1973; Reynolds and Bellward, 1989).

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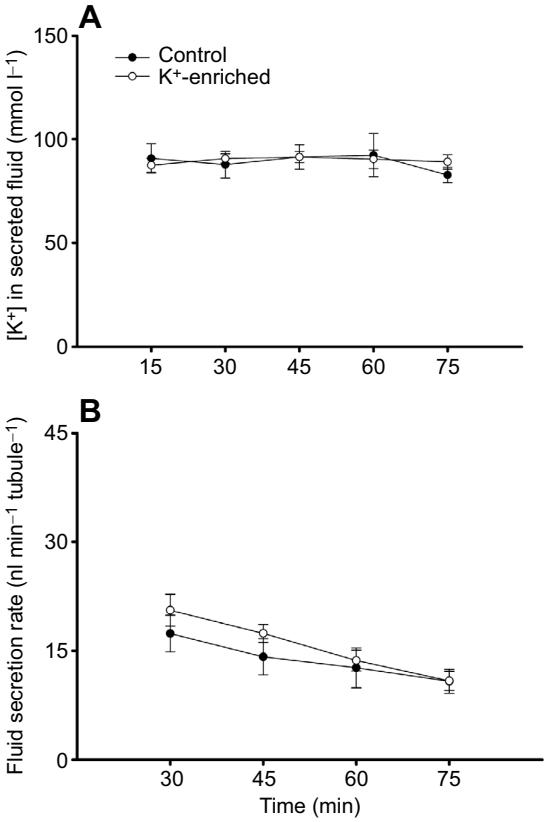

Fig. 6. The distal ileac plexus of K⁺-fed *Trichoplusia ni* secretes K⁺ and fluid. (A) Secretion of K^+; (B) secretion of fluid. When the DIP of larvae reared on a K^+-enriched diet was isolated and mounted for a Ramsay assay, it secreted K^+ and fluid at rates similar to those of the DIP isolated from control-fed animals. All data are means±s.e.m. (*N*=4).

A decreased need for ion recycling in the DIP of ion-loaded animals may stem from enhanced ion secretion by the rectal complex with increased dietary ion availability. Indeed, previous studies have demonstrated that dietary ion loading increased faecal Na^+ and K^+ content (Ramsay, 1976). Active ion transport across the rectal complex of lepidopterans has also been demonstrated (Ramsay, 1976; Reynolds and Bellward, 1989; Audsley et al., 1993). Thus the rectal lumen is the likely source for Na^+ and K^+ transport into the cryptonephridial MTs (see Fig. 1 for tubule regions). Increased availability of luminal Na^+ and K^+ in the DIP and the switch of PCs to ion reabsorption led to subsequent reabsorption of these ions into the haemolymph of ion-loaded animals in the current study.

Similar to the current study, a previous study demonstrated that *T. ni* with full guts (and thus presumably more K^+ and Na^+ available) decrease K^+ secretion and increase Na^+ reabsorption in the DIP, suggesting plasticity of epithelial ion transport in this segment of the tubule (O'Donnell and Ruiz-Sanchez, 2015). Differences in ion transport across regions of MT have been reported in dipterans, hemipterans and lepidopterans to date (Irvine, 1969; Maddrell, 1978; Haley et al., 1997; Rheault and O'Donnell, 2004; O'Donnell and Ruiz-Sanchez, 2015). In lepidopteran larvae, this heterogeneity is thought to address the need for K^+ reabsorption in downstream segments of the tubule, stemming from active secretion of large amounts of K^+ and base into the midgut during feeding (Thomas and May, 1984; Dow and O'Donnell, 1990; Moffet and Koch, 1992; Dow, 1992). Therefore,

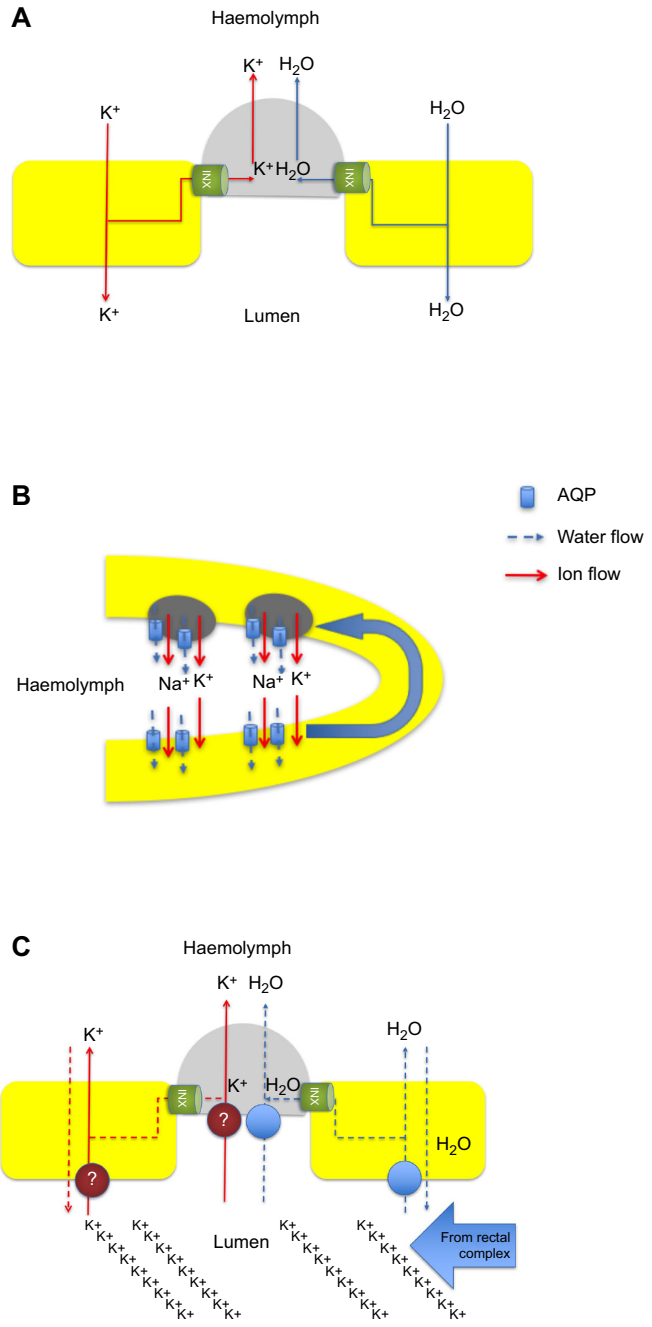

Fig. 7. Proposed GJ-based model for rapid switching of K⁺ secretion to K⁺ reabsorption in the PCs of the DIP of *Trichoplusia ni*. (A) Under normal dietary conditions *in situ* and in isolated MTs, PCs of the DIP secrete K^+ and adjacent SCs reabsorb K^+, part of which is routed through the GJ from the neighbouring PCs. (B) The juxtaposition of SCs to PCs in the loops of tubule within the plexus allows for local recycling of ions and (most likely) water under control conditions. (C) Under conditions of dietary K^+ loading, an intact preparation may experience increased supply of K^+ from the rectal complex and is likely to downregulate PC-based K^+ secretion. Instead, luminal sources of K^+ are utilized by both PCs and SCs by allowing K^+ entry via apical membrane of SCs, while GJs partially relay K^+ into the neighbouring PCs. PCs are likely to be able to switch ion secretion on and off rapidly depending on the demands of the animal. Water reabsorption across both cell types may follow in a similar manner if aquaporins are present as GJs are water permeable.

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switching to overall reabsorption of K^+ in the DIP may be aimed in part at recovering K^+ lost in digestive efforts by utilizing increased dietary and faecal K^+ availability.

We considered the possibility that part of Na^+ and K^+ reabsorption in the tubules of ion-loaded *Trichoplusia* is a paracellular leak through the septate junction. Previously reported concentrations of Na^+ (50 mmol l^{-1}) and K^+ (80 mmol l^{-1}) in secreted fluid collected from isolated DIP are higher than [Na^+] (30 mmol l^{-1}) and [K^+] (45 mmol l^{-1}) in the haemolymph or bathing saline, creating favourable conditions for a paracellular leak (Ruiz-Sanchez et al., 2015), particularly as the transepithelial potential is lumen-positive (Irvine, 1969; D.K. and M.J.O., unpublished observations). However, the reabsorption signal was maximal at the cell apex rather than at the cell–cell borders. The details will require further investigation as the molecular constituents of the septate junction complex in lepidopterans remain unknown (Jonusaite et al., 2016).

Lastly, we acknowledge the possibility that some of the results obtained from animals fed ion-rich diets may have been influenced by elevated dietary Cl^- levels as the diets were prepared using chloride salts of Na^+ and K^+. However, studies describing Cl^- levels in lepidopteran haemolymph are few, and comprehensive reports on how MTs of lepidopterans transport Cl^- are lacking. Whether transport of Na^+, K^+ and Cl^- in the MTs of lepidopterans is linked will require further mechanistic studies.

DIP of ion-fed animals reabsorbs ions only when connected to the rectal complex

Reabsorption of K^+ has been reported in the proximal segment of MTs in lepidopterans and other insects (Irvine, 1969; Moffett, 1994; O'Donnell and Maddrell, 1995; Coast, 2009; Ruiz-Sanchez et al., 2015). The current study, however, demonstrates that K^+ can be reabsorbed across more distal regions of the MT, when the need arises. Previous studies utilizing isolated tubule assays have reported that the DIP in lepidopterans is primarily secretory (see Fig. 1) and demonstrated ion and fluid secretion by *isolated* preparations (Irvine, 1969; Ramsay, 1976; O'Donnell and Ruiz-Sanchez, 2015; Ruiz-Sanchez et al., 2015). This remains in line with the current observation of isolated preparations (Fig. 5, isolated tubules; Fig. 6). The notion that the ileac plexus has secretory function in lepidopteran MTs dates back to 1969, when Irvine determined that an isolated 'rectal lead+ileac plexus' preparation secreted K^+ and fluid from the bathing saline into MT lumen (Irvine, 1969). In addition, in later studies, the rectal complex was shown to actively secrete ions and water (Ramsay, 1976; Audsley et al., 1993). A more recent study also indicated that the DIP is where the majority of ion and fluid secretion takes place (Ruiz-Sanchez et al., 2015). In other words, the rectal complex and the DIP have both been shown to possess secretory ability individually, yet the notion that separation of the two in isolated tubule assays may influence MT ion transport has never been addressed in previous studies.

In the current study, the DIP from K^+-fed animals secreted ions (Figs 5 and 6A) and fluid (Fig. 6B) when isolated from the rectal complex. As the supply of K^+ from upstream segments was effectively cut off in isolated tubules, K^+ that was secreted by the PCs and then relayed into the SCs via GJ channels provided the source for SC-based K^+ reabsorption. Similarly, *in situ* (attached to the rectal complex) DIP in *T. ni* has been reported to reabsorb Na^+ across its length (O'Donnell and Ruiz-Sanchez, 2015). However, as PCs secreted Na^+ when the DIP was isolated in the current study, Na^+ reabsorption by PCs *in situ* probably also takes place due to increased luminal supply of Na^+ by the rectal complex (Fig. 5B). PCs in the MTs of dipterans have been proposed to be donors of bicarbonate generated by the carbonic anhydrase and transported into the neighbouring SCs via GJs to enable base recovery through the basolateral Cl^-/HCO_3^- exchanger (Piermarini et al., 2010). Therefore, switching on K^+ secretion through PCs to enable SC-based K^+ reabsorption is reasonable given that the two cell types are coupled via GJs.

SC function remains unaffected in ion-loaded animals

Interestingly, regardless of dietary ion availability, Na^+ and K^+ reabsorption through the SCs was not altered by dietary ion loading and subsequent switch of PCs to ion reabsorption (Fig. 5). Thus the findings in this study may indicate that ion secretion by the PCs of the DIP in *T. ni* may be aimed at providing Na^+ and K^+ for SC-based ion reabsorption and may be 'switched off' when the upstream secretion from the rectal complex is sufficient to enable SC function. Stellate cells of dipterans (a SC equivalent) were in the past believed to lack primary active transport mechanisms on the basis of their simple cytoarchitecture and lack of cellular structures characteristic of ion-transporting cells (reviewed by Dow, 2012). However, SCs have subsequently been shown to possess many transporters absent from the neighbouring PCs related to ion, water and xenobiotic transport and their endocrine control: inward rectifier K^+ channel (Kir), multidrug resistance proteins (MRPs), chloride–bicarbonate exchangers (CBE), Na^+/K^+-ATPase (NKA), Na^+/H^+ exchanger 2 (NHE2), Na^+/H^+ antiporter 2 (NHA2) and leucokinin receptor (LK-R) (Piermarini et al., 2010, 2015; Labbe et al., 2011; Kersch and Pietrantonio, 2011; Patrick et al., 2006; Day et al., 2008; Xiang et al., 2012). Therefore, a functional designation of the PCs as ion providers in the SC-containing region is not unreasonable. It is likely, therefore, that SCs may toggle between GJ-based supply of Na^+ and K^+ from the neighbouring PCs and luminal input through their own apical membrane when secretion by the rectal complex provides sufficient luminal ion content. Further studies aimed at investigating SC-specific transporters in lepidopterans will need to be conducted to substantiate this possibility. In addition, once the transporters are identified, determining whether acid–base transport and toxic metabolite excretion are reliant on GJ coupling of SCs to PCs will be feasible.

Conclusions and future perspectives

We propose that in lepidopterans the DIP plays a role of a 'safety switch', providing ions for SC function when dietary ions are scarce (and the output from the rectal complex alone is insufficient). In addition, we propose that GJ coupling allows SCs to accept K^+ from PCs, enabling local recycling of ions and providing an additional source for SC-based ion reabsorption. The functional significance of ion and water recycling efforts in the MTs of lepidopterans may be related to: (i) base recovery (Moffett, 1994; Onken and Moffett, 2009), (ii) positive water balance needed for haemolymph expansion of growing larvae (Jungreis et al., 1973; Reynolds and Bellward, 1989) or (iii) nutrient and metabolic waste transport by the SCs and/or lower segments of the tubule (Labbe et al., 2011; O'Donnell and Ruiz-Sanchez, 2015). A recirculation system for Na^+, K^+ and osmotically obliged water in the DIP of *T. ni* may enhance clearance of wastes and toxins from the haemolymph.

How is ion transport through PCs and SCs achieved and regulated in lepidopterans? Future studies may investigate the endocrine control and the molecular mechanisms of ion transport in the two cell types of *T. ni.* Recent studies implicated bafilomycin- (H^+-ATPase pump), bumetanide- ($Na^+/K^+/Cl^-$ co-transporter) and amiloride- (H^+/K^+ and H^+/Na^+ exchangers) sensitive transporters

in the regulation of fluid secretion in *T. ni* (Ruiz-Sanchez et al., 2015). PCs and SCs in the MTs of insects studied to date are targeted by separate endocrine factors that utilize different second messenger pathways and differentially affect cation secretion by PCs and anion secretion by SCs (O'Donnell et al., 1996). A particular emphasis has been placed on the regulation of ion transport by kinins in both PCs (Coast, 2012) and SCs (Halberg et al., 2015) and by serotonin in PCs (O'Donnell and Maddrell, 1984; Coast, 2011; Ruiz-Sanchez et al., 2015). It is, however, clear from several recent (and current) studies that lepidopteran SCs may not behave (and thus may not be regulated) in the same way as the SCs of dipterans.

Lastly, there may be marked differences between the way segments of lepidopteran MTs behave *in situ* and *in vitro*. The DIP in the current study, for example, seems to be very much attuned to the feed from the (upstream) cryptonephridial tubule. *In situ* DIP of K^+-fed animals reabsorbs K^+ (probably due to K^+ being secreted in abundance by the upstream segment of the tubule). Once this supply is cut off, the DIP switches to secreting K^+ *in vitro* in order to provide a source for K^+ reabsorption by the SCs. Care should be taken when designing experiments aimed at understanding the function of individual segments of lepidopteran (and perhaps other) MTs. It is best to use a combination of *in situ* and *in vitro* assays as isolated (e.g. Ramsay) assays alone may not be sufficient to detect differences in ion transport of individual segments of the tubules.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.K., M.J.O.; Methodology: D.K., P.M.P., M.J.O.; Software: D.K.; Validation: D.K.; Formal analysis: D.K.; Investigation: D.K., P.M.P., M.J.O.; Resources: P.M.P., M.J.O.; Data curation: D.K.; Writing - original draft: D.K.; Writing - review & editing: D.K., P.M.P., M.J.O.; Visualization: D.K.; Supervision: M.J.O.; Project administration: M.J.O.; Funding acquisition: P.M.P., M.J.O.

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