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REVIEW

Blending physiology and RNAseq to provide new insights into regulation of epithelial transport: switching between ion secretion and reabsorption

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ABSTRACT

This Review addresses the means by which epithelia change the direction of vectorial ion transport. Recent studies have revealed that insect Malpighian (renal) tubules can switch from secreting to reabsorbing K^+. When the gut of larval lepidopterans is empty (during the moult cycle) or when the larvae are reared on K^+-deficient diet, the distal ileac plexus segment of the tubule secretes K^+ from the haemolymph into the tubule lumen. By contrast, in larvae reared on K^+-rich diet, ions and fluid are reabsorbed from the rectal lumen into the perinephric space surrounding the cryptonephridial tubules of the rectal complex. Ions and fluid are then transported from the perinephric space into the lumen of the cryptonephridial tubules, thus supplying the free segments of the tubule downstream. Under these conditions, some of the K^+ and water in the tubule lumen is reabsorbed across the cells of the distal ileac plexus, allowing for expansion of haemolymph volume in the rapidly growing larvae, as well as recycling of K^+ and base equivalents. RNA sequencing data reveal large-scale changes in gene transcription that are associated with the switch between ion secretion and ion reabsorption by the distal ileac plexus. An unexpected finding is the presence of voltage-gated, ligand-gated and mechanosensitive ion channels, normally seen in excitable cells, in Malpighian tubules. Transcriptomic surveys indicate that these types of channels are also present in multiple other types of vertebrate and invertebrate epithelia, suggesting that they may play novel roles in epithelial cell signalling and regulation of epithelial ion transport.

KEY WORDS: Transporting epithelia, Electrophysiology, Next-generation sequencing, Voltage-gated ion channels, Transcriptomic survey

Introduction: secretion and reabsorption by epithelia

Epithelia are sheets of cells that form a barrier between the environment and the extracellular fluids. The cells which form the epithelium are joined by junctional complexes that provide a selectively permeable barrier between the solutions bathing each surface of the epithelium and thus mark the boundary between the apical and basolateral surfaces of each cell. The hallmark of epithelial cell morphology is the asymmetric distribution of transport proteins in the apical and basolateral membranes. This asymmetry polarizes the cell so that it is capable of vectorial transport of specific solutes and osmotically obliged water from one compartment to another. Transepithelial transport of solutes is often an active process as shown in Fig. 1. Secretion of ions across animal epithelia is exemplified by NaCl secretion by the shark rectal gland, in which primary active transport by the basolateral Na^+/K^+-ATPase couples the hydrolysis of ATP to the entry of K^+ into the cell and the exit of Na^+ (Fig. 1A). The resultant lowering of Na^+ activity inside the cell energizes secondary active transport of Cl^- into the cell through the $Na^+/K^+/2Cl^-$ co-transporter. K^+ is then recycled by leakage through basolateral K^+ channels which contribute to the inside-negative basolateral membrane potential, whereas Cl^- exits the cell through apical channels into the lumen of the gland, thus contributing to a favourable lumen-negative transepithelial electrical potential which drives movement of Na^+ through the paracellular pathway from blood to lumen. Reabsorption of NaCl by epithelia can be accomplished by rearrangement of the same suite of transporters as for secretion, as in secondary active transport of Cl^- in the thick ascending limb of the loop of Henle in the mammalian kidney (Fig. 1B). The Na^+/K^+-ATPase is again sited in the basolateral membrane, but in this case the $Na^+/K^+/2Cl^-$ co-transporter and K^+ channels are located in the apical membrane and the Cl^- channels are in the basolateral membrane.

Switching between ion secretion and ion reabsorption by an epithelium is thus typically associated with a rearrangement of ion transporter distribution such as that diagrammed in Fig. 1. Such a switch might therefore be expected to be relatively slow, consistent with the time required for synthesis and/or insertion of transporter proteins into the apical and basolateral cell membranes. For instance, even though the gill epithelium of salinity-acclimated euryhaline teleosts can rapidly sense changes and mount a rapid physiological response (Kültz, 2015), transcriptomic studies suggest that up to 21 days is required for a full restructuring of the gill epithelium (Bonzi et al., 2021). Estuarine fishes such as *Fundulus heteroclitus* can quickly adjust to extremes in environmental salinity (Whitehead et al., 2012). And although *Fundulus* can take 6–48 h to fully adjust gill epithelium morphology (and presumably function) (Marshall et al., 1999; Whitehead et al., 2012), studies using their opercular epithelia demonstrated that this estuarine species can rapidly (~45–60 min) adjust their opercular epithelium conductivity (Wood and Marshall, 1994; Marshall et al., 2000; Daborn et al., 2001). Thus, although an immediate adjustment of ion transport in the fish gill can take place rapidly, a complete '180 degree' turnaround in its function takes days, owing primarily to the notion that different subsets of epithelial subtypes are responsible for giving rise to ion-secreting and ion-reabsorbing cells (Sakamoto et al., 2001; Evans et al., 2005). In contrast, studies of the Malpighian (renal) tubule epithelium in the cabbage looper caterpillar, *Trichoplusia ni*, have shown that a switch between ion reabsorption and secretion can be accomplished in less than 10 min (Kolosov et al., 2018a).

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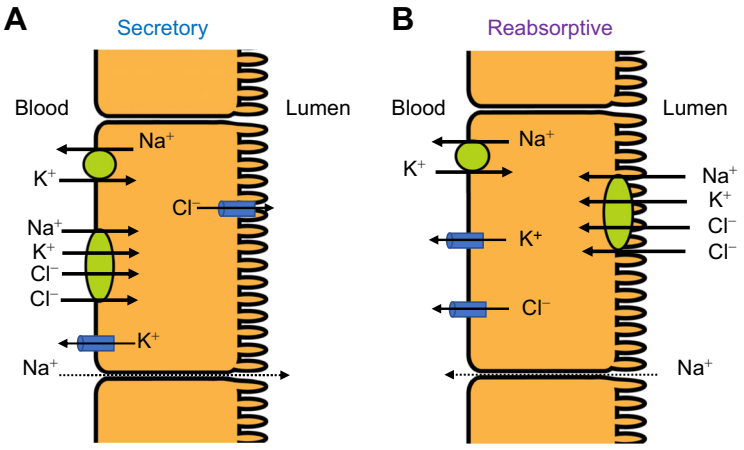

Fig. 1. Model for secretion and reabsorption of NaCl in animal epithelia involving active ion transport by Na^+/K^+-ATPase and co-transport via $Na^+/K^+/2Cl^-$ co-transporter. (A) Arrangement of transporters for NaCl secretion exemplified by shark rectal gland. (B) Arrangement of transporters for NaCl reabsorption by the thick ascending limb of the loop of Henle of the mammalian kidney. Adapted from Epstein et al. (1983).

As discussed below, recent work has indicated a role for voltage-dependent ion channels in regulating the switch (Kolosov et al., 2021; Kolosov and O'Donnell, 2019c).

In insects, the Malpighian tubules and hindgut provide the functional analogue of the vertebrate kidney. As for other tubular organs such as the kidney, pancreas and liver in vertebrates, the tubular morphology of the insect Malpighian tubule provides an increased surface area that enhances interaction with the fluids bathing the exterior and luminal surfaces and facilitates homeostatic transport of solutes and water (Jung et al., 2005). The development of the tubule has been well described in the *Drosophila* larva, in which the Malpighian tubules consist of two pairs of epithelial tubes that bud from the hindgut during embryogenesis (Jung et al., 2005). In each bud, a specialized cell called the tip cell is singled out by activation of the mechanosensitive Notch signalling pathway early in development (Jung et al., 2005). The tip cell is required for the normal pattern of cell division in the development of the tubules (Skaer, 1989). It is worth noting that the Notch pathway is also implicated in regulating the interconversion of intercalated cells and principal cells via a transitional cell type in the collecting ducts of the mammalian kidney nephron (Humphreys, 2018; Park et al., 2018). Another similarity with the vertebrate nephron is the division of the Malpighian tubules of many species into multiple segments that are adapted for specific functions. In *Drosophila* and *Rhodnius*, downstream segments are implicated in reabsorption of K^+ (Maddrell, 1978; O'Donnell and Maddrell, 1995). In other insects, such as locusts, reabsorption of solutes and water is accomplished primarily by the hindgut (Phillips et al., 1994). Tubules of the water boatman have four segments differentiated on the basis of rates of ion transport per unit length of tubule and capacity for secretion of alkaline equivalents and acidic dyes (Cooper et al., 1989), whereas larvae of many lepidopteran species have at least five morphologically distinct segments that differ functionally with regards to transport of K^+, Na^+, Cl^-, acid–base equivalents, toxins and uric acid (Irvine, 1969; Kolosov and O'Donnell, 2019c; Kolosov and O'Donnell, 2020; O'Donnell and Ruiz-Sanchez, 2015; Ruiz-Sanchez et al., 2015). Regional specializations of Malpighian tubules of insects can thus rival those of the vertebrate nephron. Even within tubule regions of apparently uniform structure, there may be dramatic functional differences. The upper region of the lower tubule of *Rhodnius prolixus* is osmotically very permeable and plays no part in reabsorbing KCl from the primary excretory fluid, whereas morphologically identical cells in the lowermost 30% of the length of the lower tubule are osmotically impermeable and capable of exceptionally high rates of KCl reabsorption from the tubule lumen when stimulated (Maddrell, 1978).

Two types of epithelial cell are common to the Malpighian tubules of endopterygote insects such as flies, ants, beetles and butterflies/moths. Principal and secondary cells have different developmental origins: in *Drosophila*, principal cells develop from the organ primordium, whereas secondary cells originate from central visceral mesoderm recruited to the tubule during organogenesis (Denholm, 2013). The maturation of principal and secondary cells in the Malpighian tubules of insects which have them is governed by two distinct transcription factors: *cut* and *tiptop/teashirt*, respectively (Denholm, 2013). The larger and more numerous principal cells are involved in electrogenic cation (Na^+, K^+) secretion that is energized by an apical vacuolar-type H^+-ATPase. Cation transport through principal cells is stimulated by diuretic hormones (DHs) which include functional homologs of the vertebrate corticotropin releasing factor (CRF) and calcitonin gene-related peptide (CGRP) families, as well as the capa peptides which share homology with the vertebrate neuromedin U peptides (Cabrero et al., 2002; Coast et al., 2001; Terhzaz et al., 2012). The smaller, less numerous secondary cells are intercalated among the tubule principal cells. In tubules of fruit flies, Cl^- and water flow through secondary cells that are called stellate cells for their distinctive shape (Cabrero et al., 2020, 2014). Cl^- transport through stellate cells is increased by kinin neuropeptides and the biogenic amine tyramine acting through increases in intracellular Ca^{2+} (Blumenthal, 2003; Cabrero et al., 2014). A striking difference in the control of tubule function is seen in beetles (Order Coleoptera). Beetle tubules lack the kinin pathway and the secondary cells are the site of action of CRF-related hormones DH37 and DH47 which stimulate K^+ flux via a cAMP-dependent mechanism (Koyama et al., 2021). In conjunction with the presence of potent antidiuretic factors that act upon beetle tubules, these differences may provide tighter control of excretory water loss in beetles, a group which thrives in osmotically stressful biomes (Koyama et al., 2021).

Changes in dietary ion availability mediate the switch from ion secretion to reabsorption in caterpillar Malpighian tubules

Lepidopteran larvae exhibit the so-called cryptonephric condition, where the distal ends of the Malpighian (renal) tubules are applied to the outer surface of the rectum and enveloped by a perinephric membrane, forming the rectal complex (Fig. 2). Emerging from the rectal complex are the free segments of the tubule; whereas the cryptonephric tubules are bathed in the fluid contained with the

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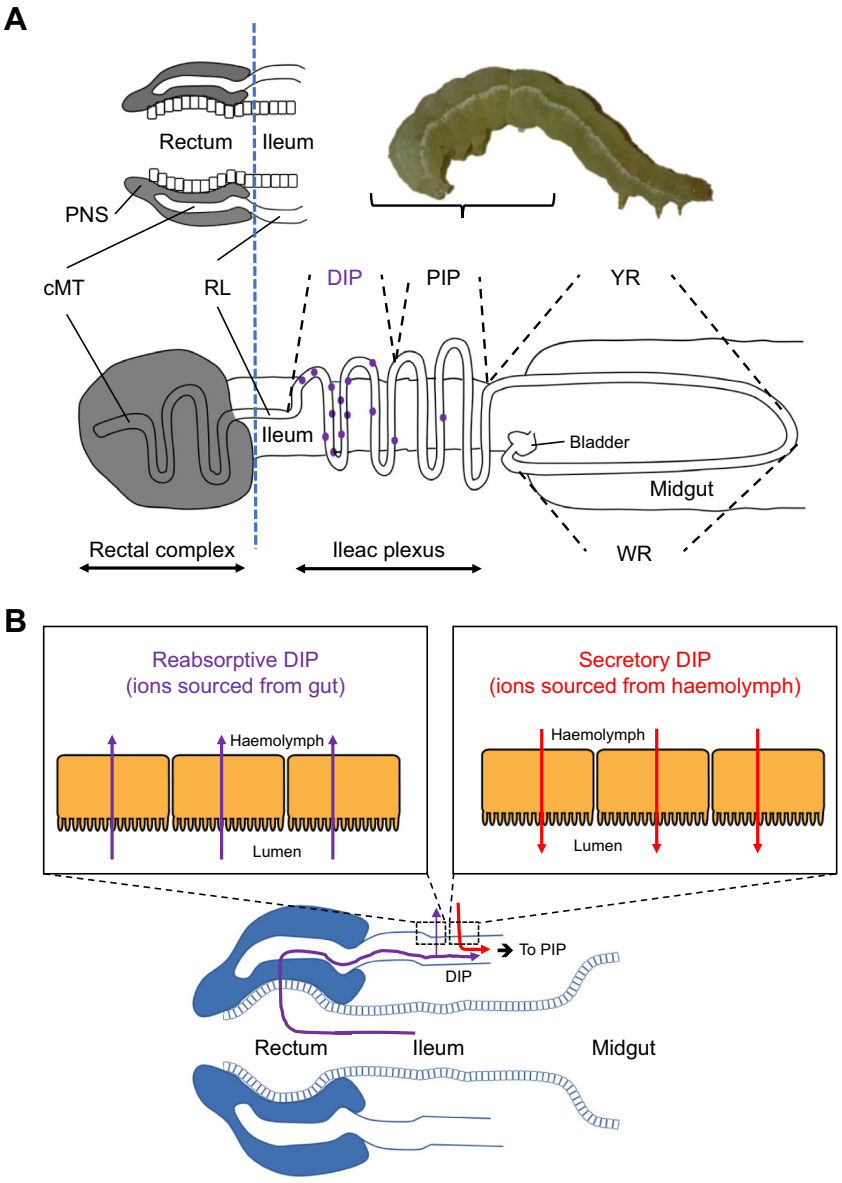

Fig. 2. Anatomical arrangements of caterpillar Malpighian tubules, their associations with regions of the gut and the functional switch between two sources of ions – haemolymph and gut. (A) In the larva, the distal end of each tubule, termed the cryptonephridial Malpighian tubule (cMT), lies within the rectal complex (in longitudinal section, dashed blue line, top panel). The short segment termed the rectal lead (RL) connects each cMT to the 'free' tubule, of which there are four distinct regions. The downstream distal ileac plexus (DIP, purple) is arranged close to the ileum and contains most of the secondary cells (purple circles). The proximal ileac plexus (PIP) then terminates in the downstream yellow and white regions (YR and WR, respectively) that are closely applied to the posterior midgut. The Malpighian tubule (MT) then terminates in the urinary bladder, which empties into the midgut–hindgut junction. The DIP, PIP, YR and WR show heterogeneity in ultrastructure and gene expression. PNS, perinephric space. (B) Larvae have two options as a source of ions and water for secreting fluid into the MT. The reabsorptive DIP epithelium (in purple) sources ions and water from the gut by way of the cMT when the caterpillars are well fed with ion-rich diets. Some ions reabsorbed from the gut are transferred into the haemolymph across the free regions of the MT. The secretory DIP epithelium (in red) sources ions and water from the larva's haemolymph, when dietary ions and water are unavailable (e.g. during moulting) or are in short supply.

perinephric space, the free tubules are bathed in haemolymph. When the larvae are not feeding (e.g. during the moult cycle) or when the diet is deficient in K^+, the distal ileac plexus region of the free tubule functions in secretory mode: secretion of K^+, Na^+, Cl^- and osmotically obliged water by the principal cells drives fluid secretion, flushing the tubule lumen so that toxins and waste can be cleared from the haemolymph. When the larvae are feeding on K^+-rich foods, transport of K^+ and HCO_3^- into the midgut lumen elevates the pH of the gut contents, breaking tannin–protein bonds to enhance digestion of the plant proteins in the diet (Berenbaum, 1980; Moffett, 1994). Water and ions are subsequently reabsorbed downstream from the rectal lumen and secreted into the lumen of the cryptonephridial segment of the Malpighian tubule. Some of the water and ions are then reabsorbed into the haemolymph across the downstream free segments of the tubule, allowing both expansion of haemolymph volume in rapidly growing larvae and recycling of K^+ and base (HCO_3^-). Switching between secretion and reabsorption by the distal ileac plexus means that either the haemolymph or the hindgut can provide a source of ions and water for fluid secretion by the tubules (Fig. 2). One advantage of this arrangement is that it allows the haemolymph to be continuously cleared of toxins and metabolic waste irrespective of dietary ion and water intake. Recent studies have examined the switch between the two ion transport modes using tissue and cell-specific functional assays, immunohistochemical identification of ion transporters, electron microscopy, quantitative PCR and transcriptomic RNAseq approaches. The following sections describe the suite of cellular and molecular changes that occur during the switch from ion secretion to reabsorption.

There is a pronounced downregulation of transcript abundance of secretory ATPases and K^+ transporters in principal cells of the distal ileac plexus during the switch to reabsorption elicited by increased levels of K^+ in the diet (Fig. 3). Shutting down secretory ATPases and changes in abundance of passive transporters allows ion transport to reverse from ion secretion to reabsorption within 24 h in response to increased ion availability in the diet (Kolosov et al., 2019a). Some transporters such as the potassium/chloride co-transporter (KCC) and sodium/proton exchangers (NHEs) may reverse the direction in which they transport ions when the tubule switches from ion secretion to reabsorption.

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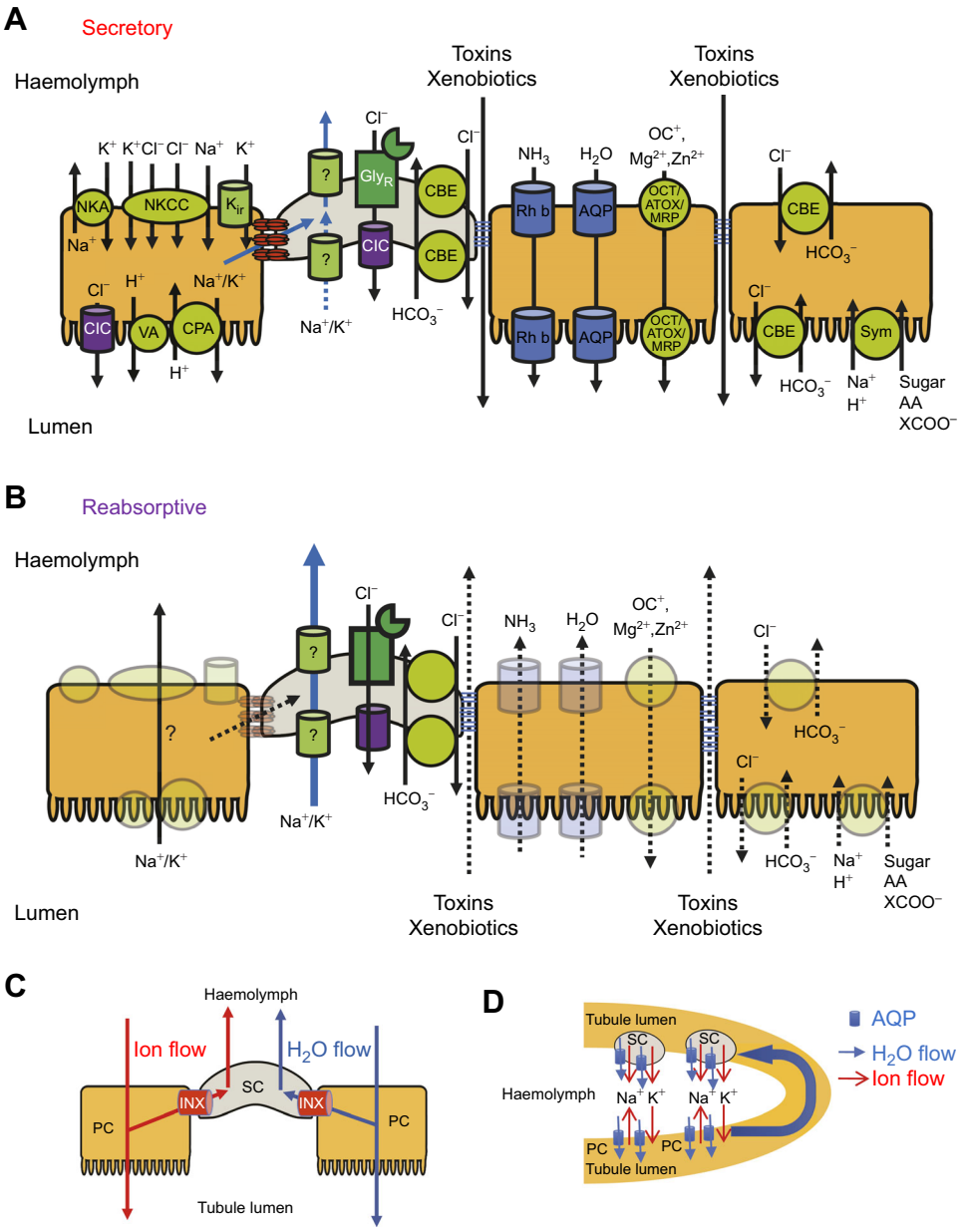

Fig. 3. Cellular and molecular changes between the distal ileac plexus in secretory mode versus reabsorptive mode. (A) In secretory mode, K^+ is secreted by the principal cells and reabsorbed by the secondary cells. K^+ secretion is enabled by a combined action of basolateral Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and inward-rectifier K^+ channels (K_{ir}) of the principal cells and apical V-type H^+-ATPase (VA) and cation–proton antiporters (CPA). Some potassium ions are diverted (blue arrows) from surrounding principal cells to the secondary cell via gap junctions (in red) and reabsorbed back into the haemolymph via the basolateral membrane of the secondary cells. Apical entry of K^+ and Na^+ from the tubule lumen through unspecified channels (labelled '?') is also possible (dashed blue arrow). Cl^- is secreted by both principal cells and secondary cells by the combined action of NKCC, Cl^- channels (ClC) and Cl^-/HCO_3^- exchangers (CBE). Base reabsorption is connected with Cl^- secretion via CBEs. Reabsorption of sugars, amino acids and carboxylates is enabled by symporters (Sym). Water is transported by aquaporins (AQP) and ammonia is transported by Rhesus protein b (Rh b). Organic cations and heavy metals are secreted by organic cation transporters (OCT), multidrug resistance proteins (MRP) and metal trafficking proteins (ATOX). Toxins and xenobiotics with no specific transporter can move into the lumen via the paracellular pathway through the septate junctions between epithelial cells. (B) In reabsorptive mode, both principal and secondary cells reabsorb K^+, although exact molecular mechanisms of this process remain largely unstudied. Dashed black lines denote reduced rates of transport through the indicated pathway or transporter. All K^+-secretory transporters are transcriptionally downregulated. Paracellular permeability and water permeability are downregulated as well to prevent the back-flux of water and toxins from the tubule lumen. Secondary processes connected to ion secretion in this segment (e.g. excretion of nitrogenous waste and nutrient reabsorption) are downregulated as well. Transcriptomic evidence suggests that while Cl^--coupled base reabsorption via principal cells is minimized during the switch-over from ion secretion to ion reabsorption, the secondary cells provide a separate pathway for base reabsorption. Gap junctional coupling between principal and secondary cells also decreases. (C) In secretory mode, increased gap junctional coupling provides a way for the secondary cells (SC) to return ions and water (secreted by principal cells, PC) back into the larva's haemolymph. (D) The MTs clear the haemolymph of waste and toxins more efficiently because of the presence of recycling loops of ions and water (in blue) as the fluid flows through the tubule.

The mRNA abundance of xenobiotic/metal transporters in the distal ileac plexus also decreases substantially when larvae are reared (for ∼2 weeks) on K^+-rich or Na^+-rich diets and are thus in reabsorptive mode (Kolosov et al., 2019a). Similarly, transcript abundance of the ammonium transporter Rhesus protein b (Rh b), and components of transporters for H^+- and Na^+-dependent reabsorption of trehalose and amino acids decreases. Together, the results suggest that less toxin excretion and nutrient reabsorption occurs when the distal ileac plexus is reabsorbing K^+ and/or Na^+ and water.

Gap junctional coupling between principal cells and secondary cells is also downregulated when larvae are reared on K^+-rich diet (for ∼2 weeks) and the principal cells are reabsorbing (Kolosov et al., 2018a). By contrast, when the tubules are in secretory mode, principal cells of the distal ileac plexus secrete K^+ whereas adjacent secondary cells reabsorb K^+, part of which is routed from the neighbouring principal cells through gap junctions (Fig. 3C). The juxtaposition of secondary cells and principal cells in the loops of tubule within the ileac plexus allows for local recycling of ions and water; K^+ reabsorbed by secondary cells may be secreted back into the lumen by principal cells of a juxtaposed loop of tubule (Fig. 3D). This recirculation system for Na^+, K^+ and osmotically obliged water may enhance clearance of waste and toxins from the haemolymph.

The switch from ion secretion to reabsorption is also associated with a reduction in paracellular permeability and water permeability. Measurements using a fluorescent extracellular permeability marker (FITC-dextran) revealed that paracellular permeability in the distal ileac plexus is reduced in larvae reared (for ∼2 weeks) on Na^+-rich or K^+-rich diet (Kolosov et al., 2019b). Electron micrographs show that the distal ileac plexus epithelium of larvae fed ion-rich diets (for ∼24 h) has longer and more convoluted septate junctions between adjacent principal cells, consistent with the observed reduction in paracellular permeability (Kolosov et al., 2019b). Reduction in paracellular permeability may be aimed at minimizing the back-flux of organic ions, waste metabolites and secreted toxins into the haemolymph. Concomitant reductions in water permeability will limit water flux accompanying K^+ reabsorption (Kolosov and O'Donnell, 2019b), thus retaining water in the lumen so that the concentrations of toxins and waste remain low, further limiting diffusive back-flux of these solutes into the haemolymph, as does the reduction in expression of xenobiotic and metal transporters.

From the experimental evidence described above, it is evident that the switch from ion secretion to ion reabsorption can take place in as little as 24 h and as long as 2 weeks, where transcriptional changes (presumably aimed at restructuring the Malpighian tubule epithelium) take place, coinciding with the changes in ion transport, paracellular permeability and water permeability. Despite all of these long-term changes, the switch from ion reabsorption to ion secretion can take as little as ∼10 min, indicating that although the phenotype of the epithelium is adjusted, it is not restructured permanently. Not much is known about the time course of this process as animal dissection, isolation and mounting of the preparation takes at least 10 min. Time course measurements will require an intricate setup with intracellular electrodes and/or luminal perfusions. We know that the first thing a tubule experiences upon excision from the animals is a drop in hydrostatic pressure in the lumen and a subsequent drop in fluid flow. We believe these may be the triggers behind the short-term changes in the direction of ion transport, which act through voltage-gated ion channels, as when the latter are blocked, robust ion secretion reverts back to ion reabsorption.

Several regions of the free Malpighian tubules can switch between Cl^- secretion and Cl^- reabsorption within minutes in a manner similar to that seen for cation transport reversal in the distal ileac plexus (Kolosov and O'Donnell, 2020). Within the distal ileac plexus, both principal cells and secondary cells secrete Cl^-, although principal cells can switch to Cl^- reabsorption when the bathing saline Cl^- concentration is increased. The mechanism underlying the reversal of Cl^- transport from secretion to reabsorption is unclear. However, an important role of the secondary cells in the distal ileac plexus is to provide a pathway for HCO_3^- reabsorption that is independent of the principal cells, which may switch between ion secretion (Na^+, K^+, Cl^-) and reabsorption depending upon dietary ion availability. HCO_3^- reabsorption likely involves chloride/bicarbonate exchangers (CBEs) in the distal ileac plexus (Kolosov and O'Donnell, 2020). Application of drugs which block Cl^- channels or CBEs leads to an increase in lumen pH, consistent with coupling between Cl^- secretion into the lumen and HCO_3^- reabsorption into the haemolymph (Kolosov and O'Donnell, 2020).

Neuropeptides may orchestrate a well-coordinated switch between ion secretion and reabsorption and provide potential links for autocrine and paracrine control of Malpighian tubule function

The widespread changes in cell structure and function in the distal ileac plexus during the switch from ion secretion to reabsorption may be orchestrated, in part, by helicokinins, which are members of the kinin family of myotropic and diuretic peptides. Helicokinin I acts as a K^+- and Cl^--sparing natriuretic on the distal ileac plexus (Kolosov and O'Donnell, 2019a; Kolosov and O'Donnell, 2020) and produces many of the effects seen during the switch from ion secretion to reabsorption when larvae are fed ion-rich diets: helicokinin I reduces K^+ and Cl^- secretion by principal cells and Na^+ reabsorption by secondary cells and it also reduces water permeability and paracellular junction permeability. These effects may be explained in part by reduced transcript abundance for a Na^+/K^+/2Cl^- co-transporter, two NHEs (nhe-7 and -8) as well as the alpha subunit of the Na^+/K^+-ATPase and aquaporin 1 (Kolosov and O'Donnell, 2019a). The effects of helicokinin I on both principal and secondary cells of the distal ileac plexus differ dramatically from the diuretic effects of kinins on dipteran Malpighian tubules. In tubules of fruit flies, for example, kinins act specifically on the stellate cells to increase fluid secretion through effects on Cl^- channels and aquaporins (Cabrero et al., 2020, 2014).

Controlling the direction of vectorial ion transport: voltage-gated, mechanosensitive and ligand-gated ion channels are implicated in the switch between ion secretion and ion reabsorption

The presence of voltage-gated ion channels in non-excitable cells challenges the paradigm that such channels are involved primarily in generating signals in nervous tissues (Pitt et al., 2020). RNAseq has identified multiple voltage-gated, mechanosensitive and ligand-gated ion channels in the Malpighian tubules of *T. ni* (Kolosov et al., 2019a,c) (Fig. 4). A recent study has shown that Ca^{2+} entry through voltage-gated calcium channels enables robust ion secretion by the distal ileac plexus (Kolosov et al., 2021) (Fig. 4A). Immunohistochemistry has revealed voltage-gated calcium channels in both apical and basolateral membranes of the principal cells. In addition, transcript abundance of the voltage-sensing subunit of a voltage-gated Ca^{2+} channel is downregulated in the distal ileac plexus of larvae reared on a K^+-rich diet, and this

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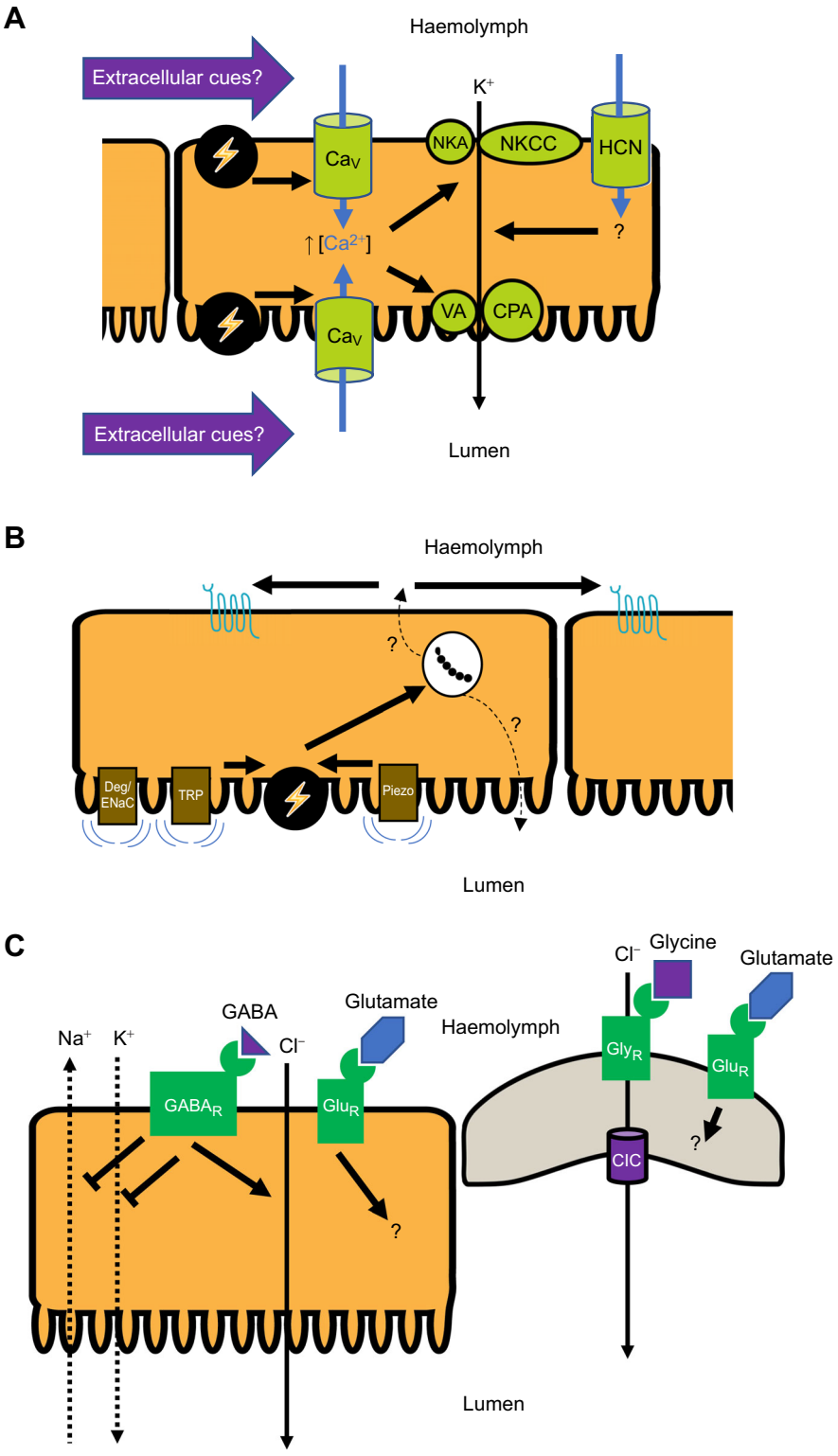

Fig. 4. Voltage-gated, mechanosensitive and ligand-gated ion channels regulate ion transport in the Malpighian tubules of larval lepidopterans. (A) Activity of voltage-gated Ca_V1 channel and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels is necessary for robust secretion in the Malpighian tubules (MT) of larval lepidopterans. Both are thought to stimulate active ion transport machinery in the distal ileac plexus. Ca_V1 relays its action via an increase in intracellular [Ca^{2+}]. The contribution of most voltage-gated ion channels to the regulation of ion transport remains unstudied (see text). (B) Mechanosensitive degenerin/epithelial Na^+ channels (Deg/ENaC), transient receptor potential (TRP) and piezo channels are expressed in the MT and may translate changes in hydrostatic pressure and/or fluid flow in the lumen into changes in membrane potential (flash sign in a black circle), which can be detected by the voltage-gated ion channels. Transcriptomic evidence also suggests that several neuropeptides (indicated by black dots in a white circle) are encoded by the epithelia and that their release may be used for autocrine/paracrine regulation of ion transport by as yet unknown pathways (indicated by '?'). (C) Several ligand-gated ion channels and metabotropic receptors for the same ligands have been detected in larval MTs. Stimulation of MTs with glycine (purple square) leads to increased Cl^- secretion by the secondary cells without affecting principal cells. In contrast, application of γ-aminobutyric acid (GABA, purple triangle) reduces K^+ secretion and Na^+ reabsorption, and increases Cl^- secretion by the principal cells. The effects of glutamate (blue hexagon) on MT ion transport remain unstudied, despite the fact that several glutamate receptors (GluR) are expressed in the MTs.

change, together with other transcriptional changes, enables the switch to ion reabsorption. *In vitro*, pharmacological inhibition of the voltage-gated Ca^{2+} channel Ca_V1 reduces intracellular Ca^{2+} concentration and reverses the direction of K^+ transport from secretion to reabsorption (Kolosov et al., 2021). Although Ca^{2+} entry through voltage-gated channels is clearly required for ion secretion by the tubule, an important question remains: as the tubules are non-excitable, how do voltage-gated calcium channels receive the depolarizing stimulus necessary to open and support Ca^{2+} influx in the absence of an action potential?

There are several mechanisms by which changes in hydrostatic pressure, ion concentration or pH inside the tubule lumen in response to dietary ion loading could lead to the switch between secretory and reabsorptive modes of the distal ileac plexus. One of these is a potential link of voltage-gated ion channels and mechanosensation in the tubule (Fig. 4B). There are three

mechanosensitive channels expressed in the distal ileac plexus: piezo, transient receptor potential channel painless (TRP painless), and a member of the degenerin/epithelial Na^+ channels (Deg/ENaC) family (Kolosov et al., 2019a). In studies of other epithelia, stretch activation of piezo in alveolar type I cells of the mammalian lung leads to increases in intracellular Ca^{2+} concentration and release of ATP that in turn acts as a paracrine stimulation of surfactant secretion by alveolar type II cells (Diem et al., 2020). Piezo1 is also implicated in urinary dilution and the decrease in urea concentration in the kidney following rehydration in mammals, possibly through Piezo1-dependent changes in intracellular [Ca^{2+}], leading to decreased cAMP levels and enhanced retrieval of aquaporin 2 from the plasma membrane (Martins et al., 2016). An important aspect of future work will be to determine how piezo channels in the distal ileac plexus of caterpillar Malpighian tubules are activated so as to lead to Ca^{2+} entry. Increased fluid secretion rates in response to higher levels of intracellular Ca^{2+} will require both higher levels of active ion transport by the basolateral Na^+/K^+-ATPase and the apical V-ATPase in the principal cells, as well as increased K^+ and Cl^- entry through channels, co-transporters or exchangers. In this context, it is worth noting that there are Ca^{2+}-gated K^+ and Cl^- channels expressed in the distal ileac plexus (Kolosov and O'Donnell, 2020), and these can be activated by fluctuations in intracellular calcium concentration. In addition to piezo, TRP painless channels are non-selective cation channels which could also mediate Ca^{2+} entry in response to membrane stretch (Goodman, 2003). Similar to piezo, studies connect TRP V family ion channels to Ca^{2+}-mediated activation of BK channels in K^+-secreting renal collecting duct cells in mice (Li et al., 2018). Although Deg/ENaC channels are not voltage gated and are sodium selective (Eastwood and Goodman, 2012), enhanced Na^+ current through the channel could lead to a depolarization that would open voltage-gated Ca^{2+} channels in the apical membrane and thus lead indirectly to a Ca^{2+} influx.

RNAseq has also revealed transcripts for hyperpolarization-activated cyclic nucleotide gated (HCN) channels in the distal ileac plexus. HCN channels are unusual in that they are activated by hyperpolarization, leading typically to an influx of Na^+, primarily, with smaller amounts of K^+ and Ca^{2+} (Wahl-Schott and Biel, 2009) (Fig. 4A). HCN channels are also regulated by intracellular and extracellular pH, raising the possibility that pH changes in the tubule lumen during feeding could alter their opening. HCN channels have been directly implicated in the regulation of ion transport by the distal ileac plexus; application of the HCN blocker ZD7288 to isolated tubules results in a switch from K^+ secretion to K^+ reabsorption by the principal cells, while secondary cells remained unaffected (Kolosov and O'Donnell, 2019c). Current flow through voltage-dependent HCN channels could also be altered as a secondary response to the operation of electrogenic chloride/bicarbonate exchangers ($Cl^-/2HCO_3^-$). For example, increased bicarbonate concentration in fluid reabsorbed from the rectal lumen and then into the cryptonephridial tubules in feeding larvae could increase lumen pH in the distal ileac plexus, thus modulating pH-sensitive HCN channels.

Several inward-rectifier K^+ channels are also expressed in the region of the tubule that switches between ion secretion and ion reabsorption. Basolateral $K_{ir}1$ channels that are blocked by the small molecule inhibitor VU591 play an important role in enabling secretion (Kolosov et al., 2018b). As in vertebrate epithelia such as the kidney nephron, leakage of current through paracellular shunt pathways allows for crosstalk between the apical and basolateral cell membranes of Malpighian tubules (Pannabecker et al., 1992) (Fig. 5). Back-flux of K^+ from the tubule lumen to the haemolymph through the paracellular pathway provides a mechanism by which the basolateral membrane voltage can generate a current that flows through the septate junctions and charges up the apical membrane, and vice versa (Fig. 5). For example, depolarization of the basolateral membrane by K^+ inflow through K_{ir} channels in the principal cells could lead through crosstalk to a depolarization of the apical membrane that may, in turn, open voltage-gated Ca^{2+} channels, allowing an influx of Ca^{2+} and consequent intracellular signalling. The contributions of other K_{ir} channels to ion transport regulation and their cellular/membrane localization remain unstudied in the distal ileac plexus of *T. ni*.

The paracellular pathway provides another means by which voltage-gated ion channels may regulate vectorial ion transport in the tubules. MTs of *Aedes aegypti* transport Cl^- (in part) via septate junctions (Beyenbach, 2003), which can in principle offset the apical membrane potential enough for voltage-gated ion channels to react to this change.

There are receptors for multiple neurotransmitters in the distal ileac plexus, including glycine, γ-amino butyric acid (GABA), serotonin, glutamate (metabotropic and ionotropic), acetylcholine and catecholamines (α1A adrenergic) (Kolosov et al., 2019a). Application of serotonin to the yellow region isolated together with the ileac plexus increases the secretion of fluid and Na^+ and decreases secretion of K^+ (Ruiz-Sanchez et al., 2015). Both glycine and GABA alter Cl^- secretion by the distal ileac plexus. Glycine stimulates Cl^- secretion by the secondary cells (Kolosov and O'Donnell, 2019c), whereas GABA acts upon the principal cells, reducing K^+ secretion and Na^+ reabsorption and increasing Cl^- secretion (Kolosov and O'Donnell, 2019c). The advantage of such a multiplicity of messengers is unclear, but it is worth noting that multiple peptides and amines also stimulate fluid secretion in tubules of the adult tobacco hawkmoth, *Manduca sexta* (Skaer et al., 2002). In view of the large number of compounds that stimulate fluid secretion by the tubules, Skaer et al. (2002) suggest that rather than single compounds eliciting stereotypical responses from a single tissue, there is continuous broadcast of information in the form of a 'chemical language' within the extracellular fluids; this language coordinates the functions of multiple tissues, including the Malpighian tubules.

The discussions above indicate that there are multiple mechanisms which play a role in the switch between ion secretion and ion reabsorption. The switch takes place under physiologically relevant conditions (e.g. ingesting ion-rich diet, moulting), and some of the above-described mechanisms are able on their own to induce a change in the direction of ion transport. For instance, pharmacological closing of gap junctions (Kolosov et al., 2018a) and inhibition of HCN channels (Kolosov and O'Donnell, 2019c) induce switching from ion secretion to ion reabsorption in the distal ileac plexus. In contrast, pharmacological inhibition of Ca_V1 (Kolosov et al., 2021), application of HK-1 (Kolosov and O'Donnell, 2019a) and stimulation of ligand-gated ion channels (Kolosov and O'Donnell, 2019c, 2020) regulate transport of specific ions without switching the distal ileac plexus between ion secretion and ion reabsorption. There are several potential reasons for this complexity. Firstly, the magnitude of the cellular response may matter – blocking gap junctions and hyperpolarization-activated channels is likely to change the membrane potential of the whole cell and cause a downstream response, resulting in the ion transport switch from ion secretion to ion reabsorption. In contrast, pharmacologically blocking or activating ion channels that conduct a single ion species may change the membrane potential and fine-

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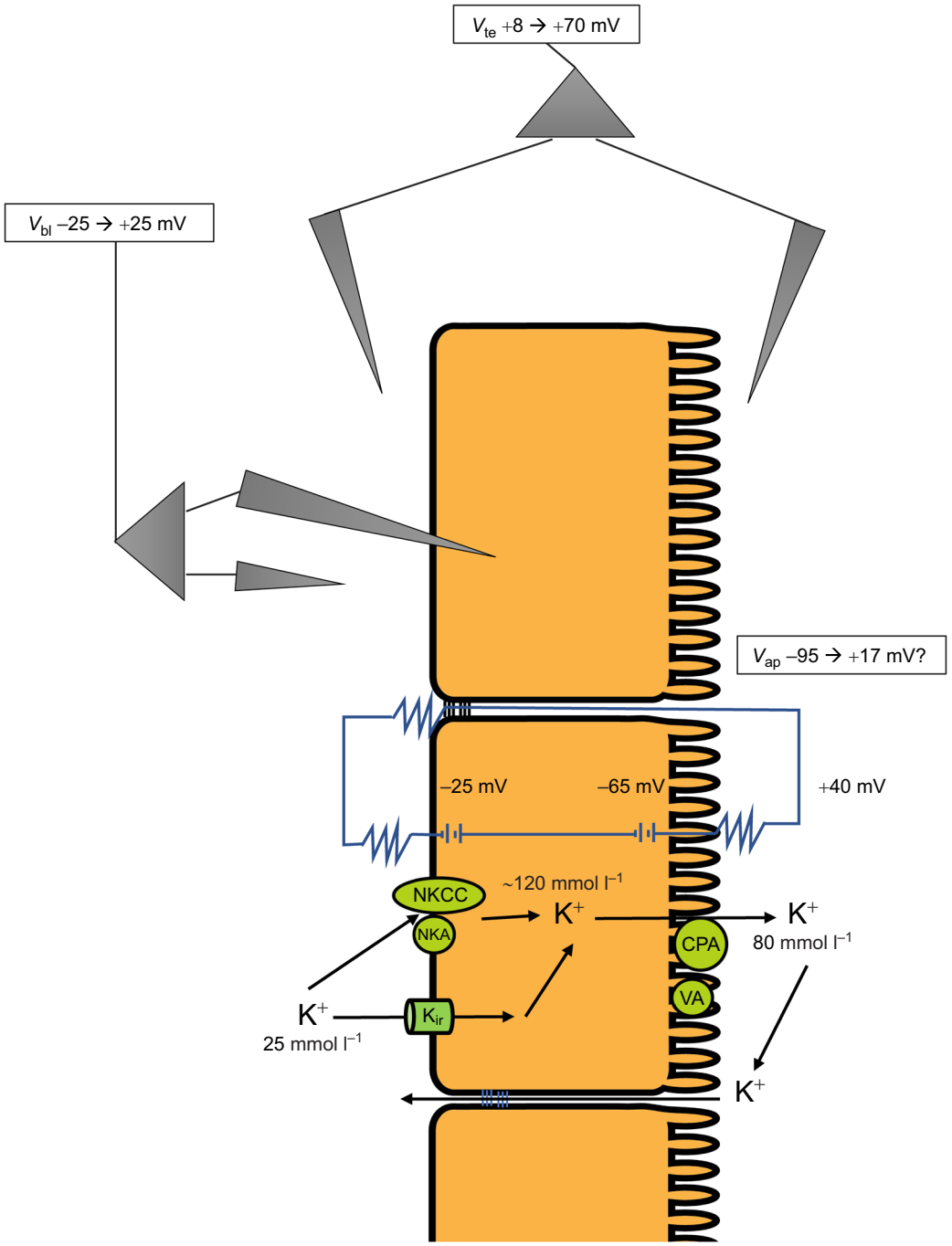

Fig. 5. Cross-talk between apical and basolateral membrane and typical membrane potential values and luminal, haemolymph and intracellular K^+ concentrations. K^+ is secreted across principal cells in the distal ileac plexus of larval lepidopterans. Typical haemolymph [K^+] is 25 mmol l^{-1}. Basolateral entry can be active via combined action of Na^+/K^+-ATPase (NKA) and $Na^+/K^+/2Cl^-$ co-transporter (NKCC), and/or passive via inward-rectifier K^+ (K_{ir}) channels. Active transport across the apical membrane occurs by combined action of V-type H^+-ATPase and K^+/H^+ antiporters (CPA). As K^+ accumulates in the lumen (~80 mmol l^{-1}), paracellular back-flux via septate junctions can allow for cross-talk between apical and basolateral membrane potential (V_{ap} and V_{bl}), so changes in membrane potential on either membrane can affect each other. Transepithelial potential (V_{te}) across the distal ileac plexus can fluctuate between +8 mV and +70 mV lumen-positive and V_{bl} can fluctuate between –25 mV inside-negative to +25 mV inside-positive. Therefore, V_{ap} may fluctuate between –95 mV inside-negative and +17 mV inside-positive. These wide voltage ranges across basolateral and apical membranes span the activation ranges for many voltage-dependent ion channels. Intracellular [K^+] has not been measured directly in tubules of *T. ni*, but most reports in insect tubules indicate values of 60 to 120 mmol l^{-1}. Resistance across septate junctions and both membranes is illustrated with resistor symbols. The ion pumps, exchangers, co-transporters and channels in the apical and basolateral membranes behave as an electromotive force, indicated by the battery symbols, that makes the cell interior negative.

tune ion transport without bringing about the more widespread cellular changes required for switching the direction of ion transport. Secondly, the downstream/upstream position in the signalling cascade that controls the switch-over from ion secretion to ion reabsorption, may be important – depending on how far downstream the molecular machinery is, its experimental manipulation may result in a complete or partial switch from transepithelial ion secretion to reabsorption.

'Big Data' provide new insights into regulation of epithelial ion transport: the impact of RNAseq on studies of epithelial transport in less-studied species

For epithelia in model species such as *Drosophila*, earlier microarray analyses of gene expression have been greatly extended by the more recent application of RNAseq (Chintapalli et al., 2007; Leader et al., 2018; Robinson et al., 2013). Mammalian epithelia such as the kidney were early candidates for RNAseq (Mimura et al., 2014), as were model organisms and tissues used in studies of vertebrate transporting epithelia, such as the pufferfish gill (Cui et al., 2014). The decreasing cost of next-generation sequencing in recent years and the availability of genomic data for non-model species has made it possible to design transcriptomic experiments to uncover novel and previously unaccounted for ion transport and regulatory mechanisms in epithelia other than those of model insect species such as *Drosophila*.

For example, transcriptomic analysis of Malpighian tubules of larval and adult mosquitoes (*A. aegypti*) identified Na^+ and Cl^- channels whose expression is highly enriched in the tubules of the blood-feeding adult females (Li et al., 2017). These channels are candidates for transporters hypothesized in a current model of fluid transport by the tubules, namely a basolateral sodium channel, which allows the entry of Na^+ into the principal cells from the haemolymph, and an apical chloride channel, which allows the movement of Cl^- from the stellate cells to the tubule lumen (Hine et al., 2014). RNAseq studies of the Asian tiger mosquito, *Aedes albopictus*, indicate that blood feeding alters the Malpighian tubule epithelium from one specializing in active transepithelial fluid secretion to one specialized for detoxification and metabolic waste excretion (Esquivel et al., 2014). Digestion of haemoglobin from the blood meal leads to the production of haeme, a toxic metabolite that causes cell and tissue damage through oxidative stress, so activation of multiple mechanisms for haeme detoxification is adaptive.

Previous studies of the control of fluid secretion by Malpighian tubules of the caterpillar *T. ni* have identified multiple molecules with diuretic effects, including amines and peptides. RNAseq of the tubules led to the serendipitous discovery of transcriptomic evidence for a glycine receptor in the distal ileac plexus segment of the tubule. This discovery prompted subsequent functional tests which revealed glycine modulation of ion transport (Kolosov and O'Donnell, 2020). The progression of understanding in epithelial physiology often fits a sequence: observation of a phenomenon, followed by identification of the tissues and cells involved, pharmacological evidence for involvement of particular proteins (e.g. ion transporters), and identification of the genes which code for those proteins. RNAseq offers the opportunity to turn this sequence of events around, so that identification of genes that are richly expressed or unexpectedly present in a tissue (e.g. glycine receptors) may lead to functional assays that reveal previously unobserved phenomena (e.g. glycine stimulation of tubule Cl^- secretion).

Functions of voltage-gated, ligand-gated and mechanosensitive ion channels in epithelia of other clades

Voltage-gated, ligand-gated and mechanosensitive ion channels have been detected in epithelia of several vertebrates and invertebrates using publicly available RNAseq datasets (Kapoor et al., 2021). Studies on vertebrate (and most often mammalian) epithelia have reported expression of voltage-gated ion channels in epithelia of the lung, intestine (Barshack et al., 2008), kidney (Siroky et al., 2017) and skin (Pitt et al., 2021). Expression of voltage-gated channels selective for Ca^{2+}, Na^+ and K^+ has been identified, as well as expression of voltage-gated channels that are non-selective and cation permeable. Many of these channels are involved in the regulation of intracellular Ca^{2+} signalling, osmotic stress response, extracellular ion sensing and modulation of vectorial ion transport (Abbott, 2014; Bleich and Warth, 2000; Demolombe et al., 2001; Morera et al., 2015; Nilius and Droogmans, 2001; Schönherr et al., 2000; Shi et al., 1997; Siroky et al., 2017; Yang and Cui, 2015; Zhu et al., 2010).

A likely advantage of the presence of voltage-gated, ligand-gated and mechanosensitive ion channels in animal epithelia is the ability of these channels to respond quickly to changing environmental and systemic variables. In contrast to motile cilia in specialized cell types, the immotile, primary cilia that protrude from the surface of most types of mammalian epithelial cell receive stimuli from the environment and transduce the information into an intracellular response (Wachten and Mick, 2021). Primary cilia on renal cells are thought to act as mechanosensors that detect fluid flow. Although molecular mechanisms underlying the sensory function of primary cilia are not well understood, G-protein-coupled receptors (GPCRs) that produce changes in the concentrations of intracellular second messengers (cAMP, Ca^{2+}) are generally thought to be involved. In intestinal epithelia, GPCRs that are responsive to a range of nutrients have been identified (Moran et al., 2021). One of these GPCRs, gustducin, can stimulate both phosphodiesterase, to cause cAMP degradation, and phospholipase C, leading to inositol trisphosphate-mediated Ca^{2+} release. The subsequent increase in cytoplasmic Ca^{2+} activates a Ca^{2+}-sensitive transient receptor potential (TRP) channel M5, thus triggering membrane depolarization and opening of voltage-gated Ca^{2+} channels, amplifying the Ca^{2+} signal. In kidney and intestinal epithelia, the calcium-sensing receptor (CaSR) is a GPCR that senses several key nutrients. The primary ligand for CaSR is extracellular Ca^{2+}, but in intestinal epithelia it can also be activated allosterically by L-amino acids (Moran et al., 2021). In the proximal tubule of the kidney, activation of CaSR by an increase in luminal Ca^{2+} concentration leads to an increase in sodium-dependent proton extrusion and fluid reabsorption (Capasso et al., 2013).

Future directions

In summary, although expression of voltage-gated, mechanosensitive and ligand-gated ion channels in epithelia of different animal clades is well reported, there is very little understanding of: (i) whether the role of a particular ion channel is conserved across epithelia of different animal clades, (ii) whether these novel ion transport regulators are connected to any other aspects of epithelial function (e.g. junctional or water permeability), and (iii) what precisely activates voltage-gated ion channels in animal epithelia, and specifically whether mechanosensation and voltage-gated ion channels are connected or are parts of two separate signalling networks aimed at adjustment of epithelial ion transport.

For example, switching between ion secretion and ion reabsorption in the Malpighian tubules of larval lepidopterans involves simultaneous fine-tuning of ion transport, water permeability, paracellular permeability and gap junctional coupling. There is a gap in our understanding of whether voltage-gated ion channels regulate only ion transport, or whether they regulate all four aspects of the switch-over. Any links between the presence of voltage-gated, ligand-gated and mechanosensitive ion channels and the regulation of paracellular permeability, gap junctional coupling and water permeability even in the Malpighian tubules of larval lepidopterans still remain largely speculative, and certainly deserve attention in epithelia of other animal groups.

Additionally, mechanisms of voltage-gated ion channel activation in animal epithelia remain largely unexplored – it is unclear whether these channels respond directly to the changes in epithelial cell membrane potential resulting from altered intracellular and extracellular ion concentrations, or whether they are activated by other upstream mechanisms (mechanosensors, changes in intracellular/extracellular pH, etc.). For instance, mechanosensitive ion channels seem ubiquitously expressed in animal epithelia, but the mechanisms of their activation and their influence upon epithelial ion transport remain relatively unexplored. Typically, mechanosensitive ion channels are activated by mechanical stretch of the cell membrane – as a result they open up and depolarize the cell membrane potential by conducting Ca^{2+}, Na^{+} and K^{+} through the channel pore. Mechanosensitive ion channels can also be gated by push/pull from extracellular or intracellular macromolecules and can respond to changes in cell volume (Jentsch, 2016). Thus, an intriguing possibility is that voltage-gated and mechanosensitive ion channel machinery in epithelia is a link between epithelial ion transport and mechanosensitive osmotic cell volume regulation, which can attune the cell to the availability of extracellular ions and water and engage cell volume-regulating ion transporters in vectorial ion transport. In the Malpighian tubules of caterpillars specifically, fluid flow and hydrostatic pressure are likely to change when the distal ileac plexus undergoes the switch between ion secretion and ion reabsorption. As a speculative example, increased luminal fluid flow from the rectal complex may keep principal cell volume low by pushing the cells against the basal lamina that encases the tubule cells. When luminal fluid flow is decreased, cell volume may increase, triggering piezo and recruiting cell volume-regulating transporters for vectorial ion secretion.

In summary, it is our belief that studies of voltage-gated, mechanosensitive and ligand-gated ion channels in epithelia of different animal clades will provide new insights into the fundamental properties and regulation of epithelial cells.

Competing interests

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