Investigations of the signaling cascade involved in diuretic hormone stimulation of Malpighian tubule fluid secretion in *Rhodnius prolixus*

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**A B S T R A C T**

In insects, the excretory system is comprised of the Malpighian tubules (MTs) and the hindgut, which collectively function to maintain ionic and osmotic balance of the haemolymph and rid the organism of toxic compounds or elements in excess. Secretion by the Malpighian tubules of insects is regulated by a variety of hormones including peptidergic factors as well as biogenic amines. In *Rhodnius prolixus*, two endogenous diuretic hormones have been identified; the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) and the corticotropin releasing factor-related peptide, RhoprCRF. Both factors significantly increase secretion by MTs and are known to elevate intracellular levels of cAMP. Interestingly, applying sub-maximal doses of these two diuretic factors in combination on isolated MTs in vitro reveals synergistic effects as rates of fluid secretion are significantly higher than would be expected if rates of secretion from MTs treated with each factor alone were summed. This observed synergism suggests that different downstream targets may be activated by the two diuretic factors, but that some cellular elicitors may be shared since cAMP is elevated in response to either diuretic hormone.

This study investigated the signaling cascade involved in the diuretic hormone regulation of Malpighian tubule fluid secretion. Bioassays were performed in physiological as well as modified salines (e.g. calcium-free) alone or in the presence of a variety of pharmacological compounds that interfere with prospective intracellular targets, such as the apical cation/H+ exchanger. Intriguingly, only amiloride yielded differential effects on the two diuretics with 5HT-stimulated secretion being blocked, whereas in contrast, RhoprCRF-stimulated secretion was unaffected. In addition, experiments examining the role of extracellular and intracellular calcium on fluid secretion rate showed that both diuretics are dependent on intracellular calcium availability. Finally, fluid secretion stimulated by either diuretic hormone was also sensitive to inhibition of cAMP-dependent protein kinase A. Taken together, these results suggest that each diuretic hormone activates pathways dependent upon intracellular calcium and cAMP.

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**1. Introduction**

The haematophagous insect, *Rhodnius prolixus*, and related species within the triatomine subfamily are commonly referred to as kissing bugs due to their tendency to feed on the faces of people (Milne et al., 2009). Since *R. prolixus* requires a blood meal at each larval stage and also during its adult life (Friend et al., 1965), it poses a serious threat to those living in Central and South America as it is a vector of the protozoan *Trypanosoma cruzi*, which causes Chagas disease in humans (Vallejo et al., 2009). Chagas disease is characterized by myocardial damage which can lead to abnormal heart rhythms that can cause sudden death (Laranja et al., 1956).

*R. prolixus* ingests large blood meals, up to 10 times the unfed body weight, thus securing sufficient nutrients for growth or reproduction (O’Donnell, 2009). However, ingesting such a huge bloodmeal poses challenges to homeostasis due to excess water, ions and toxins present in the diet (O’Donnell, 2009). Fed insects must therefore excrete the plasma portion of the ingested blood to concentrate the nutrients and eliminate unnecessary weight, which limits mobility and increases the risk of predation. Subsequently, they must rid their body of ions such as K+ and other toxins produced by metabolism of their meal (O’Donnell, 2009). However, it is the immediate disposal of the nutrient-poor, Na+-rich plasma portion of its blood meal that facilitates the transmission of *T. cruzi* that causes Chagas disease in humans.

Production of hypoosmotic urine is accomplished by the Malpighian tubules (MTs) (Maddrell and O’Donnell, 1992). MTs are a main component of the excretory system in insects that are blind ended and comprised of a single layer of epithelial cells, which...
are bathed in haemolymph with their open end attached to the apical membrane of MTs in *R. prolixus* has been proposed (Ianowski and O’Donnell, 2006). In the fluid secretory upper tubule, an apical Na\(^+\)/H\(^+\) exchanger pumps H\(^+\) into the lumen to drive the secondary transport of Na\(^+\) or K\(^+\) from cell to lumen via apical cat-

**2. Materials and methods**

### 2.1. Animals

Fifth-instar *R. prolixus* were kept in incubators at 28 °C with high humidity maintained using a water bath. Insects of both sexes were used in experiments approximately 6–8 weeks post-feeding (3–5 weeks post emergence as fifth instars). Dissections were carried out at room temperature under a microscope.

### 2.2. Ramsay assay

Sylgard-coated Petri dishes with pre-made wells and two min-

- Fresh saline was then applied to tubules. Tubules were washed three times with either physiological saline (described below). The dorsal cuticle, dorsal and ventral fat body, trachea, foregut, and anterior midgut were removed prior to dissection of tubules. MTs were removed two at a time and transferred into 20 μL saline droplets in the Sylgard plate using fine glass probes. Fine glass probes were used to pull the proximal (open) end of the tubules out of the saline droplet and wrap them around minute pins while leaving the distal end of tubule submersed in the saline droplet. Drugs and stimulants (5HT or RhoprCRF) were applied to the saline droplets.

- Tubules were nicked near the junction of the fluid secretory upper MTs and the KCl-reabsorbing lower MTs to allow droplet formation. Each treatment lasted 30 min and droplets were collected with a fine micropipette tip connected to an aspirator and the droplet diameter measured with the aid of an eyepiece micrometer. Tubules were washed three times with either physiological or calcium-free saline between each treatment to ensure removal or dilution of the drugs and/or stimulants from the first 30 min treatment. Saline with the drug or stimulant was first removed and fresh saline was then applied to tubules.

- The droplet volume was calculated using the formula \( V = \frac{\pi d^2}{4} \), where \( d \) is the diameter of the droplet. Secretion rate was calculated by dividing the volume of secreted droplet over the time allowed for secretion (30 min). In each set of treatments, one treatment (either first or second) was used to check viability of tubules by stimulation with 1 μmol L\(^{-1}\) 5HT or 1 μmol L\(^{-1}\) RhoprCRF.

### 2.3. Saline and drugs

The composition of the physiological saline was: NaCl, 150 mmol L\(^{-1}\); KCl, 8.6 mmol L\(^{-1}\); CaCl\(_2\), 2 mmol L\(^{-1}\); NaHCO\(_3\), 20 mmol L\(^{-1}\).

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4 mmol l\(^{-1}\); glucose, 34 mmol l\(^{-1}\); MgCl\(_2\), 8.5 mmol l\(^{-1}\); HEPES, 5 mmol l\(^{-1}\) and titrated to pH 7.0. Drugs amiloride, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), benzamil, harnaline, cimetidine, clonidine, ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetra-acetic acid (EGTA), 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), N-[2-(p-bromocinnamylamo)-ethyl]-5-isouquinoline-sulfonamide (H89) and 5HT were obtained from Sigma–Aldrich (Oakville, ON). RhoprCRF was obtained as described previously (Paluzzi et al., 2012). EIPA, cimetidine, clonidine and harnaline were dissolved in DMSO. Final concentrations of DMSO (<1%) did not have any significant effects on secretion by tubes stimulated by either 5HT or RhoprCRF (data not shown).

2.4. Statistics

All data are expressed as mean ± standard error of mean (S.E.M.). Statistically significant differences (\(p < 0.05\)) were determined using one-way ANOVA or unpaired t-tests.

3. Results

3.1. Effects of amiloride and amiloride-derivatives on secretion of MTs stimulated by 5HT and RhoprCRF

In order to assess whether a Na\(^{+}/\)H\(^{+}\) exchanger (NHE) is involved in the transepithelial transport of cations by \(R.\ prolixus\) MTs stimulated by the two endogenous diuretic hormones 5HT and RhoprCRF, MTs were challenged with a well known NHE inhibitor, amiloride (Masereel et al., 2003). As observed earlier (Maddrell and O’Donnell, 1992), amiloride significantly inhibits MT fluid secretion stimulated with 1 mmol l\(^{-1}\) RhoprCRF (Fig. 1A). A higher concentration of amiloride was also tested on MTs treated with 1 mmol l\(^{-1}\) RhoprCRF. Results show that even a ten-fold higher concentration of amiloride, 500 mmol l\(^{-1}\), did not hinder fluid secretion stimulated by RhoprCRF (Fig. 1B).

EIPA, an amiloride-derivative with higher selectivity for NHEs relative to epithelial Na\(^{+}\) channels, was tested on tubes treated with either 5HT or RhoprCRF. Tubes were first treated with 5HT or RhoprCRF alone, washed with saline and then treated with 1 mmol l\(^{-1}\) 5HT + 100 mmol l\(^{-1}\) EIPA or 1 mmol l\(^{-1}\) RhoprCRF + 100 mmol l\(^{-1}\) EIPA. Both diuretic hormone treatments showed significantly lowered fluid secretion rates in the presence of EIPA (Fig. 2A).

Benzamil, an amiloride-derivative with higher selectivity for epithelial Na\(^{+}\) channels relative to NHEs, was tested on tubes treated with 5HT or RhoprCRF. Fluid secretion stimulated by either diuretic hormone was significantly lowered when tubes were treated with 100 mmol l\(^{-1}\) benzamil (Fig. 2B).

3.2. Effects of non-amiloride-based NHE inhibitors on fluid secretion by MTs stimulated by 5HT and RhoprCRF

In order to determine the possible involvement of an amiloride-insensitive NHE that may be activated by either 5HT and/or RhoprCRF, tubes were challenged with three additional NHE inhibitors, namely harnaline, cimetidine and clonidine, which are not amiloride derivatives. All three of these inhibitors were effective at significantly reducing fluid secretion stimulated by either of the diuretic hormones (Fig. 3A–C).

3.3. Interference with classical signaling components of insect diuretic hormones: the role of calcium and protein kinase A (PKA)

In order to evaluate the involvement of extracellular calcium on diuretic hormone stimulated fluid secretion by MTs, tubes were dissected under Ca\(^{2+}\)-free saline that was supplemented with MgCl\(_2\) to maintain saline osmolarity. Tube secretion assays were also carried out using Ca\(^{2+}\)-free saline and tubes were treated either with 1 mmol l\(^{-1}\) 5HT + 1 mmol l\(^{-1}\) EGTA (a calcium chelator) or 1 mmol l\(^{-1}\) RhoprCRF + 1 mmol l\(^{-1}\) EGTA. There were no significant differences in fluid secretion rates observed between tubes stimulated with either diuretic hormone carried out in regular saline or those carried out in Ca\(^{2+}\)-free saline in the presence of the calcium chelator, EGTA (Fig. 4A). In order to investigate the involvement of intracellular calcium in diuretic hormone stimulated fluid secretion by MTs, tubes were dissected under Ca\(^{2+}\)-free saline as noted above. Tubes were stimulated initially with either 1 mmol l\(^{-1}\) 5HT or 1 mmol l\(^{-1}\) RhoprCRF, followed by Ca\(^{2+}\)-free saline washes before being challenged with Ca\(^{2+}\)-free saline containing 1 mmol l\(^{-1}\) 5HT in the presence of 1 mmol l\(^{-1}\) EGTA and TMB-8, an intracellular calcium antagonist that blocks calcium mobilization, or 1 mmol l\(^{-1}\) RhoprCRF with 1 mmol l\(^{-1}\) EGTA and TMB-8. A variety of concentrations of TMB-8 were tested, beginning at 10 mmol l\(^{-1}\). Fluid secretion rates treated with 1 mmol l\(^{-1}\) 5HT and 1 mmol l\(^{-1}\) RhoprCRF were unaffected in the presence of 10–100 mmol l\(^{-1}\) TMB-8 (data not shown). However, at 1 mmol l\(^{-1}\) TMB-8, fluid secretion rates in tubes stimulated with 1 mmol l\(^{-1}\) RhoprCRF showed a significant decrease while no significant differences were seen in tubes stimulated with 1 mmol l\(^{-1}\) 5HT and 1 mmol l\(^{-1}\) TMB-8 (Fig. 4B). Further, MTs treated with 10 mmol l\(^{-1}\) TMB-8 in the presence of 1 mmol l\(^{-1}\)
5HT and 1 μmol l⁻¹ RhoprCRF both showed significant decreases in fluid secretion rate (Fig. 4c).

To investigate the potential involvement of protein kinase A (PKA) in the signaling cascade elicited by the two diuretic hormones, a known PKA inhibitor, H89, was applied to tubules stimulated with 1 μmol l⁻¹ 5HT or 1 μmol l⁻¹ RhoprCRF. At a concentration of 10 μmol l⁻¹, fluid secretion rates were not significantly different than controls (Fig. 5a). However, at a fivefold higher dose of 50 μmol l⁻¹, H89 significantly inhibited fluid secretion rates stimulated by either diuretic hormone (Fig. 5b).

4. Discussion

Identification of the two endogenous diuretic hormones in R. prolixus, 5HT and RhoprCRF, (Lange et al., 1989; Maddrell et al., 1991; Te Brugge et al., 2002, 2011), has facilitated studies of the signaling cascade involved in the regulation of fluid secretion by the MTs.

In 1992, Maddrell and O’Donnell found that 5HT-stimulated secretion in MTs was inhibited by amiloride, a known NHE inhibitor (Maddrell and O’Donnell, 1992). The pH of the secreted fluid also decreases in response to amiloride, consistent with continued secretion of H⁺ into the lumen by the apical vacuolar H⁺-ATPase after inhibition of the apical NHE. Among the numerous NHE isoforms identified, at least five are known to be present in insects: NHE3, NHE7.9, NHE8, NHE9 and NHE10 (Pullikuth et al., 2003). NHE3 is known to be resistant to amiloride (Maserel et al., 2003). NHE7, 9 and NHE8 are amiloride-sensitive while NHE9 and 10 have no known amiloride binding pocket (Blaesse et al., 2010).

In the current study, the results are in accordance with what was found previously in that amiloride, at a concentration of 50 μmol l⁻¹, inhibited secretion stimulated by 1 μmol l⁻¹ 5HT (Fig. 1a) (Maddrell and O’Donnell, 1992). In addition, amiloride
was tested against the recently elucidated peptidergic diuretic hormone, RhoprCRF (Te Brugge et al., 2011), and using a concentration of 50 μmol l⁻¹, secretion rate was not inhibited in tubules stimulated by 1 μmol l⁻¹ RhoprCRF. In order to rule out the possibility that this may be a dose-dependent result, the concentration of amiloride was raised 10-fold and tested at 500 μmol l⁻¹ against 1 μmol l⁻¹ RhoprCRF. Again, secretion rate was not affected (Fig. 1b). This result parallels the observation made in an earlier study where MTs stimulated by cAMP were insensitive to amiloride (Maddrell and O’Donnell, 1992).

Although amiloride is a non-specific inhibitor, with inhibitory actions on the sodium channel and the sodium calcium exchanger as well (Kleyman and Cragoe, 1988), studies involving electrical measurements rule out the presence of a sodium channel on the apical membrane or upper MTs in R. prolixus (Gomez et al., 2012). Further, amiloride’s effects on the sodium channel are greatest when doses are less than 1 μmol l⁻¹. The non-specific effect of amiloride is greatest at concentrations around 1 mmol l⁻¹ where the sodium channel, NHE as well as the sodium calcium exchanger are affected while its effects on only the NHE are greatest at 100 μmol l⁻¹ doses (Petzel, 2000).

Given the different responses of 5HT-stimulated and RhoprCRF-stimulated tubules to amiloride, two amiloride-derivatives with different efficacies on different NHE isoforms were tested against 5HT and RhoprCRF. EIPA is one of the more potent NHE blockers and has been found to have a 10–500-fold greater potency than amiloride (Kleyman and Cragoe, 1988). Unlike the results obtained with native amiloride, EIPA at a concentration 100 μmol l⁻¹ inhibited secretion stimulated by both diuretic hormones to a similar extent (Fig. 2a). EIPA was also tested at a concentration of 10 μmol l⁻¹ to determine if the diuretic hormones were differentially sensitive to EIPA inhibition. The results were similar with EIPA equally inhibiting secretion by tubules stimulated by either of the diuretic hormones (data not shown). However, the results are in accordance with what is known regarding the greater inhibitory potency of EIPA than amiloride (Kleyman and Cragoe, 1988), which may partially explain why RhoprCRF-stimulated tubules were inhibited by EIPA but not amiloride.
Benzamil, a well-known sodium channel blocker, is another amiloride derivative where the 2-carboxylguanidino is substituted with a benzyl group (Kleyman and Cragoe, 1988). It has been shown that this substitution actually decreases the inhibitory activity on the vertebrate NHE (Kleyman and Cragoe, 1988). However, tubules stimulated with either of the diuretic hormones in the presence of 100 μmol l⁻¹ benzamil showed similar results to experiments involving EIPA (Fig. 2b). Fluid secretion rate was significantly reduced when benzamil was co-applied with either of the diuretic hormones.

In an attempt to explain the puzzling results observed from the EIPA and benzamil treatments, NHE inhibitors that are not amiloride derivative were also tested. Harmaline blocked fluid secretion in tubules stimulated by either diuretic hormone, which is consistent with the results seen in treatments conducted with EIPA and benzamil (Fig. 3a). Two additional non-amiloride derivatives that inhibit NHE’s, which were previously used to differentiate between housekeeping and epithelial (apical localization) NHE types in mammals (Ramamoorthy et al., 1991), were therefore tested. In this case however, clobbine (Fig. 3b) and cimeditine (Fig. 3c) significantly inhibited fluid secretion by both diuretic hormones.

Current models of insect epithelial ion transport propose that NHEs are energized by the Na⁺/K⁺-ATPase, whereas sodium:proton antiporters (NHAs) are electrogenic (Na⁺/nH⁺) and are energized by the voltage created by the apical V-type H⁺-ATPase (Wieczorek et al., 1999; Xiang et al., 2012). Using FlyAtlas (Chintapalli et al., 2007) and immunohistochemical analysis of D. melanogaster MTs, Day and colleagues demonstrated that two homologs of the bacterial K⁺ efflux (Kef) family genes are localized to the apical membrane of tubules, unlike the other NHE gene products which are either ubiquitously expressed or are enriched in non-epithelial tissues (Day et al., 2008).

More recently, work on Anopheles gambiae MTs showed that two alkalai metal cation:proton antiporters, AgNHA1 and AgNHA2, localize to the apical surface of either the principal cells (AgNHA1, which co-localizes with the V-type ATPase) and several other epithelial cells (Rheault et al., 2007; Xiang et al., 2012) or stellate cells (AgNHA2) (Xiang et al., 2012). The early study of electrogenic K⁺/H⁺ exchange in vesicles prepared from the midgut of larval Manduca sexta showed that the exchanger was sensitive to amiloride (Wieczorek et al., 1991). Studies of the pharmacological sensitivity of the Drosophila melanogaster Malpighian tubule to a range of amiloride derivatives are consistent with effects on an exchanger, rather than a Na⁺ channel (Giannakou and Dow, 2001). The order of inhibition of fluid secretion in the D. melanogaster Malpighian tubules is EIPA > 2,4-dichlorobenazim > DMA > amiloride = benzamil, similar to those obtained in MTs of Aedes aegypti (Petzel, 2000).

We suggest that amiloride is a weak inhibitor of sodium:proton exchange in R. prolixus MTs or that it is less permeable than the other inhibitors. In support of the latter proposal, amiloride was ineffective when applied to the bath side of 5HT-stimulated R. prolixus MTs but reduced secretion rate by 77% when applied from the lumen side (Gutierrez et al., 2004; Gamez et al., 2012). It is also worth noting that amiloride, but not derivatives such as EIPA, has been shown to antagonize 5HT receptors (Pauwels, 1997). Antagonism of the 5-HT receptor would account for the restoration of secretion by cAMP (Maddrell and O’Donnell, 1992). Similarly, generation of intracellular cAMP in response to RhoprCRF would be relatively unaffected by amiloride. The other more potent blockers of cation:proton antiporter activity inhibit fluid secretion in response to either 5HT or RhoprCRF.

Calcium often plays an important role in many signaling systems, sometimes as a second messenger and sometimes holding regulatory as well as modulatory roles. Calcium has been found to be an important mediator of diuretic hormone stimulation of insect MTs (O’Donnell et al., 1998; Rosay et al., 1997; Tobe et al., 2005). For instance, extracellular and intracellular calcium have roles in basal secretion rates as well as diuretic hormone-stimulated secretion rates in locust MTs (Morgan and Mordue, 1985). It has also been found that free calcium levels may affect adenylate cyclase activity (Clark and Spring, 1992). In contrast to 5HT-stimulated fluid secretion in locusts, where tubules deprived of extracellular calcium secrete at reduced rates (Morgan and Mordue, 1985), the results of treatments where tubules were challenged by 5HT and extracellular calcium chelated by EGTA showed that extracellular calcium plays no role in 5HT-stimulated secretion by MTs in R. prolixus (Fig. 4a). Similarly, RhoprCRF-stimulated secretion was also independent of extracellular calcium, which is consistent with results found in locust MTs (Morgan and Mordue, 1985).

To investigate whether intracellular calcium plays a role in fluid secretion by either of the two diuretic hormones in R. prolixus, tubules were treated with the intracellular calcium antagonist, TMB-8. At a dose of 1 μmol l⁻¹ TMB-8, secretion rates were decreased only in tubules stimulated by RhoprCRF (Fig. 4b). However, at a ten-fold higher dose (10 μmol l⁻¹), both 5HT and RhoprCRF-stimulated secretion was significantly reduced by TMB-8. This supports the notion that intracellular calcium plays a role in fluid secretion in the MTs and may interact with cAMP in the signaling cascade.

Intracellular messengers such as cAMP activate protein kinases which then go onto phosphorylate proteins to be activated or translocated. Since cAMP levels are increased by application of 5HT and RhoprCRF to the MTs (Astroin, 1975; Montoreano et al., 1990; Te Brugge et al., 2002), the possibility that protein kinase A (PKA) is involved in the signaling cascade is highly likely. To test this, H89, a known PKA inhibitor was applied to tubules stimulated to secrete by the two diuretic hormones. Fluid secretion by MTs stimulated by either hormone was not affected by 10 μmol l⁻¹ H89 (Fig. 5a). However, a fivefold higher dose (50 μmol l⁻¹) significantly inhibited fluid secretion by each diuretic hormone (Fig. 5b).

Similarly, a concentration of 30 μmol l⁻¹ H89 partially reversed the 5HT-induced TEP changes in the blowfly salivary glands (Rein et al., 2008). Collectively, our results demonstrate that the two endogenous diuretic hormones require components of both the calcium and cAMP signaling cascade. In other species, diuretic hormones can dose-dependently activate alternative signaling pathways. In the mosquito Aedes aegypti, the CRF-related diuretic peptide acts via calcium signaling at low concentration (1 nmol l⁻¹) to activate chloride conductance. At higher doses (100 nmol l⁻¹), CRF-related peptides act via calcium and cAMP pathways to stimulate chloride conductance as well as cAMP-dependent transport of sodium by principal cells (Clark et al., 1998). In the cricket, Acheta domesticus, cAMP stimulates fluid secretion whereas increases in intracellular Ca²⁺ are inhibitory (Clark and Spring, 1992).

In summary, our results support the involvement of both intracellular Ca²⁺ and cAMP in mediating the effects of 5HT and RhoprCRF in R. prolixus upper Malpighian tubule. Continued secretion in calcium-free saline in the presence of the chelator EGTA, however, rules out a role for extracellular Ca²⁺ in stimulation by 5HT or RhoprCRF. Amiloride derivatives such as benzamil and EIPA and chemically unrelated NHE inhibitors reduce fluid secretion by MTs stimulated with either 5HT or RhoprCRF. We propose that inhibition of secretion by amiloride in tubules stimulated by 5HT but not by RhoprCRF or cAMP can be explained on the basis of two characteristics of amiloride: it is known to antagonize 5HT receptors (Pauwels, 1997) but it is a relatively weak inhibitor of sodium:proton exchange in insect Malpighian tubules. Thus, amiloride blocks stimulation by 5HT, and this blockade can be reversed by application of exogenous cAMP (Maddrell and O’Donnell, 1992).
or by stimulation with RhoprCRF, which acts through a receptor distinct from that for SHT.

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