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Evidence for intercellular communication in mosquito renal tubules: a putative role of gap junctions in coordinating and regulating the rapid diuretic effects of neuropeptides

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Abstract

Adult female mosquitoes require a blood meal from a vertebrate host to successfully reproduce. During a single blood feeding, a female may ingest more than the equivalent of her own body mass, resulting in an acute stress to osmotic and ionic homeostasis. In response to this stress, the renal (Malpighian) tubules mediate a rapid diuresis that commences as soon as blood is ingested. The diuresis is regulated by neuropeptides (e.g., kinins, calcitonin-like peptide) that act on receptors in the Malpighian tubule epithelium. Interestingly, the expression of these receptors is discontinuous throughout the epithelium, which raises the question as to how Malpighian tubules mount such a rapid and synchronized response to neuropeptide stimulation. Here we propose a hypothesis that gap junctions functionally couple the epithelial cells of Malpighian tubules, resulting in a coordinated physiological response to the binding of neuropeptides. We review recent, relevant literature on the electrophysiology, physiology, and molecular biology of mosquito Malpighian tubules that indicate the presence of gap junctions in the epithelium. We also provide new physiological and immunochemical data that are consistent with the proposed hypothesis.

Keywords

Malpighian tubules; diuresis; innexins; kinin; calcitonin-like peptide

1.1 Introduction

The adult female is the only life stage and sex of the mosquito that feeds on vertebrate blood; she requires the nutrient-rich blood meal for the proper development of her eggs. Ingesting blood leads to remarkable molecular and physiological changes in the mosquito,

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including dramatic effects on the antennae, fat body, flight muscle, stomach (midgut), and ovaries (Attardo et al., 2005; Bonizzoni et al., 2011; Clements, 2000; Gonçalves et al., 2009; Price et al., 2011; Rinker et al., 2013; Sanders et al., 2003; Scaraffia et al., 2005; Thangamani and Wikel, 2009). However, there is arguably no more rapid and dramatic effect of blood feeding than of that observed on the renal excretory system of mosquitoes.

As soon a yellow fever mosquito *Aedes aegypti* begins feeding on a vertebrate host, she will produce and excrete urine; this post-prandial diuresis may last for 2 hours and contributes to the elimination of excess NaCl, KCl and water that are absorbed into her extracellular fluid (hemolymph) from the ingested blood plasma and cells (Beyenbach, 2003b; Coast, 2009; Williams et al., 1983). Moreover, the diuresis lightens her flight payload to facilitate escape from the host. A similar excretory phenomenon occurs in malarial mosquitoes of the genus *Anopheles*, but the alimentary canal of these mosquitoes has evolved a unique ability to filter the ingested blood leading to the direct excretion of plasma by the hindgut. This so-called 'pre-diuresis' 1) minimizes the absorption of plasma-derived NaCl and water into the hemolymph, and 2) maximizes the midgut's capacity to hold blood cells, which are enriched with KCl relative to the plasma. After the pre-diuresis, *Anopheles* mosquitoes exhibit a rapid post-prandial diuresis during which KCl and water are primarily excreted (Briegel and Rezzonico, 1985; Coast, 2007; Coast, 2009; Nijhout and Carrow, 1978).

Diuresis in mosquitoes is mediated by the five Malpighian (renal) tubules, which are blind-ended tubular structures that are bathed by the hemolymph and produce urine via transepithelial fluid secretion. The tubules empty their secretions into the alimentary canal near the junction of the midgut and hindgut. For detailed reviews on the general structure and transport mechanisms of mosquito Malpighian tubules, we refer the reader to the following references (Beyenbach and Piermarini, 2009; Beyenbach and Piermarini, 2011; Beyenbach et al., 2010). In brief, the Malpighian tubule epithelium consists of two cell types: principal and stellate cells. Principal cells are large fusiform cells that comprise over 90% of the tubule mass. They are responsible for the active transepithelial transport of Na^+ and K^+ , which is energized by a V-type H^+ -ATPase localized in the luminal brush border. The active transport of cations by principal cells generates a favorable electrochemical gradient for Cl^- to follow passively via a paracellular (between cell) pathway (Beyenbach and Piermarini, 2011).

Stellate cells are small star-like cells with a nucleated cell body from which 3 or 4 arm-like projections radiate. These cells intercalate between principal cells, primarily along the distal 2/3 of the tubule (towards the blind end). Direct roles of stellate cells in the transepithelial secretion of ions and water by mosquito Malpighian tubules are not yet established due to the difficulty of measuring the physiology of these small cells, but electrophysiological evidence indicates that they may contribute to the secretion of Cl^- via apical chloride channels (O'Connor and Beyenbach, 2001). Furthermore, immunochemical evidence suggests that stellate cells have the potential to play roles in the transepithelial transport of 1) water via aquaporins (Liu et al., 2011) and 2) Na^+ via a Na/H antiporter (Xiang et al., 2012) and a Na,K-ATPase (Patrick et al., 2006).

The post-prandial diuresis in mosquitoes is regulated by neuropeptides, which are synthesized by neurosecretory cells and released into the hemolymph (Wheelock et al., 1988); the release is presumably activated by the stimulation of stretch receptors in the abdominal wall during engorgement (Nijhout and Carrow, 1978). In Malpighian tubules, the neuropeptides bind to G protein-coupled receptors, which signal the stimulation of metabolic and ion transport pathways, thereby enhancing transepithelial fluid secretion. Among the known neuropeptides with diuretic activity in isolated mosquito Malpighian tubules are kinin peptides (leucokinin VIII; aedeskinin I, II, III) (Hayes et al., 1989; Schepel et al., 2010; Veenstra et al., 1997), a calcitonin-like peptide (a.k.a. DH₃₁) (Coast et al., 2005; Petzel et al., 1985), and a corticotropin releasing factor (CRF)-related peptide (a.k.a. DH₄₄) (Clark et al., 1998a; Clark et al., 1998b; Coast et al., 2005). All three types of peptides elicit rapid effects on the physiology of isolated mosquito Malpighian tubules, but those of the CRF-related peptide appear to be the weakest in magnitude compared to those of the kinin and calcitonin-like peptides (Coast et al., 2005). Moreover, recent functional genetic studies have provided compelling evidence implicating roles for the kinin and calcitonin-like peptide receptors in the post-prandial diuresis of mosquitoes (Kersch and Pietrantonio, 2011; Kwon et al., 2012). Thus, we focus our attention on the kinin and calcitonin-like peptides.

Kinins act with switch-like speed on isolated mosquito Malpighian tubules by opening the paracellular pathway for chloride transport, thereby reducing the primary impedance to fluid secretion (Beyenbach, 2003a; Beyenbach, 2012). Moreover, kinins promote the assembly of the apical V-type H⁺-ATPase in principal cells (Beyenbach et al., 2009). Thus, kinins activate both passive paracellular and active transcellular transport pathways, leading to a non-selective enhancement of NaCl, KCl, and H₂O secretion (Beyenbach, 2012). The signaling pathway for kinins involves an increase of intracellular calcium (Pietrantonio et al., 2005; Radford et al., 2004; Yu and Beyenbach, 2002) and requires the activity of protein kinase C (Miyachi et al., 2013). The cDNA encoding the kinin receptor has been cloned and functionally characterized in *Ae. aegypti* and *An. gambiae* (Pietrantonio et al., 2005; Radford et al., 2004). In Malpighian tubules, immunoreactivity for the receptor is expressed predominately on the basolateral membrane of stellate cells (Lu et al., 2011; Radford et al., 2004), but physiological expression of the receptor is also detected in principal cells (Yu and Beyenbach, 2004). Thus, the rapid diuretic effects of kinins on mosquito Malpighian tubules likely require the coordinated actions of both cell types.

In contrast to kinins, the calcitonin-like peptide (CLP) opens a conductance for Na⁺ on the basolateral membrane of the mosquito Malpighian tubule epithelium, leading to a pronounced natriuresis—i.e., the selective secretion of NaCl and water (Coast et al., 2005; Petzel et al., 1985). In Malpighian tubules of *D. melanogaster*, the CLP also activates the V-type H⁺-ATPase in principal cells (Coast et al., 2001). The signaling pathway for CLP involves an increase of intracellular cAMP (Beyenbach et al., 2009; Petzel et al., 1987). Furthermore, in isolated Malpighian tubules of *Ae. aegypti*, membrane-permeant analogs of cAMP (e.g., dibutryl-cAMP) 1) mimic the physiological effects of CLP (Coast et al., 2005; Petzel et al., 1985; Williams and Beyenbach, 1983) and 2) enhance the biochemical activity of the V-type H⁺-ATPase (Tiburcy et al., 2013). Such analogs also stimulate the activity of a

bumetanide-sensitive Na,K,2Cl cotransporter (Hegarty et al., 1991), but the activation of this transporter in tubules treated with CLP has not yet been verified.

The cDNA encoding the CLP receptor has been cloned from at least one mosquito species, *Ae. aegypti* (Kwon et al., 2012); immunoreactivity for the receptor is detected on the basolateral membrane of principal cells in the distal segment, but in a 'variegated' manner (Kwon et al., 2012). That is, not every principal cell of the distal segment appears to express the receptor. Despite the patch-work immunochemical expression of the receptor, CLP elicits discrete effects on the basolateral membrane voltage of principal cells, transepithelial fluid secretion in isolated tubules, and intracellular concentrations of cAMP (Coast et al., 2005; Petzel et al., 1987; Petzel et al., 1985). Thus, there appears to be a synchronized response of the epithelium to CLP treatment, even with an apparent discontinuous expression of the receptor throughout the epithelium.

How does an epithelium composed of two distinct cell types act in such rapid, robust, and coordinated manners in response to neuropeptides when their receptors are not found continuously throughout the epithelium? The goal of the present mini-review is to propose a unifying hypothesis for these observations; i.e., we hypothesize that gap junctions allow for the remarkably rapid and coordinated actions of diuretic neuropeptides on mosquito Malpighian tubules. Gap junctions are intercellular channels that allow for the transport of metabolites, second messengers, and electrical currents between neighboring cells. These structures play critical roles in the synchronization of excitable cells, but their roles in epithelial function are not as well characterized, especially in insects. In the following pages, we review direct and indirect lines of evidence that support the putative roles of gap junctions in synchronizing and regulating the actions of neuropeptides on mosquito Malpighian tubule function.

1.2 Evidence for gap junctions in mosquito Malpighian tubules

1.2.1 Electrophysiological coupling of epithelial cells

The phenomenon of electrical coupling among epithelial cells in insect Malpighian tubules has been known for nearly half a century. In fact, the tubules of a larval midge (*Chironomus thummi*) were one of the first renal tissues for which the phenomenon of intercellular communication was reported (Loewenstein et al., 1965). However, direct functional evidence for gap junctions in mosquito Malpighian tubules was not uncovered until nearly four decades later in a two-electrode voltage clamping (TEVC) study of principal cells by the Beyenbach laboratory (Masia et al., 2000). In this study, they demonstrated that the transepithelial electrical conductance of a single principal cell is over five times greater than the value expected based on earlier measurements of transepithelial conductance in isolated microperfused tubules (Masia et al., 2000). The discrepancy between the observed and expected values could be explained by a single principal cell having electrical connections to over 5 epithelial cells (Masia et al., 2000).

A subsequent study by the Beyenbach laboratory confirmed the above phenomena by demonstrating that the 1) natural oscillations of the basolateral membrane voltage of principal cells are perfectly synchronized among principal cells at least 3 cells apart (Figure

1A), and 2) injection of current into one principal cell (cell #1 in Figure 1B) results in voltage deflections in the neighboring principal cell (cell #2 in Figure 1B) and at least one cell beyond the neighboring cell (cell #3 in Figure 1B) (Weng et al., 2008). These electrophysiological findings are consistent with earlier anatomical studies by Tim Bradley and colleagues who found ultrastructural evidence for gap junctions between principal cells in mosquito Malpighian tubules (Bradley et al., 1982). With such robust electrical coupling in the Malpighian tubule epithelium provided by gap junctions, one can easily envision how a neuropeptide agonist binding to a single principal or stellate cell can elicit a synchronized response in several cells downstream of the one receiving the primary message. In other words, a discontinuous pattern in the expression of a neuropeptide receptor could still result in a coordinated physiological response of the entire epithelium.

1.2.2 Metabolic regulation of principal cells by stellate cells

Indirect evidence for gap junctions in mosquito Malpighian tubules derives from immunochemical and physiological studies of the Cl/HCO_3 anion exchanger (AE). We cloned the AE cDNA from Malpighian tubules of adult female mosquitoes (*Ae. aegypti*) and demonstrated that the encoded protein mediates DIDS¹-sensitive, Na^+ -independent Cl/HCO_3 exchange (Piermarini et al., 2010). As shown in Figure 2A, immunoreactivity for this exchanger is expressed exclusively in stellate cells, where it localizes to the basolateral membrane (Piermarini et al., 2010). Linser and colleagues have also revealed that stellate cells of adult female *An. gambiae* express the AE (Linser et al., 2012).

We also demonstrated that the spontaneous rates of fluid secretion in isolated Malpighian tubules are not significantly affected when DIDS is added to the peritubular bath (Piermarini et al., 2010). Thus, inhibiting the AE, which is only expressed in stellate cells (Figure 2A), does not appear to influence the basal rate of transepithelial fluid secretion in Malpighian tubules. However, when we added DIDS to the peritubular bath of isolated tubules stimulated to secrete at diuretic rates by aedeskinin-III or the calcitonin-like peptide, then the rates of transepithelial fluid secretion are significantly inhibited to basal levels (Piermarini et al., 2010). Thus, inhibiting the AE of stellate cells appears to selectively block diuresis, suggesting a critical role of stellate cells in the tubule epithelium when its metabolism is enhanced.

We have recently attempted to determine if these in vitro findings translate into in vivo observations by performing diuresis assays on adult female mosquitoes (*Ae. aegypti*). As shown in Figure 2B, mosquitoes injected with 900 nl of a phosphate-buffered saline (PBS) into their hemolymph excrete urine at rates that are 16 times greater than uninjected (control) mosquitoes. However, when the mosquitoes are coinjected with PBS and DIDS (1 mM), the diuresis is completely inhibited (Figure 2B). Thus, DIDS inhibits diuretic rates of urine excretion in vivo, which is consistent with our in vitro results in isolated Malpighian tubules (Piermarini et al., 2010).

How can the inhibition of a transporter expressed in stellate cells influence the active transepithelial secretion of fluid, which is primarily mediated by principal cells? We

¹DIDS = 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid

interpret these findings as evidence of metabolic coupling between stellate and principal cells via gap junctions (Beyenbach and Piermarini, 2011; Piermarini et al., 2010). That is, the binding of diuretic neuropeptides to the epithelium stimulates oxidative metabolism in the mitochondria of principal cells to generate ATP for fueling the apical V-type H^+ -ATPase. This enhanced metabolism also leads to the production of intracellular CO_2 , which is rapidly hydrolyzed to H^+ and HCO_3^- by carbonic anhydrase. The H^+ can be utilized as a substrate for transport by the apical V-type H^+ -ATPase, leaving intracellular HCO_3^- behind. It makes physiological sense for the principal cells to export the HCO_3^- , because an excess of this anion could buffer intracellular H^+ away from the proton pump and/or inhibit the mitochondrial production of ATP (Lodeyro et al., 2001), thereby leading to a lower activity of the V-type H^+ -ATPase (Figure 2C).

The neighboring stellate cells, which are equipped with a large basolateral surface area containing the AE, offer a convenient sink for the HCO_3^- . Gap junctions would provide a direct conduit for shuttling HCO_3^- to the stellate cells thereby 1) allowing for the reabsorption of a valuable physiological buffer into the hemolymph and 2) avoiding an accumulation of HCO_3^- in the cytoplasm of principal cells (Figure 2C). Thus, gap junctions may not only provide a means for electrical coupling among epithelial cells (e.g., Figure 1), but also for the intercellular regulation of principal cell metabolism by stellate cells.

If such a metabolic hypothesis is accurate, then diuresis should be inhibited by pharmacological blockers of gap junctions. As shown in Figure 2B, we have begun to test this hypothesis in mosquitoes using the *in vivo* diuresis assay. Notably, the coinjection of mosquitoes with PBS and a commercially available inhibitor of gap junctions (CAR) significantly inhibits the diuresis to a similar degree as DIDS (Figure 2B). Thus, pharmacological blockade of the AE or gap junctions in mosquitoes impairs their capacity to excrete urine at diuretic rates, which is consistent with our proposed ‘metabolic’ hypothesis. However, parallel studies with gap junction inhibitors on isolated Malpighian tubules are still required to validate this hypothesis.

1.2.3 Molecular and immunochemical expression of innexins

Another line of direct evidence for gap junctions in mosquito Malpighian tubules derives from molecular and immunochemical studies on innexins, which are genes that encode the gap-junctional proteins of invertebrates. This gene family has evolved independently from the connexins, which are genes that encode the gap-junctional proteins of vertebrates. On the cellular and molecular level, gap junctions are formed by the binding of opposing hemichannels on the lateral membranes of neighboring cells. Each hemichannel consists of six monomeric subunits encoded by an innexin gene. The subunits that form the hemichannels can be derived from the same gene (homomeric) or from more than one gene (heteromeric). Likewise, the resulting gap junctions can be categorized as homotypic if the opposing hemichannels are identical or heterotypic if the opposing hemichannels are different.

The genome of the yellow fever mosquito possesses six innexins named after their orthologs in *Drosophila*: Inx1, Inx2, Inx3, Inx4, Inx7, and Passover/*shakB* (Weng et al., 2008). We have previously demonstrated that Malpighian tubules of *Ae. aegypti* express transcripts for

Inx1, Inx2, Inx3, and Inx 7 (Weng et al., 2008). Thus, the tubules have the molecular potential to express a variety of homotypic and heterotypic gap junctions. Resolving which of the various combinations are physiologically possible will require functional characterization of the innexin proteins in a heterologous expression system (e.g., *Xenopus* oocytes) and immunochemical localization of the innexin proteins in the Malpighian tubule epithelium.

Towards achieving the latter goal, we have recently developed an antibody against Inx3 of *Ae. aegypti*. Figure 3A shows that the antibody detects a single band of ~50 kDa in crude lysates of adult female *Ae. aegypti*, which is close to the expected size of ~45 kDa. Notably, the antibody labels discrete regions near the lateral cell boundaries of principal cells, consistent with an intercellular localization (Figure 3B, arrows). Moreover, the antibody labels several punctate intracellular structures, which may reflect trafficking of the protein in vesicles (Figure 3). Preliminary observations indicate that the apparent intercellular labeling occurs in both 1) the distal segment of tubule, which is comprised of principal and stellate cells, and 2) the proximal segment of the tubule, which is comprised solely of principal cells. Thus, Inx3 has the potential to form gap junctions between principal and stellate cells in the distal segment and likely forms gap junctions between principal cells in the proximal segment. Higher resolution approaches (e.g., immunogold labeling) will be required to determine whether the expression of Inx3 occurs in the lateral membranes of stellate cells. It also remains to be elucidated whether Inx3 contributes to the aforementioned electrical and/or metabolic coupling in the epithelium.

1.3 Summary

In the above pages, we have put forth a new hypothesis that intercellular communication via gap junctions allows the Malpighian tubule epithelium of mosquitoes to respond to diuretic neuropeptides in a rapid and coordinated manner. We have summarized electrophysiological, physiological, and molecular lines of evidence that are consistent with this hypothesis. We believe that the stage is now set for further experimental studies utilizing RNA interference, which can directly test the specific roles of innexins in the 1) responses of Malpighian tubules to neuropeptides and 2) post-prandial diuresis of adult female mosquitoes. We encourage other researchers to consider our hypothesis in interpreting the rapid actions of neuropeptides on other epithelial systems in insects, including the alimentary canal, reproductive tract, salivary glands, and tracheal system.

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Highlights

- the mosquito Malpighian tubule epithelium is electrically coupled via gap junctions
- stellate cells regulate the metabolism of principal cells via gap junctions
- Malpighian tubules express four genes encoding innexins (gap-junctional proteins)
- innexin 3 immunoreactivity localizes to the lateral boundaries of principal cells
- gap junctions synchronize the effects of diuretic peptides on Malpighian tubules

Figure 1.

Electrophysiological evidence for gap junctions in mosquito Malpighian tubules. A) electrical synchronization of principal cells in isolated Malpighian tubules of the yellow fever mosquito (*Ae. aegypti*). Three different cells are impaled with a voltage microelectrode to measure the basolateral membrane voltage (V_{bl}). Note that the oscillations of V_{bl} are perfectly synchronized among the cells, even in those that are three cells apart (e.g., cells 1 and 4). B) illustration of the experimental setup used to measure the electrical coupling of principal cells in isolated Malpighian tubules. Cell 1 is impaled with two microelectrodes to allow for voltage clamping of its V_{bl} , whereas cells 2 and 3 are each impaled with a single microelectrode to measure their V_{bl} . Shown in the circles are idealized results of V_{bl} based on actual observations in which the injection of current into cell 1 causes a downward deflection of V_{bl} (hyperpolarization) not only in cell 1, but also in cells 2 and 3. All data and illustrations are reproduced from Weng et al., 2008.

Figure 2.

Indirect ‘metabolic’ evidence of gap junctions in Malpighian tubules. A) immunolabeling of the Cl/HCO₃ anion exchanger (AE) in isolated Malpighian tubules of *Ae. aegypti*. Only the stellate cells express detectable AE immunoreactivity (green labeling). Unpublished data. B) effects of DIDS and a gap-junction blocker (carbenoxylone, CAR) on the urine excretion rates of intact adult female *Ae. aegypti* using the approach of Raphemot et al (Raphemot et al., 2013). In brief, mosquitoes are injected with 900 nl of a phosphate-buffered saline (PBS) and the amount of urine excreted is measured 2 h later (see Raphemot et al., 2013 for technical details). In some cases, DIDS (1 mM) or CAR (1.3 mM) is added to the PBS. Control mosquitoes are not injected. Lower-case letters indicate statistical categorization of the means using a one-way ANOVA and Newman-Keuls multiple comparison test ($P < 0.05$). Values are means $\pm$ SEM. Unpublished data. C) illustration of the metabolic support hypothesis (modified from Beyenbach and Piermarini, 2011). See text for details.

Figure 3.

Immunochemical evidence for gap junctions in mosquito Malpighian tubules. A) Western blot of adult female mosquitoes (*Ae. aegypti*) using a rabbit anti-innexin 3 (Inx3) antibody (see antibody details below). A single band of ~50 kDa is detected, which is close to the expected size of ~45 kDa. B) immunolabeling of Malpighian tubules isolated from adult female mosquitoes (*Ae. aegypti*). The green staining indicates Inx3 immunoreactivity. The arrows indicate discrete labeling of lateral cell boundaries. Punctate intracellular labeling is also observed within principal cells, which is consistent with expression in vesicles. The anti-Inx3 antibody was raised and affinity-purified against a synthetic peptide corresponding to residues 'EMAPIYPEIGKYGKDTAI' of the deduced Inx3 protein in *Ae. aegypti* (NCBI Accession #XP_001661531). No labeling was observed in tubules incubated with pre-immune sera or the affinity-purified antibody preadsorbed with the antigenic peptide (data not shown). Unpublished data.