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Helicokinin alters ion transport in the secondary cell-containing region of the Malpighian tubule of the larval cabbage looper *Trichoplusia ni*

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ABSTRACT

Excretion in insects is accomplished by the combined actions of the Malpighian tubules (MTs) and hindgut, which together form the functional kidney. MTs of many insect groups consist of principal cells (PC) and secondary cells (SC). In most insect groups SCs are reported to secrete ions from haemolymph into the tubule lumen. Paradoxically, SCs in the MTs of the lepidopteran cabbage looper *T. ni* are used to *reabsorb* Na^+ and K^+ back into haemolymph. The current study was designed to investigate the effects and mode of action of the lepidopteran kinin, Helicokinin (HK), on ion transport in the SC-containing region of MT of *T. ni*. We identified a HK receptor (HK-R) homologue in *T. ni* and detected its expression in the SC-containing region of the MTs. The mRNA abundance of *hk-r* altered in response to changes in dietary K^+ and Na^+ content. HK-R immunolocalized to both PCs and SCs. Ramsay assays of preparations of the isolated distal ileac plexus (DIP) indicated that [HK] = 10^{-8} M: (i) decreased fluid secretion rate in unstimulated and serotonin-stimulated preparations, and (ii) increased $[Na^+]/[K^+]$ ratio in the secreted fluid. Scanning ion-selective electrode technique measurements revealed that HK reduced: (i) K^+ secretion by the PCs, and (ii) Na^+ reabsorption by the SCs in intact tubules. In vitro incubation of the DIP with HK resulted in reduced mRNA abundance of *hk-r* as well as Na^+/K^+-ATPase subunit *α* (*NKAα*), $Na^+/K^+/Cl^-$ co-transporter (*nkcc*), Na^+/H^+ exchangers (*nhe*) *7* and *8*, and aquaporin (*aqp*) *1*. Taken together, results of the current study suggest that HK is capable of altering fluid secretion rate and $[Na^+]/[K^+]$ ratio of the fluid, and that HK targets both PCs and SCs in the DIP of *T. ni*.

1. Introduction

1.1. Insects couple excretion of metabolic wastes to ion and fluid secretion in the Malpighian tubules

Insects represent more than three-quarters of terrestrial animals and are of extreme importance as pollinators, disease vectors, invasive species and agricultural pests (Gullan and Cranston, 2014). Lepidopterans (butterflies and moths) comprise a group of insects that contains many agricultural pests (e.g., tobacco hornworm *Manduca sexta* and cabbage looper *Trichoplusia ni*) (Gullan and Cranston, 2014). The caterpillar larval stage can be a significant pest, consuming three times its own weight daily, feeding on a wide variety of cultivated plants (McEwen and Hervey, 1960). Physiological consequences of ingesting such large amounts of food include a need for continual active excretion of metabolites and the potential for ionoregulatory disturbance due to substantial excretory loss of ions.

Excretion in insects is accomplished by the combined actions of the Malpighian tubules (MTs) and hindgut, which together form the functional kidney (Wigglesworth, 1961). Active ion transport from haemolymph (insect blood) into the MT drives fluid secretion by osmosis (Nation, 2016). The primary urine is then modified in downstream segments of the tubule or the hindgut; much of the water and ions 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 in abundance through their diet. Current models propose a role for a vacuolar-type H^+-ATPase and

Abbreviations: MT(s), Malpighian tubule(s); PC(s), principal cell(s); SC(s), secondary cell(s); DIP, distal ileac plexus; HK, helicokinin; HK-R/*hk-r*, Helicokinin receptor protein/*transcript*; IHC, immunohistochemistry; PFA, paraformaldehyde; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PBT, phosphate-buffered saline with Triton-X; TRITC, tetramethylrhodamine isothiocyanate; cDNA, cloned DNA; qPCR, quantitative real-time PCR; GPCR, G protein-coupled receptor; *nkcc*, $Na^+/K^+/Cl^-$ co-transporter; *nhe-6/8*, Na^+/H^+ exchangers; *NKAα*, Na^+/K^+-ATPase subunit *α*; *aqp-1*, aquaporin-1; *kir*, inward-rectifier K^+ channel; *kcc*, K^+/Cl^- co-transporter; *VA*, vacuolar-type H^+-ATPase; *inx-2/3/7*, innexin-2/3/7

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Na^+/K^+-ATPase pumps coupled to Na^+/H^+ or K^+/H^+ exchangers and $Na^+/K^+/Cl^-$ co-transporter to drive K^+ and Na^+ from hemolymph into the cell and then into the tubule lumen (Beyenbach and Wieczorek, 2006; Piermarini et al, 2015). Protons transported into the lumen across the lumen-facing (apical) membrane are exchanged for Na^+ or K^+. One advantage of this arrangement is that insects feeding on K^+-rich diets can secrete K^+-rich fluids. Caterpillars of butterflies and moths like *Trichoplusia ni* feed on K^+-rich plant-based diet. Therefore, it is expected that the majority of their ion transport (which drives excretion) in the MT will be K^+-based. Despite this, fluid secreted by the MTs of lepidopterans is known to contain appreciable levels of Na^+ (Irvine, 1969; Ramsay, 1976; Ruiz-Sanchez et al, 2015).

1.2. Regulation of Na^+ and K^+ levels in the fluid secreted by the MTs lepidopterans

Adult lepidopterans supplement their poor dietary Na^+ intake with an interesting behavioural phenomenon termed "puddling" (Smedley and Eisner, 1996). Adult males aggregate around puddles formed from water runoff and waste fluids of vertebrate origin ingesting and expelling (in some species) up to 400 times of their own weight of collected fluid. Adult females receive collected Na^+-rich deposits as a nuptial gift during copulation (Adler and Pearson, 1982). It is thought that one of the purposes of this behaviour is to sequester environmental Na^+ to supplement the otherwise Na^+-poor diet (Capinera, 2008). Little, however, is known about how larval lepidopterans (that cannot 'puddle') handle their Na^+ balance and how the excretion of dietary Na^+ is regulated. Recent studies have described Na^+ and K^+ transport in the MTs of *Trichoplusia ni*, with particular focus on the distal ileac plexus region (O'Donnell and Ruiz-Sanchez, 2015; Ruiz-Sanchez et al, 2015; Kolosov et al, 2018a; Kolosov et al, 2018b). Dietary availability of Na^+ and K^+ in lepidopterans is known to vary depending on the crops they feed on (source: https://ndb.nal.usda.gov/), and endocrine mechanisms may aid regulation of the ion content. In locusts, a corticotropin-releasing factor related peptide increases Na^+ secretion by MTs at the expense of K^+ secretion (Coast, 1995), but mechanistic studies regarding the neuropeptide control of Na^+ and K^+ transport in the MTs of the larval lepidopterans are all but absent.

1.3. Kinins regulate ion and fluid secretion in the MTs of insects

Transport of cations and anions is known to be regulated separately in epithelia of insect MTs (O'Donnell et al., 1996). In dipterans (mosquitoes and flies), cation and anion secretion take place in different cell types and employ distinct second messenger pathways. Kinins are hormones isolated in many groups of insects and other invertebrates and target the MTs and other tissues (Coast et al., 1990; Thompson et al., 1995; Holmes et al., 2000; Torfs et al., 1999; Veenstra et al., 1997; Scherkenbeck and Zdobinsky, 2009). The effects of kinins on the MTs includes depolarization of the transepithelial voltage and increased fluid secretion consistent with increased permeability to Cl^- (Hayes et al., 1989). Additionally, while most kinins are diuretic in a dose-dependent manner, inhibition of fluid secretion has been reported for Leucokinin-VIII (LK-VIII) in *Aedes* MTs at concentrations $\leq 10^{-9}$ M (Hayes et al., 1989).

1.4. Kinin family of neuropeptides target secondary cells in many groups of insects

MTs of many insect groups consist of principal cells (PCs) and secondary cells (SCs) (O'Donnell et al., 1996; Halberg et al., 2015). SCs are credited with being a home to many important ion transporters, aquaporins and endocrine control mechanisms, and serve in ion secretion (Dow and Davies, 2006; Dow, 2012). Immunohistochemical studies and incubation of tubules with fluorescently-labelled kinin indicate that the kinin receptor localizes to the SCs in the MTs of many insects groups. (Radford et al., 2002, 2004; Lu et al., 2011; Kersch and Pietrantonio, 2011; Halberg et al., 2015). In fact, the SC-based kinin receptor in mosquitoes has been demonstrated to be critical in post-prandial fluid excretion following a blood meal (Kersch and Pietrantonio, 2011). However, the PCs of mosquitoes (in the regions of the MT devoid of SCs) have been credited with responding to kinins as well (Yu and Beyenbach, 2002; Beyenbach, 2003; Yu and Beyenbach, 2004). It has been suggested that the presence of more than one kinin receptor, as well as the sharing of second messengers via gap junctions, may be responsible for the observed kinin action on the PCs in dipterans (Lu et al., 2011).

1.5. Effects of Helicokinin on ion transport in the MTs of lepidopterans remains unstudied

Three diuretic kinins have been isolated from the ventral nerve cord of adult *Helicoverpa zea* and named Helicokinins (HK) (Blackburn et al., 1995) and all were reported to increase the fluid secretion rate in a dose-dependent manner in *Heliothis veriscens* larvae (Seinsche et al., 2000). Given that HK peptide mimetics have potential applications as a pest control measure for lepidopterans (Scherkenbeck et al., 2009), further studies of these neuropeptides are warranted. Although HK has been described as a diuretic hormone, there are no studies describing how it affects ion transport, and specifically cation (Na^+ and K^+) transport, in the MT of lepidopterans. This study was designed to investigate the effects of HK-1 application on cation transport by the MTs of *Trichoplusia ni* and on the paradoxical reabsorption of cations through the SCs in particular.

2. Materials and methods

2.1. Experimental animals

Trichoplusia ni (Hübner 1800) eggs were supplied by the Great Lakes Forestry Centre (Sault St. Marie, ON). Larvae were allowed to develop to fifth instar at 23–25 °C and 40–50% relative humidity while 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 as described in Kolosov et al., 2018a. 5th instar larvae were treated with High-K^+ or High-Na^+ diets for 24 h. 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. Kinin receptor search and identification

Homologues of *Drosophila melanogaster*, *Aedes aegypti* leucokinin receptor were sought in lepidopterans using NCBI databases. *Heliothis veriscens* helicokinin receptor (HK-R, accession #DD231174.1) was identified in the NCBI as a target and was used for a search in *T. ni* genome (http://cabbagelooper.org). Newly identified sequence TNI005965-RA was 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) were sent for sequencing at Farncombe Institute (McMaster University, Canada).

2.4. RNA extraction, cDNA synthesis and PCR protocols

Total RNA was extracted using Trizol and manufacturer's instructions (e.g., Kolosov and Kelly, 2016; Kolosov et al., 2018a). For the tissue expression profile using PCR, rectal complex, salivary glands, midgut, ileum, and white, yellow, distal and proximal ileac plexus regions of the MT were dissected out from three caterpillars and pooled together so as to avoid missing a transcript based on its absence from a single larva. The quality of extracted RNA was assessed from the A260/A280 ratio generated by a NanoDrop ND-1000 spectrophotometer. From every sample, 2 µg of RNA was taken, and topped up to 8 µl with DEPC-treated water. Total RNA was then treated with DNase I (Amplification 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).

Helicokinin receptor transcript in distinct tissues mentioned above was detected by RT-PCR in a BioRad PCR machine for RT-PCR (PTC-2000; Bio-Rad Laboratories Canada Ltd). 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) in a 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 was synthesized during reactions, a dissociation curve analysis was carried out after each qPCR run. Amplicons of PCR product were sequenced to ensure that amplicon from the desired transcript was amplified and their size was confirmed to be in line with designed primers. 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 originating from animals raised on different diets to confirm that no statistically significant changes occurred in different groups as a result of dietary treatment ($P = 0.842$, One-way ANOVA).

2.5. Whole-mount immunohistochemistry (IHC) procedures

Whole-mount IHC procedures in *T. ni* have been described in detail by Kolosov et al. (2018a) and modelled after Patrick et al. (2006). Briefly, the DIP was dissected out in lepidopteran saline and fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH = 7.4). The following day, tissues were then washed in PBS and dehydrated in methanol/PBS series. 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 antibody overnight at 4 °C at 1:100 dilution in PBT/1%-BSA. Affinity purified polyclonal rabbit antibody against *Aedes aegypti* leucokinin receptor (*Ae*KR) was used to label *Trichoplusia* HK-R (the antibody was a generous gift from P.V. Pietrantonio, Texas A&M University). The peptides correspond to amino acid residues 328–345 of the C-terminal domain of *Ae*KR (Luet al., 2011). Phylogenetic analysis and epitope comparison confirmed the homology of HK-R found in *Trichoplusia,* other kinin receptors, and *Aedes* kinin receptor (Fig. 1A). An additional preparation where primary antibody was omitted was included with each IHC analysis to act as negative control.

Tissues were washed after 16–18 h in PBT/1%-BSA supplemented with normal goat serum to remove unbound primary antibody. Following washes, tissues were incubated with 1:1000 of secondary goat anti-rabbit TRITC-conjugated secondary antibody (Cedarlane, Burlington, ON, Canada) in the dark at room temperature for 2 h. Following incubation with secondary antibody, tissue was washed 3 times in PBT/1%-BSA for 15 min each time. Tubules were then mounted on slides using ProLong Antifade® reagent (ThermoFisher Scientific) and left to cure in the dark. Images of HK-R were obtained using a laser-scanning confocal image acquisition system CTR-6500 coupled to a Leica DM6000CS microscope at McMaster University imaging facilities.

2.6. Synthetic Helicokinin

Homologues of *Helicoverpa armigera* leucokinin precursor KC340929.1 were identified in the recently released *Trichoplusia ni* genome (Fu et al., 2017; http://cabbagelooper.org) to ensure that *T. ni* is capable of producing its own kinins. Kinin sequence identified from the *T. ni* genome was translated and aligned with three known Helicokinin (HK) peptides isolated from *Helicoverpa zea* (Blackburn et al., 1995). Previously-described HK-1 YFSPWG demonstrated 100% sequence identity with *T. ni* kinin precursor. Thus, its active (YFSPW-G$_{amide}$) version was purchased from GenScript (Piscataway, USA). Purified HK peptide was reconstituted in normal lepidopteran saline at superstock concentrations and stored at −20 °C until used.

2.7. Ramsay assay and effects of HK on secretion rate and ion content of the fluid secreted by the DIP

Droplets of fluid secreted by isolated DIP set up in the Ramsay assay were collected and ion concentrations in the droplets were measured as described previously (Ruiz-Sanchez et al., 2015) with several modifications described in detail by Kolosov et al. (2018a). Briefly, each DIP was separated from tracheae and transferred into a 70 µl droplet of lepidopteran saline under paraffin oil. 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.

Table 1
Primer sets used for PCR and qPCR analysis of ion transporter transcript expression and abundance.

Transcript amplified	Forward primer	Reverse primer	NCBI accession number	T_a, °C
NKAα	CAACAACGCTTACCTGGAACTC	AGGGTCAATCATACTCATCAATCC	MH048889	55
Kir	GGATACTGGTGAATACGAGGTTG	CAGGGTTGTGATTTCTATGTGTC	MH048890	55
VAb	GCTTACCAGTGCGAGAAACAC	GAATAGGATGGGTGATGTCGTC	MH048891	55
nhe-7	ACGCAAAAGCAACACTTCTACATC	GTTCCACATTCACTATTTCACCTC	MH048892	54
nhe-8	CTATTTTGTGGTGTTGTGATGTCTC	AAGGTGAAGCGATAAAGCGTAG	MH048893	55
nkcc	GAGGTCAAAGGAGGTGGTA	CCCAACAGCACAGATAATA	MH048895	51
kcc	CTGTGTATGTTGGCATCTTCG	AAACTTGGTTGTAGGTGGGTCTC	MH048896	54
aqp-1	CATCTCCATCTCAGCATCCA	GCCTCCCACTTTGTCTTCTG	MH230174	57
hk-r	AGTGTTGGCAGTGGTTGGAA	GCTGAGTGGCGTAATGATA	TNI005965-RA	59

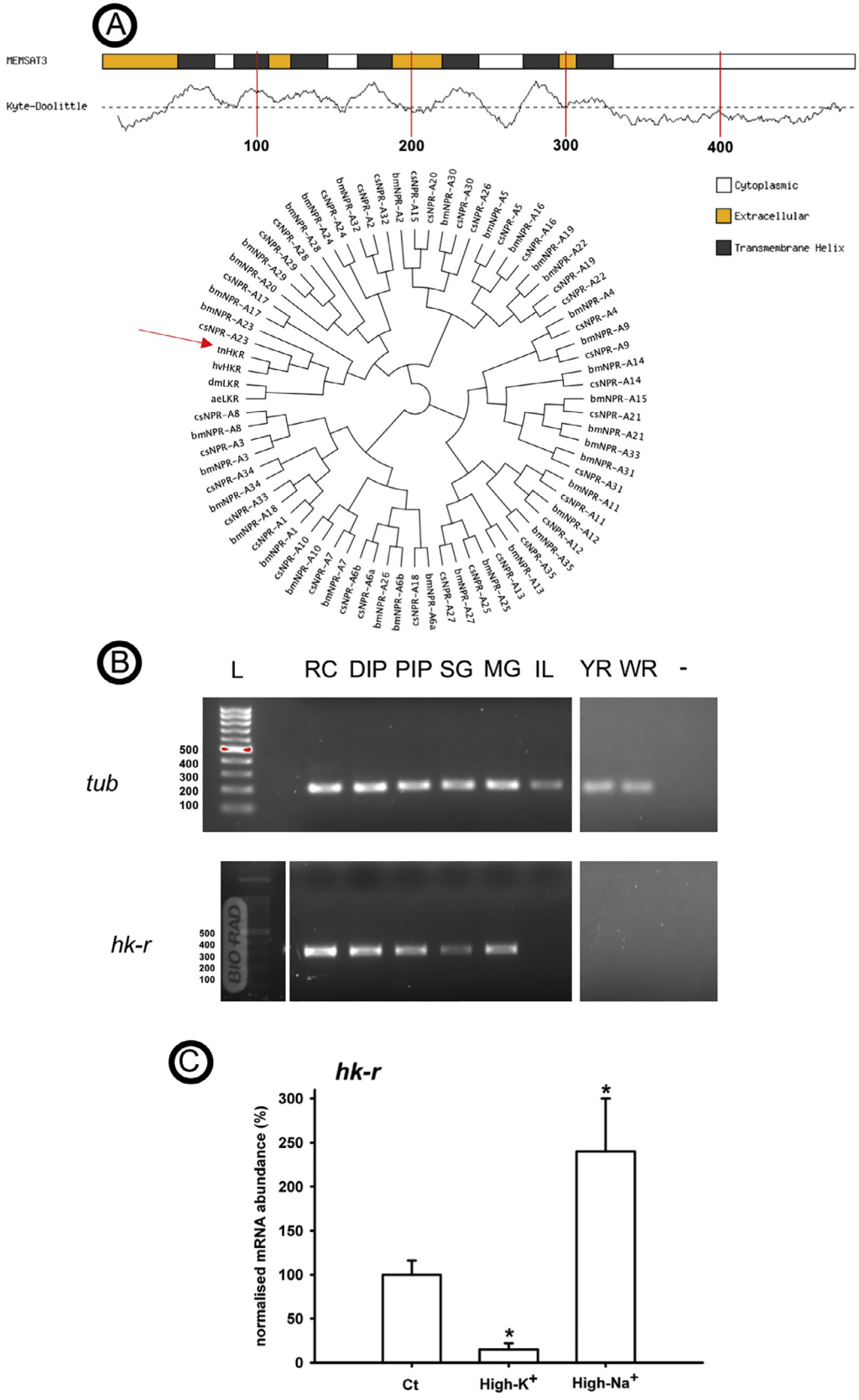
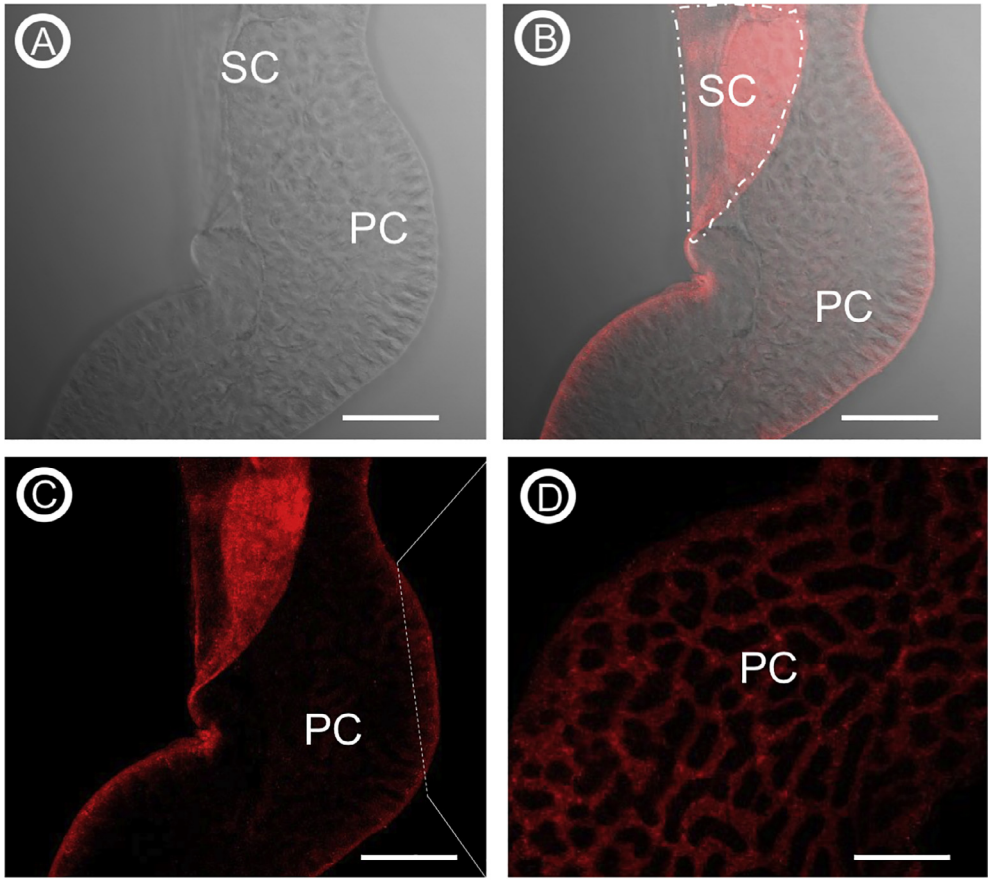


Fig. 1. Protein transmembrane tendency, phylogenetic tree, expression profile and transcript abundance changes of *Trichoplusia ni* Helicokinin receptor. (A) Hydrophobicity analysis of the *Trichoplusia ni* Helicokinin receptor (HK-R), red lines correspond to the residue positions numbered underneath, and phylogenetic tree of *T. ni* HK-R (tnHKR, red arrow), *Heliothis veriscens* HK-R (hvHKR), *Drosophila melanogaster* leucokinin receptor (dmLKR), *Aedes aegypti* leucokinin receptor (aeLKR), and all of the A-family GPCR neuropeptide receptors identified in *Bombyx mori* (bmNPR-A1-34) and *Chilo suppressalis* (csNPR-A1-34). (B) *hk-r* mRNA is expressed in the rectal complex (RC), distal ileac plexus (DIP) and the proximal ileac plexus (PIP), salivary glands (SG), and midgut (MG). *hk-r* mRNA was not detected in the ileum (IL), the yellow region (YR) and the white region (WR) of the Malpighian tubules of *Trichoplusia ni*. Tubulin was run as a positive control. (C) *hk-r* mRNA abundance decreased in the DIP of K^+-fed larvae and increased in the DIP of Na^+-fed larvae. All data in (C) are presented as mean values ± s.e.m. (N = 5). An asterisk indicates a significant difference due to dietary ion loading as determined by a one-way ANOVA tested against the control group.

After collection of the second droplet (following 30 min of fluid secretion) 10% of the saline volume was exchanged with pre-diluted HK-containing saline in half of the tubules isolated from the same caterpillar and an equal volume of saline was exchanged in the paired control tubules, resulting in the final [HK] of 10^{-13}–10^{-6} M in the treated droplets. Collected droplets were placed at the bottom of a separate Petri dish filled with oil and sized using an ocular micrometer to calculate droplet volume and fluid secretion rate. [HK] = 10^{-8} M was



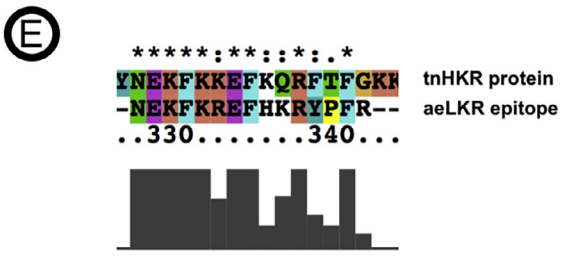


Fig. 2. Immunolocalization of Helicokinin receptor in the principal and secondary cells of the distal ileac plexus of *Trichoplusia ni.* (A) Brightfield image of the distal ileac plexus with principal cells (PC) and secondary cells (SC) visible. (B) Overlap of the brightfield image and fluorescent image of the prep stained with aeLKR antibody demonstrated localization in the PC and SC of the distal ileac plexus of *Trichoplusia ni.* (D) Tangential section of the PC membrane demonstrates localization to the basolateral infoldings. (E) Alignment of *T. ni* HK-R peptide sequence with the sequence of the epitope used to raise the aeLKR antibody which has been used to immunolocalize *T. ni* HK-R in the current study. Scale bars = 50 µm in (A–C) and 25 µm in (D).

found to decrease the fluid secretion rate (see Section 3). This active dose was applied to unstimulated DIP, as well as the DIP preparations stimulated with 1 µM 5-hydroxytryptamine (5-HT) to investigate whether the antidiuretic effect of the active dose persists in stimulated tubules. The active dose of HK was also applied to isolated DIP preparations to determine changes in $[Na^+]$ and $[K^+]$ content of the secreted fluid. Droplet $[Na^+]$ and $[K^+]$ were measured using Na^+- and K^+ -selective microelectrodes prepared as described in Donini and O'Donnell, 2005.

2.8. Scanning ion-selective electrode technique (SIET)

Hardware, software and methodology for acquiring 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; Kolosov et al., 2018a). SIET was used to measure ion fluxes in intact DIPs stimulated with 10^{-8} M HK. Feeding fifth instar larvae were dissected and a DIP was separated from tracheal connections and pulled away from the ileum. 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 detected. 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.

2.9. In vitro exposure of the DIP to HK

In order to simulate prolonged exposure of the DIP to active internal ligand concentration in the haemolymph, DIPs were dissected out from feeding 5th instar larvae and placed in Petri dishes filled with lepidopteran saline. Paired setups were used, where two DIPs were isolated from the same larva – one treated with 10^{-8} M HK at room temperature for 4 h and a paired preparation sham-treated with the same volume of saline used to deliver HK. Use of paired setups allowed for control in variations in transcription rates between individual larvae. Following the 4-hour exposure, DIPs were collected for RNA extraction, cDNA synthesis, and qPCR analysis (see Section 3.4 RNA extraction, cDNA synthesis and PCR protocols) of previously-identified molecular targets of ion transport (see Table 1). Tubulin was used as a housekeeping gene and lack of changes in its expression with experimental treatment was

verified using a Student's *t*-test (P = 0.404). Tubulin and gap junction innexin primers used in the current study were published in Kolosov et al. (2018a).

2.10. Statistical analysis

Significant differences due to experimental treatment were determined using a Student's *t*-test or a one-way ANOVA coupled with a Dunnett's post-hoc test in SigmaPlot (version 11) statistical software. Statistical significance was based on the observation of a $P < 0.05$.

3. Results

3.1. Trichoplusia ni Helicokinin receptor (HK-R) in the distal ileac plexus of Malpighian tubules (MT)

A search in the *Trichoplusia ni* genome identified an encoding for a G-protein-coupled receptor (GPCR) with seven membrane-spanning domains, extracellular N-terminus, three extracellular loops and a cytosolic C-terminus (Fig. 1A). The newly identified GPCR from *T. ni* forms a high-confidence clade with well-characterized kinin receptors from *Heliothis vericens, Drosophila melanogaster* and *Aedes aegypti* in a phylogenetic tree constructed from sequences of all published A-family GPCRs from rice stem borer *Chilo suppressalis* and silkworm *Bombyx mori* (Fig. 1A). Transcript encoding *hk-r* was detected in the rectal complex, the distal and proximal ileac plexus, salivary glands and the midgut, but absent from the ileum and yellow and white regions of the MTs (Fig. 1B). Lastly, transcript abundance of *hk-r* decreased in animals exposed to high-K^+ diet and increased in animals exposed to high-Na^+ diet (Fig. 1C).

3.2. HK-R is expressed in the principal cells (PCs) and secondary cells (SCs) of the MTs

HK-R immunolocalized to both PCs and SCs (Fig. 2A–C). In tangential section through a PC, HK-R immunofluorescence localized to what appeared to be basolateral membrane infoldings (Fig. 2D). Alignment of anti-*Aedes* LK-R antibody epitope and *T. ni* HK-R sequence indicated significant homology (Fig. 2E).

3.3. Helicokinin (HK) reduces fluid secretion in isolated DIP with a narrow active dose range

HK reduced fluid secretion rate in MTs mounted for a Ramsay assay when applied at concentration of 10^{-8} M for 30 min in unstimulated (Fig. 3A) and serotonin-stimulated (Fig. 3B) preparations. No other concentration of HK in the bathing saline produced such an effect (a range of 10^{-13} M–10^{-6} M was tested).

3.4. HK is a K^+-sparing natriuretic in Trichoplusia ni

At 10^{-8} M, HK increased [Na^+] (Fig. 4A) and decreased [K^+] (Fig. 4B) in secreted fluid after 30 min of application. This resulted in an increased [Na^+]/[K^+] ratio (Fig. 4C).

3.5. HK decreases K^+ secretion via PCs and Na^+ reabsorption via SCs

The mode of action of HK on the DIP was investigated using SIET. K^+ secretion by the PCs of MTs *in situ* was found to decrease following 10 min of exposure to 10^{-8} M HK (Fig. 5A), while Na^+ reabsorption by the SCs decreased (Fig. 5D). Na^+ reabsorption by PCs (Fig. 5B) and K^+ reabsorption by the SCs (Fig. 5C) remained unaffected by HK treatment.

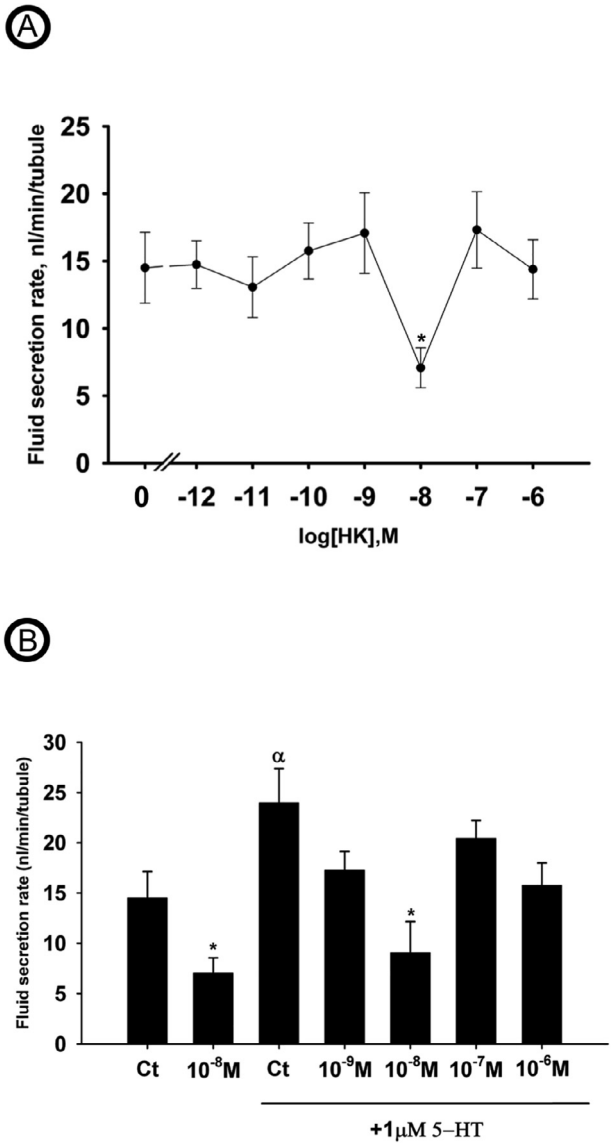


Fig. 3. Helicokinin reduces fluid secretion rate in isolated distal ileac plexus of *Trichoplusia ni.* (A) Ramsay assays were used to assess the effects of Helicokinin (HK) on the fluid secretion rate in the isolated distal ileac plexus (DIPs) of *T. ni* following 30 min exposure to the hormone at indicated concentrations. Notably, 10^{-8} M HK reduced fluid secretion rate significantly compared to unstimulated DIPs (labelled with "0"). (B) The effects of active concentration (10^{-8} M) of HK and several flanking concentrations on the fluid secretion rate in the isolated DIP stimulated with 1 μM serotonin (5-HT) (Ct and 10^{-8} M HK are from panel A and are included for comparison). All data points are presented as mean values ± s.e.m. (N = 5). Asterisks in (A) and (B) indicate significant difference due to stimulation with HK (as compared to "0" unstimulated groups in (A), and control (Ct) groups in (B). α in (B) is used to indicate a significant increase in the fluid secretion rate due to stimulation with serotonin. All significant differences were determined by a one-way ANOVA tested against the control group.

3.6. In vitro exposure to an active dose of HK leads to reduced mRNA abundance of its receptor, nkcc, nhe-7 and -8, NKAα, and aqp-1

In order to simulate the exposure to active HK levels in the animal, DIPs were excised and exposed to 10^{-8} M of HK for 4 h. This resulted in reduced transcript abundance of *nkcc*, *nhe-7* and *-8*, *NKAα*, and *aqp-1*, as well as *hk-r* (Fig. 6). The treatment did not affect mRNA abundance of *kir, kcc, VAb* or *inx-2, -3* and *-7.*

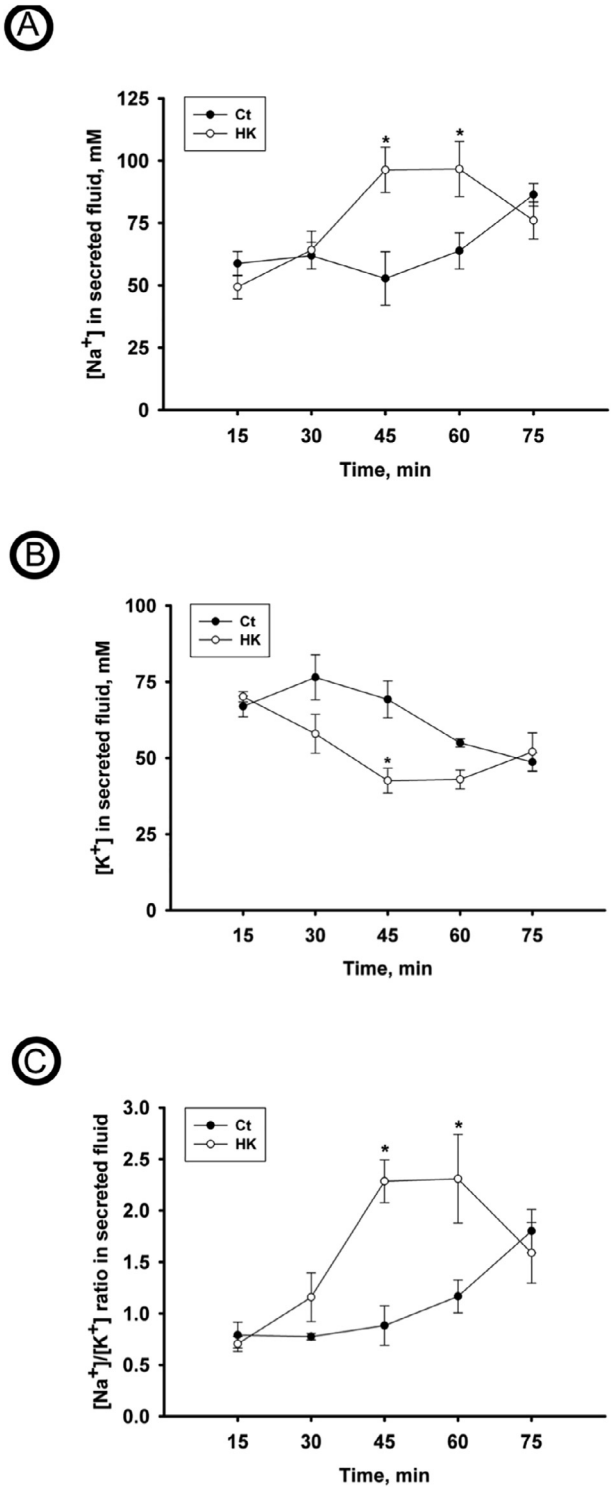


Fig. 4. Helicokinin is a K^+-sparing natriuretic in the distal ileac plexus of *Trichoplusia ni.* Application of 10^{-8} M Helicokinin (HK) results in (A) increased $[Na^+]$, (B) decreased $[K^+]$, and consequently (C) increased $[Na^+]/[K^+]$ in secreted fluid following 30 min exposure, as compared to unstimulated control (Ct) DIPs. HK was added after obtaining the 15-min data points. All data points are presented as mean values ± s.e.m. (N = 5). Asterisks indicate significant difference due to stimulation with HK. All significant differences were determined by a Student's *t*-test.

4. Discussion and conclusions

4.1. Overview of findings

Several lines of evidence in the current study indicate a role for Helicokinin (HK) in regulating Na^+ and K^+ transport in the distal ileac plexus (DIP) of the larval *Trichoplusia ni*. Firstly, HK receptor (HK-R/*hk-r*) was identified, *hk-r* expression was detected in the DIP, and its mRNA abundance was shown to decrease in K^+-fed animals and increase in Na^+-fed animals. Secondly, when HK was applied to DIP preparations at 10^{-8} M, it resulted in: (i) reduced fluid secretion coupled with reduced *aqp-1* mRNA abundance, (ii) reduced K^+ secretion by the PCs and Na^+ reabsorption by the SCs, coupled with an increased $[Na^+]/[K^+]$ ratio in the secreted fluid. In vitro exposure to 10^{-8} M HK reduced transcript abundance of *nhe-7, nhe-8, NKAα* and *nkcc*. These ion transporters have been associated with secretion of K^+ by the PCs in a recently published study (Kolosov et al., 2018b). Lastly, the reduction in mRNA abundance of these transporters observed in the current study in the HK-treated DIP (in vitro), is correlated with reductions in mRNA abundance for these and other K^+ transporters in the DIP of K^+-fed animals (Kolosov et al., 2018b), suggesting another link between HK and Na^+/K^+ balance in *Trichoplusia ni.* Overall, it appears that HK is an antidiuretic with a very narrow dose range capable of acting on the DIP of *T. ni*, producing Na^+-rich and K^+-poor fluid, while the likely mechanisms of the long term HK action at the tissue level involve alterations in HK receptor and ion transporter abundance.

4.2. HK-R expression in the DIP, localization to PCs and SCs, and altered mRNA abundance in the DIP of larvae fed ion-rich diets suggest that HK may directly target the DIP

In order to demonstrate that HK receptor (HK-R) identified in *Trichoplusia ni* genome was similar to a previously identified HK-R of *Heliothis veriscens* and leucokinin receptors from dipterans *Drosophila melanogaster* and *Aedes aegypti,* a phylogenetic tree was constructed using sequences of all known A-family GPCRs from lepidopterans *Chilo suppressalis* and *Bombyx mori* using methodology described by Xu et al. (2006). *Trichoplusia ni* HK-R was found to cluster with A23 group of receptors that included all previously identified kinin receptors of insects.

Transcript encoding *hk-r* was detected in the rectal complex and the ileac plexus, as well as the midgut and the salivary gland. *Aedes* leucokinin receptor has been localized in similar tissues (Kersch and Pietrantonio, 2011). HK has been shown to alter contractions of isolated whole gut in a lepidopteran *Sporodoptera frugiperda* (Howarth et al., 2002). Multiple neuropeptide receptors have been detected in the salivary glands of *T. ni* and have been shown to alter in mRNA abundance with diet (Rivera-Vega et al., 2017). Since both midgut and salivary gland are involved in feeding, and MTs are involved ionoregulation, the finding that all three tissues express HK-R suggests that endocrine regulation of feeding and ionoregulation in *T. ni* are linked.

Immunohistochemistry detected aeLK-R-like immunoreactivity in both PCs and SCs (Fig. 2). By contrast, kinin receptors in dipterans *Drosophila melanogaster, Anopheles gambiae* and *Aedes aegypti* have been shown to localize exclusively to the stellate cells, the dipteran counterpart of SCs of lepidopterans (Radford et al., 2002; Radford et al., 2004; Kersch and Pietrantonio, 2011). However, the principal cells of mosquitoes (in the regions of the MT devoid of SCs) and crickets (that lack SCs) have been credited with responding to kinins as well (Chung et al., 1995; Yu and Beyenbach, 2002; Beyenbach, 2003; Yu and Beyenbach, 2004). It has been suggested that the presence of more than one kinin receptor, as well as the sharing of second messengers via gap junctions may be responsible for the observed kinin action on the PCs in dipterans (Lu et al., 2011). A study employing fluorescently-labelled drosokinin demonstrated that it bound to multinucleated dome-like cells in tubules of *Manduca sexta* larvae and *Bombyx mori* larvae

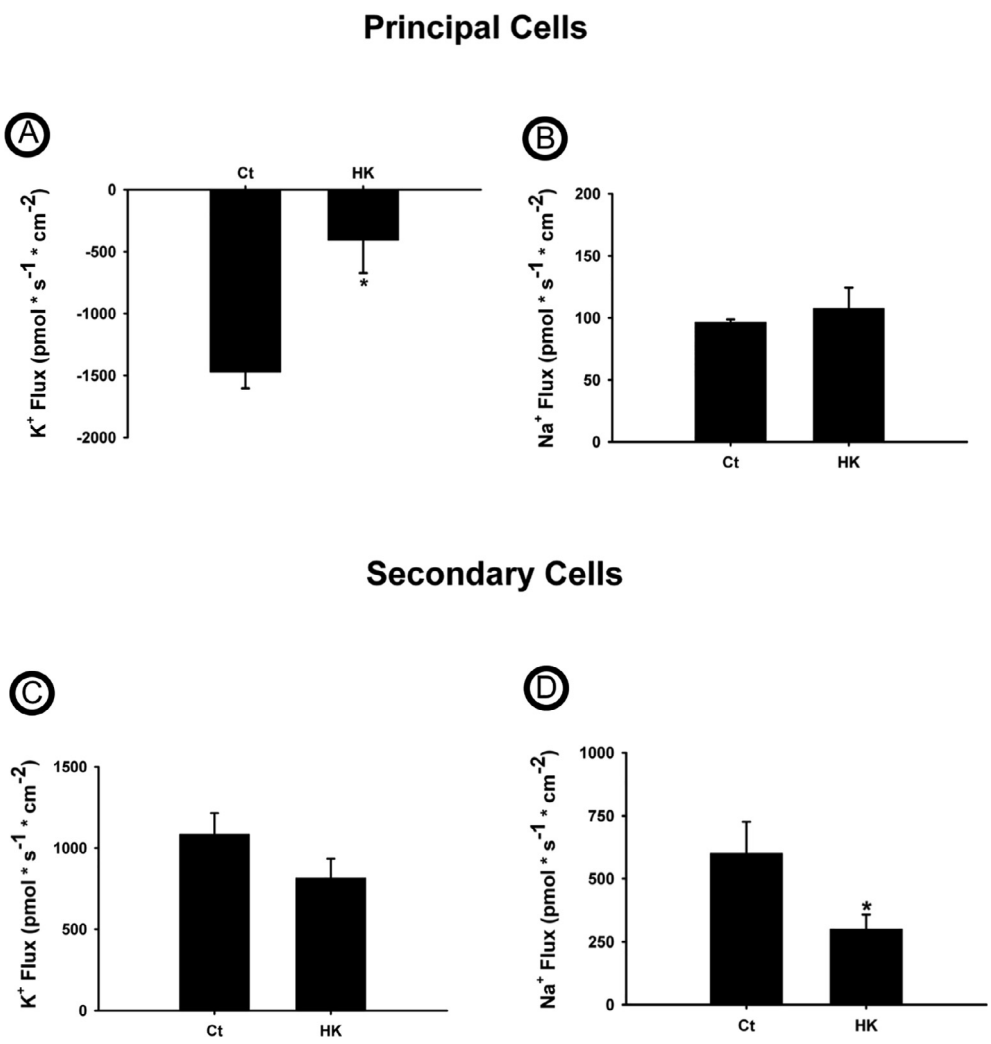


Fig. 5. Helicokinin reduces K^+ secretion and Na^+ reabsorption by the principal and secondary cells, respectively, in the intact distal ileac plexus of *Trichoplusia ni*. Addition 10^{-8} M Helicokinin (HK) to the bathing saline (A) reduced K^+ secretion by the PCs, (B) without affecting Na^+ reabsorption by the PCs, or (C) K^+ reabsorption by the SCs. Simultaneously, (D) Na^+ reabsorption by the SCs decreased. All data are mean values ± s.e.m. (N = 5–6). Asterisks denote significant differences due to HK application as determined by a Student's *t*-test. Negative values indicate ion secretion and positive values indicate ion reabsorption.

(Halberg et al., 2015). These cells are likely to be principal cells (PCs) as secondary cells of lepidopterans typically have one nucleus (e.g., Traut et al., 2007; Labbe et al., 2011; Kolosov et al., 2018a). However, drosokinin peptide sequence (CNSVVLGKKQRFHSWG$_{amide}$) is quite distinct from HK sequence encoded by lepidopterans, including *Trichoplusia* (YFSPWG$_{amide}$), and the differences may have influenced its receptor-binding affinity. Additionally, leucokinin I and drosokinin have been reported to increase the fluid secretion rate in Malpighian tubules of adult and larval *Manduca sexta*, respectively, albeit at much higher concentrations, consistent with reduced affinity for the receptor (Skaer et al., 2002; Halberg et al., 2015).

Altered transcript abundance of *hk-r* in the DIP of the larvae fed ion-enriched diet indicated that HK may be acting directly on the DIP and affecting ion transport in this tissue during systemic disturbances of ion homeostasis. Alterations in peptide receptor abundance have been linked to the changes in the circulating levels of their ligands in many endocrine systems of animals (Sealfon, 1998). In fact, Skaer and colleagues have suggested that a "continuous broadcast" of information relayed via hormones and signalling molecules exists in haemolymph circulation and tissue-level response is adjusted instead of adjusting circulating hormone levels (Skaer et al., 2002).

4.3. HK has a very narrow range of antidiuretic action in the DIP

In the current study, 10^{-8} M HK lowered fluid secretion rate in isolated DIP (both unstimulated and stimulated with 5-hydroxytryptamine, further 5-HT) (Fig. 3A). In unstimulated tubules HK induces K^+-sparing natriuresis and reduces fluid secretion. Molecular evidence suggests that the long-term targets of HK action are NKA, NKCC and NHEs, reducing ion transport by PCs and SCs and aqp-1 which reduces water permeability. The short-term effects of HK must be relayed via a second-messenger pathway that likely inhibits the same molecular targets. Alteration of intracellular Ca^{2+} levels is a likely suspect in accordance with previous publications which describe this as a second messenger for kinin action in PCs and SCs (e.g., O'Donnell et al., 1996; Yu and Beyenbach, 2002; Radford et al., 2004). The effects of HK on 5-HT-stimulated Malpighian tubules are similar to cGMP-mediated action of antidiuretic CAPA in *Aedes aegypti*, where it reduces fluid secretion in 5-HT- and DH_{31}-stimulated Malpighian tubules (Sajadi et al., 2018). Both 5-HT- and DH_{31} receptors are thought to be localized in the PCs in dipterans and act via cAMP as a second messenger (Clark and Bradley, 1996; Coast et al., 2005). A recent study, however, additionally implicated cAMP-mediated intracellular Ca^{2+} waves in the action of 5-HT on *Rhodnius* tubules (Gioino et al., 2014). Therefore, it is possible that HK shares a part of cAMP/Ca^{2+} signalling

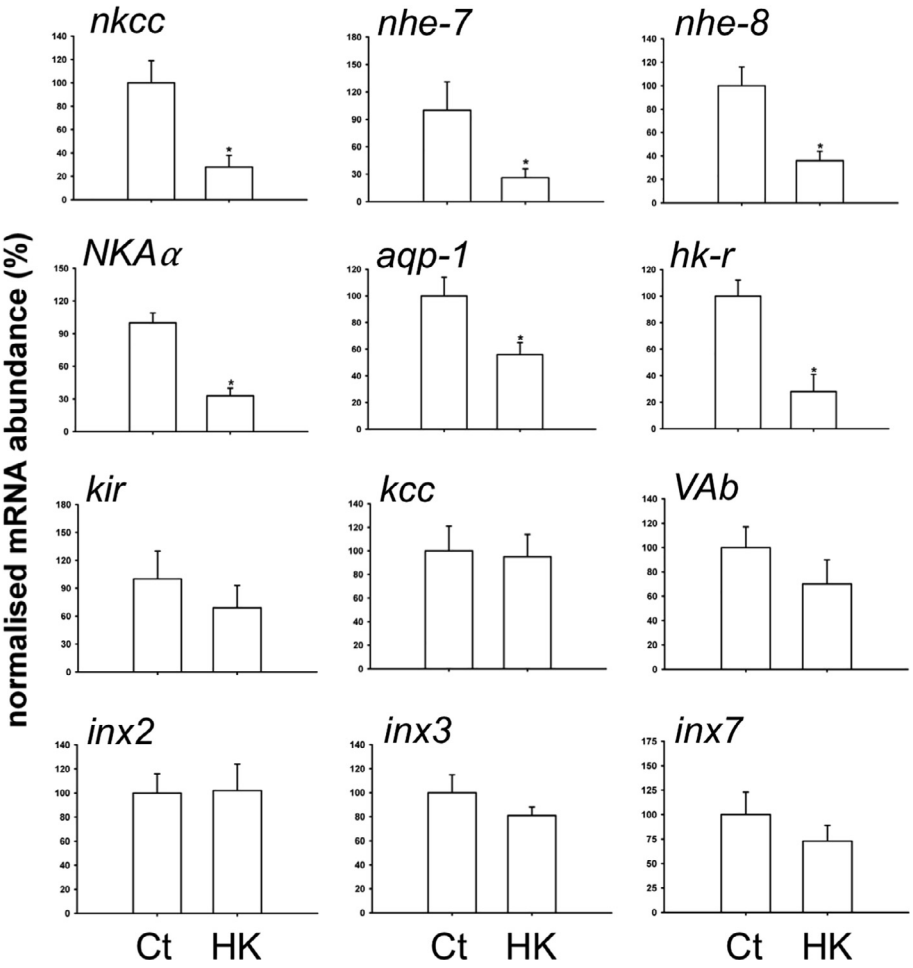


Fig. 6. In vitro incubation with Helicokinin reduces mRNA abundance of several secretory ion transporters and aquaporin-1 in the distal ileac plexus of *Trichoplusia ni*. Exposure of isolated distal ileac plexus feeding fifth instars of *Trichoplusia ni* to 10^{-8} M Helicokinin decreased transcript abundance of $Na^+/K^+/Cl^-$ co-transporter (*nkcc*), Na^+/H^+ exchangers (*nhe*)-*7* and -*8*, Na^+/K^+-ATPase subunit α (*NKA*), aquaporin-1 (*aqp-1*), and HK receptor (*hk-r*), and did not alter mRNA abundance of inward-rectifying K^+ channel (*Kir*), K^+/Cl^- co-transporter (*kcc*), V-type H^+-ATPase subunit b (*VA*b), and innexin (*inx*) *-2*, *-3* and *-7*. All data are presented as mean values ± s.e.m. (N = 5). Asterisks indicate significant differences due to HK treatment as determined by a Student's *t*-test.

pathway with 5-HT and thus HK effects resemble effects of cAMP/serotonin on ion transport in *T. ni*. Alternatively, HK may interfere with the 5-HT-cAMP signalling pathway downstream of cAMP by relying entirely on another second messenger; cGMP-mediated effects of CAPA in *Aedes* tubules suggest that it may be cGMP. Detailed examination of second messenger pathways employed by HK in PCs and SCs will be addressed in the future studies.

Additionally, in vitro exposure to 10^{-8} M HK lowered *aqp-1* mRNA abundance, adding another mechanism of action to its antidiuretic activity in isolated DIP of *T. ni*. Aqp-1 has been previously detected in the MTs of dipterans (Marusalin et al., 2012) and lepidopterans (Azuma et al., 2012). Aqp-1 mRNA abundance was reduced in the MTs of blood-fed mosquitoes after completion of diuresis, demonstrating its importance in water transport in this tissue (Drake et al., 2015).

Interestingly, compared to previous reports of HK action on the MTs of lepidopterans, no diuretic activity of HK was found across the 10^{-12}–10^{-6} M range (Fig. 3A). Previous studies have reported HK to be diuretic in the distal tubule of adult *Manduca sexta,* which lacks the cryptonephric complex and DIP of the larva (Blackburn et al., 1995). HK is also diuretic in larval *Heliothis veriscens* (Seinsche et al., 2000; Scherkenbeck et al., 2009), but the latter authors do not describe which segments of the tubule (as termed by Ramsay, 1976) were used for the fluid secretion assay. Previous studies have demonstrated that segments of the lepidopteran tubule differ in ion and water transport capacity (Irvine, 1969; Ramsay, 1976; Moffett, 1994; Ruiz-Sanchez et al., 2015; O'Donnell and Ruiz-Sanchez, 2015). Therefore, the current study employed isolated DIP preparations to gain insight into HK action on the two cell types comprising the tubule within this region. Our results suggest that HK may be active at doses immediately surrounding 10^{-8} M – however, this narrow active dose range does not extend to 10^{-7} M or 10^{-9} M. CRF-like diuretic peptide has been shown to have dose-dependent receptor-mediated effects targeting discrete transport pathways via discrete second messenger pathways in *Culex* tubules (Clark et al., 1998). The drastically different effects of this peptide on *Culex* tubules took place at 10^{-7} M and 10^{-9} M concentrations. Thus, higher doses of HK may trigger a response in the distal ileac plexus that obliterates its action on this segment at doses $\geq 10^{-7}$ M. The narrow range of the tissue-level response to HK as well as decreased *hk-r* mRNA abundance in HK-treated DIP in vitro, may be indicative of high circulating levels of HK in the DIP of K^+-fed larvae, decreasing the overall response of the tissue to HK.

4.4. HK reduces K^+ secretion by the PCs and Na^+ reabsorption by the SCs, resulting in a Na^+-rich and K^+-poor fluid

Fluid secretion in the MTs of insects is driven by the secretion of ions (Nation, 2016). In plant-eating insects, the majority of the fluid secretion is sustained by the secretion of K^+ into the tubular lumen (Beyenbach and Wieczorek, 2006). However, fluid secretion is also a function of available aquaporins necessary for water to cross cell membranes (see Section 5.3). We suggest that (i) antidiuretic and (ii) K^+-sparing natriuretic actions of HK occurred simultaneously in the isolated DIP of *T. ni*. HK reduces K^+ secretion into the lumen (Figs. 4B and 5A) and decreases the fluid secretion rate (Fig. 3A) in the DIP of *Trichoplusia ni.* Simultaneously though, SCs reabsorb less Na^+ from the fluid, thus leading to natriuresis. Moreover, HK reduced *aqp-1* mRNA abundance, presumably since fewer water channels (aquaporins) are required when ion transport rates are reduced.

4.5. HK is likely to be a part of endocrine response to changes in the dietary intake of Na^+ and K^+

Taken together, the results of the current study demonstrate (i) the localization of HK-R to both PCs and SCs, and (ii) independent alterations in K^+ and Na^+ transport in the two cell types by HK and (iii) changes in mRNA abundance of DIP ion transporters by HK. These results suggest that HK may play an important role as a K^+-sparing natriuretic in the DIP of *Trichoplusia ni* as the dietary K^+ and Na^+ content varies. DIP of *T. ni* fed K^+-rich diet, demonstrate reduced K^+ secretion by the PCs (Kolosov et al., 2018a) and reduced mRNA abundance of *nkcc, nhe-7* and *-8, NKAα,* and *aqp-1* (Kolosov et al., 2018b), all of which are consistent with HK action demonstrated by the current study (Fig. 5A and 6). However, SC function remains unaffected – in particular, reduced Na^+ reabsorption demonstrated in the current study (Fig. 5D) does not take place in K^+-fed larvae (Kolosov et al., 2018b). This may be explained by the fact that both PCs and SCs express HK-R and, thus, the receptor abundance may be regulated by dietary ion loading independently in the two cell types. Therefore, reduced mRNA abundance of *hk-r* observed in the DIP of K^+-fed larvae in the current study (Fig. 1C) may take place in the SCs only, so that they respond less to circulating HK (see Fig. 7 for diagrammatic representation). Lastly, our observations are consistent with high circulating levels of HK in K^+-loaded animals, given that we see similar mRNA changes in the DIP of K^+-fed larvae and in the isolated DIP treated with HK.

Conversely, feeding larvae Na^+-rich diet causes increased reabsorption of Na^+ by the SCs (Kolosov et al., 2018a), which is the opposite of HK action on the SCs demonstrated in the current study (Fig. 5D) and would be consistent with low circulating levels of HK. However, if increased *hk-r* mRNA abundance observed in the DIP of Na^+-fed larvae in the current study (Fig. 1C) takes place in the PCs, then when circulating HK levels are reduced, most HK would be absorbed by the PCs, while SCs would bind less HK, leading to a phenotypic result opposite to that of HK in vitro (see Fig. 7 for diagrammatic representation). Importantly, PCs with increased HK-R levels may be slightly stimulated even by the lower levels of HK, which is consistent with the observation that PCs in Na^+-fed larvae secrete slightly less K^+ (Kolosov et al., 2018b).

What makes this mode of action more plausible is the observation that mRNA abundance encoding for gap junction (GJ) proteins Innexins have been shown to decrease in the DIP of larvae fed ion-rich diets (Kolosov et al., 2018a). Therefore, it seems likely that reduced GJ-coupling between PCs and SCs reduces second-messenger cross talk between the two cell types, allowing HK to target them independently. Adjusting HK-R levels in PCs and SCs independently is also likely to play a role in fine-tuning the targets of HK-based ion transport regulation in ion-loaded animals, depending on which ion is supplied in abundance through the diet.

PCs and SCs of dipterans have been demonstrated to be attuned to different actions of different diuretic hormones by employing different second messenger pathways. Cation transport in the MTs of *Drosophila*, *Culex* and *Aedes* is stimulated by diuretic hormones via cyclic nucleotide action, while kinin-stimulated anion transport is mediated by changes in intracellular calcium (Cabrero et al., 2014; Clark et al., 1998; O'Donnell et al., 1996; O'Donnell et al., 2003; Petzel et al., 1987; Yu and Beyenbach, 2002). Therefore, if HK targets both PCs and SCs independently, its action may be relayed via different second messenger pathways in these cell types, additionally minimising the cross-talk through the gap junction. If HK is to target mainly PCs in the DIP of K^+-fed larvae (as postulated above), it is possible that its action is relayed through a second messenger pathway different from that targeting SCs (see Fig. 7 for diagrammatic representation). In fact, natriuretic K^+-sparing action of HK in the current study is similar to that of serotonin action on *Rhodnius* tubules, where Na^+:K^+ also increased and serotonin action was relayed via cAMP- and PKA-mediated calcium waves (Ianowski and O'Donnell, 2006; Gioino et al., 2014). The same study, however, showed that these calcium waves were able to propagate to the neighbouring cells (presumably) through the gap junction, highlighting the importance of regulation of this structure in the signalling cross-talk between epithelial cells in the MTs (Gioino et al., 2014). Serotonin and cAMP have been shown to stimulate fluid secretion and ion transport in lepidopterans by previous studies (Skaer et al., 2002; Ruiz-Sanchez et al., 2015). Interestingly, cAMP in *Trichoplusia* has been shown to induce K^+-sparing natriuresis, similar to the action of HK on the DIP observed in the current study (Ruiz-Sanchez et al., 2015). Sharing parts of the signalling pathway by HK and serotonin may help explain how HK was able to reduce fluid secretion even in serotonin-stimulated DIP in the current study.

4.6. Significance of findings

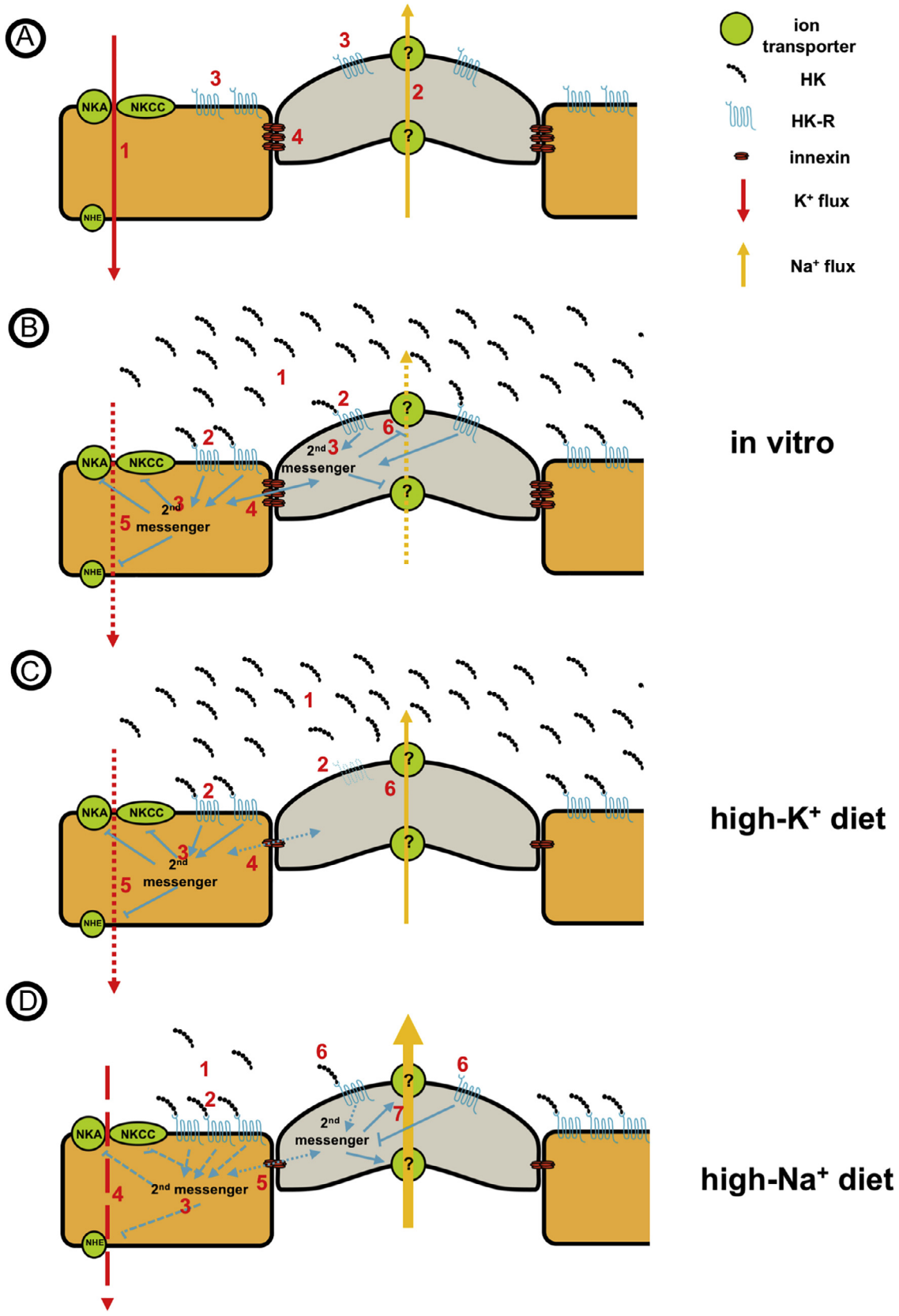
Insect kinins are thought to relay their action on MTs mostly by acting on Cl^- channels and septate junctions adjacent to the SCs (O'Donnell et al., 1996; Beyenbach, 2003; Coast, 2012). Interestingly, kinins of vertebrates are known to relay their effects in ion-transporting epithelia through NKA, NKCC, Cl^- channels, and K^+ channels (Cuthbert, 2001). In the current study we have clearly demonstrated the ability of Helicokinin to impact cation transport in the DIP of a larval lepidopteran and its impact on the expression of NKA, NKCC and NHEs (Fig. 8).

Classical studies indicate that dipteran kinins stimulate stellate-cell based chloride secretion via Ca^{2+} as a second messenger while CAPA acts on the PCs via cAMP/cGMP pathway (O'Donnell et al., 1996). Therefore, there appears to be virtually no cross-talk between the two cell types in dipterans in regards to second messenger pathways or ligands. In contrast, in the current study we have demonstrated the ability of HK to inhibit fluid secretion in 5-HT-stimulated tubules. HK may use a separate second-messenger pathway to reduce fluid secretion in the DIP stimulated by diuretics. The ability to reduce the amount of fluid reabsorbed in this segment of the tubule in ion-loaded animals may be an important adaptation in lepidopteran larvae. The absence of this ability would lead to osmotic backflow of water from the tubule lumen back into haemolymph when cations are reabsorbed, impeding haemolymph clearance by the Malpighian tubules.

The ability of HK to target both PCs and SCs in a non-overlapping manner may give lepidopteran tubules a higher range of fine-tuning ability, making them more successful at regulating the water and ion content of the secreted fluid. What is of particular interest is that HK signalling appears to prompt the DIP to reabsorb more of the ion that is abundantly supplied in the diet – larvae fed high-Na^+ diet reabsorb more Na^+ (putative low circulating HK), while K^+-fed larvae reabsorb more K^+ (putative high circulating HK). This seems intuitive if the function of the ileac plexus (besides fluid secretion) is recycling of cations and water. Thus, HK provides the ability to adjust the $[Na^+]/[K^+]$ ratio of the fluid that passes through the DIP in the larvae that experience Na^+:K^+ imbalance. The antidiuretic effect on observed in isolated DIP may indicate the ability of HK to alter water transport by this segment of the tubule. This phenomenon may provide a means for fluid retention in the lumen of intact DIP of K^+-fed animals, where both Na^+ and K^+ are reabsorbed, and fluid would otherwise naturally follow by osmosis and would also be reabsorbed. Therefore, in an intact tubule the effect of HK on the fluid transport across isolated DIPs may actually result in luminal fluid retention and increased diuresis reported in earlier studies.

4.7. Perspectives and future studies

HK signalling may be behind the ability of lepidopteran larvae to toggle between Na^+ and K^+ secretion in the DIP of the MTs. However, the natriuretic K^+-sparing action of HK may depend on the ability of this peptide to stimulate ion transport in PCs and SCs independently. Therefore, further studies are necessary to provide insight into how the



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signalling pathways and cross-talk between two cells are regulated in HK-stimulated DIP.

Although we did not observe an effect of HK on innexin mRNA abundance, this finding does not rule out GJ closure as one of the mechanisms of HK action. As postulated above, HK action in the DIP of ion-loaded larvae may rely on the minimization of the GJ-based

Fig. 7. Working model of HK action on PCs, SCs and the putative signaling pathways within the two cell types. (A) Distal ileac plexus (DIP) consists of principal cells (PCs, orange) and secondary cells (SCs, grey). In situ, PCs secrete K^+ (1), and SCs reabsorb Na^+ (2). Both cell types express HK-R (3) and are connected by innexin-based gap junctions (GJ) (4). (B) In vitro exposure of the DIP to HK: increased ligand abundance (1) stimulates HK-R on PCs and SCs (2), likely resulting in elevated levels of an intracellular 2nd messenger (3) that readily diffuses through the GJ (4). The 2nd messenger reduces K^+ secretion by the PCs (5), which likely involves Na/K-ATPase, Na/K/Cl co-transporter and Na/H exchangers, as well as reducing Na^+ reabsorption via SCs (6). (C) Mode of action of HK on the in situ DIP in larvae fed high-K^+ diet is similar to the in vitro exposure, except that reduced HK-R abundance at the SCs (2) leaves SCs unstimulated by HK. Reduced GJ coupling to the PCs (inferred from decline in innexin mRNA levels in Kolosov et al., 2018a) minimizes second messenger cross-talk, resulting in unaltered Na^+ reabsorption via SCs (6). (D) Mode of action of HK on the in situ DIP in Na^+-fed animals. Increased number of HK-R (2) in the PCs is consistent with reduced circulating HK levels (1), resulting in low levels of receptor activation and modest increase in 2nd messenger levels (3), resulting in a slight reduction in K^+ secretion by the PCs (4). Reduced GJ cross-talk (inferred from decline in innexin mRNA levels in Kolosov et al., 2018a) allows for independent effects of HK on the SCs, where unaltered receptor levels bind lower circulating levels of HK (as most of it has been cleared by adjacent PCs) (6). Presence of unstimulated HK-Rs leads to increased Na^+ reabsorption by the SCs (7), i.e., the opposite of what is seen in isolated in vitro preparations from control-fed animals in panel (B). Additionally, some increase in Na^+ reabsorption by the SC may be explained by the likely higher levels of Na^+ in the lumen following reabsorption across rectal complex.

Endpoint	dipterans	DIP of larval lepidopteran
Water transport	increase	decrease
Ion secretion	increased Cl^-	K^+-sparing natriuretic
Molecular targets	Cl^- channels aquaporins SC-based septate junctions	NKA, NKCC, NHEs aquaporins HK-R

Fig. 8. Summary of the differences in the effects of kinins on the Malpighian tubules of dipterans and lepidopterans. Kinins have been reported to increase fluid and chloride secretion in the Malpighian tubules of dipterans, specifically targeting aquaporins, chloride channels and secondary-cell based septate junctions. In contrast, lepidopteran kinin is reported to be a potassium-sparing natriuretic and has been reported to reduce fluid secretion, depending on the methodology and life stage. We have demonstrated that reduction in ion-secretory transporters and aquaporin-1 occur in HK-treated Malpighian tubules of *Trichoplusia ni*.

coupling between the two cell types in order to affect them individually. GJ can exhibit rectification and can be gated by a variety of factors including Ca^{2+}, pH and voltage (Oshima, 2014; Skerrett and Williams, 2016). Thus, the effects of HK on the opening-closure of the GJ will have to be investigated separately using electrophysiological techniques.

Lastly, kinins have been proposed to open up the paracellular Cl^- shunt through their action on the septate junctions surrounding stellate cells in the MTs of mosquitoes (Beyenbach and Piermarini, 2011). Mechanistic studies regarding Cl^- transport and septate junction in the MTs of the larval lepidopterans are much needed to understand the intricacies of the kinin-based regulation of ion transport in this diverse and economically-important group of insects.

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