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Natriuresis and diuretic hormone synergism in *R. prolixus* upper Malpighian tubules is inhibited by the anti-diuretic hormone, RhoprCAPA-α2

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ABSTRACT

Insects contain an array of hormones that coordinate the actions of the excretory system to achieve osmotic and ionic balance. In the hematophagous insect, *Rhodnius prolixus*, two diuretic hormones have been identified, serotonin (5HT) and a corticotropin releasing factor-related peptide (RhoprDH), and both lead to an increase in fluid secretion by Malpighian tubules (MTs). However, only 5HT activates reabsorption by the lower MTs to recover K^+ and Cl^-. An anti-diuretic hormone (RhoprCAPA-α2) is believed to coordinate the cessation of the rapid diuresis following blood meal engorgement. However, the role of RhoprCAPA-α2 on fluid secretion by MTs stimulated by RhoprDH was previously unknown. Here we demonstrate that, unlike the inhibitory effect on 5HT-stimulated secretion by MTs, RhoprCAPA-α2 does not inhibit secretion stimulated by RhoprDH although it does abolish the synergism that occurs between the two diuretic hormones. In addition, we show that the natriuresis elicited by either diuretic hormone is reduced by RhoprCAPA-α2. Using electrophysiological tools, we investigate the possible mechanism by which this complex regulatory pathway is achieved. Analysis of the pH of secreted fluid as well as the triphasic response in transepithelial potential in MTs treated with diuretic hormones, suggests that RhoprCAPA-α2 does not inhibit the V-type H^+ ATPase. Taken together, these results indicate that RhoprCAPA-α2 functions to reduce the rapid diuresis following blood feeding, and in addition, it inhibits the natriuresis associated with diuretic hormone stimulated MTs. This may reflect an important regulatory mechanism related to the slow diuresis that occurs as the K^+-rich blood cells are digested.

1. Introduction

Insect neurohormones play an integral role in the physiology of insects. One chief function involves the hormonal regulation of osmotic and ionic homeostasis (Coast, 2009). Tissues principally involved in maintaining ionic balance include the Malpighian (renal) tubules (MTs) and hindgut, which together form the functional kidney. Numerous diuretic factors have been identified in insects that belong to a variety of peptide families and also include at least two biogenic amines (Baldwin et al., 2001; Blackburn et al., 1991; Blumenthal, 2003; Coast et al., 2001, 2005; Furuya et al., 1995, 1998, 2000a,b; Kean et al., 2002; Maddrell et al., 1991; Orchard, 2006; Te Brugge et al., 2011b). Although less studied, anti-diuretic factors which act upon the hindgut to increase reabsorption of selected ions and water have also been identified (Audsley et al., 1992; Fournier and Girardie, 1988; Meredith et al., 1996; Phillips et al., 1980; Spring and Phillips, 1980). In addition, peptidergic anti-diuretic factors which inhibit fluid secretion by MTs (Coast et al., 2010, 2011; Eigenheer et al., 2003; Paluzzi and Orchard, 2006; Paluzzi et al., 2008; Quinlan et al., 1997) and absorption by the anterior midgut (Ianowski et al., 2010; Orchard and Paluzzi, 2009) have been described.

In *Rhodnius prolixus*, two diuretic hormones have been identified, including the biogenic amine serotonin (5-hydroxytryptamine, 5HT) and a vertebrate corticotropin releasing factor-related peptide, RhoprDH (Lange et al., 1989; Maddrell et al., 1991; Te Brugge et al., 2002, 2011b). Both diuretic hormones increase fluid secretion by MTs (Maddrell et al., 1991; O'Donnell and Maddrell, 1984; Te Brugge et al., 2002, 2011b) but only 5HT leads to reabsorption of K^+ and Cl^- by the lower region of the proximal Malpighian tubule (Donini et al., 2008; Haley and O'Donnell, 1997; Maddrell and Phillips, 1975; O'Donnell et al., 1982). In addition, both RhoprDH and 5HT increase absorption by the anterior midgut (Te Brugge et al., 2009, 2011b), which together with the MTs, plays a central role in the rapid post-prandial diuresis (see Orchard, 2009).

Electrophysiological studies on isolated upper MTs of *R. prolixus* have established that a characteristic triphasic response in transepithelial potential that follows 5HT-treatment is the result of sequential activation of apical Cl^- channels, apical V-type H^+

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ATPase and a basolateral NKCC cotransporter (Ianowski et al., 2002, 2004; Ianowski and O'Donnell, 2001). The lumen-negative TEP in secreting MTs of *R. prolixus* reflects the combined contributions of the V-type H^+ ATPase, which drives the lumen to more positive values, and the increased apical chloride conductance, tending to drive the lumen to more negative values (Ianowski and O'Donnell, 2001). Studies utilizing a closely related diuretic peptide isolated from the termite *Zootermopsis nevadensis* (Baldwin et al., 2001) and structurally related to RhoprDH, identified a similar triphasic change in transepithelial potential (TEP) as is seen following 5HT treatment (Donini et al., 2008). In addition, the two diuretic hormones likely operate via similar signaling mechanisms since both increase the levels of the intracellular messenger, cyclic AMP (cAMP) (Te Brugge et al., 2002). This suggests that both hormones act via a common signaling cascade to activate the same transporters to initiate and maintain the rapid post-feeding diuresis. However, the application of both hormone types (either crude extracts or structurally related peptides) has revealed some inconsistencies in whether or not the diuretic factors act in synergy (Maddrell et al., 1993; Te Brugge et al., 2002). In other insects such as the locust, *Locusta migratoria*, two diuretic factors act synergistically but utilize two distinct signaling mechanisms; one that involves cAMP and another that is cAMP-independent (Coast, 1995). If the factors were acting via identical signaling schemes and cellular transporters, a synergistic effect would not be expected. Therefore this suggests that, although similar, some important differences in the signaling mechanisms and cellular targets of these two diuretic hormones remain unresolved.

An endogenous anti-diuretic hormone, RhoprCAPA-α2, inhibits 5HT-stimulated fluid secretion by the MTs (Paluzzi et al., 2008) and also inhibits absorption by the anterior midgut (Ianowski et al., 2010; Orchard and Paluzzi, 2009). However, the endogenous diuretic peptide has only recently been identified (Te Brugge et al., 2011b), and the effect of RhoprCAPA-α2 on RhoprDH-stimulated secretion by MTs is unknown.

In this study, we investigate the activity of RhoprCAPA-α2 on fluid secretion by MTs stimulated by RhoprDH. In addition we analyze the effect of RhoprCAPA-α2 on the synergism occurring between the two diuretic hormones. Using electrophysiological techniques, we examine the role of RhoprCAPA-α2 on the natriuresis elicited by upper MTs stimulated with diuretic hormones and also investigate the modulation of ion transporters implicated in the rapid diuresis following blood meal engorgement.

2. Materials and methods

2.1. Animals and saline

R. prolixus Stål which had previously been fed defibrinated rabbit's blood (Cedarlane, Burlington, ON) as fourth instars were obtained from a colony at the University of Toronto, Mississauga. Insects were housed in an incubator at 25 °C under high humidity and unfed fifth instar insects approximately 4–6 weeks post-emergence were used in all experiments. All experiments were carried out at room temperature (~22–23 °C) using saline composed of 129 mM NaCl, 8.6 mM KCl, 8.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10.2 mM $NaHCO_3$, 4.3 mM NaH_2PO_4, 8.6 mM Hepes, 20 mM glucose and titrated to pH 7. Insects were secured on dental wax and whole MTs were excised with the aid of fine glass probes and micro scissors.

2.2. Malpighian tubule secretion assay

Whole MTs were transferred into 20 μL droplets of saline in a Sylgard-lined petri dish and submerged in water-saturated paraffin oil. The lower tubule was pulled out from the saline droplet and wrapped around a minuten pin so that the upper and lower tubule junction was half way between the saline droplet and the minuten pin. The initial bathing saline was replaced with fresh saline containing test compounds, which included RhoprDH (Te Brugge et al., 2011b), serotonin (5-hydroxytryptamine, 5HT), or RhoprCAPA-α2 (Paluzzi et al., 2008) at the concentrations indicated in the results. MTs were nicked at the junction between the upper and lower tubules and the diameter of secreted droplets was measured with the aid of an eyepiece micrometer. Fluid secretion rates were determined as previously described (Donini et al., 2008). As a control, tubules were washed with saline following the above treatment and were then incubated with a saturating dose of 5HT (1 μM) to determine maximal fluid secretion rates.

2.3. Measurement of Na^+ and K^+ concentrations of secreted fluid

Na^+ and K^+ ion-selective microelectrodes were prepared as previously described (Donini et al., 2008; Naikkhwah and O'Donnell, 2011). In brief, microelectrodes were pulled from 1.5 mm outer-diameter unfilamented borosilicate glass capillary tubing using a vertical micropipette puller (Narishige, Tokyo, Japan) and silanized as described previously (Naikkhwah and O'Donnell, 2011). Na^+-selective microelectrodes were initially backfilled with 150 mM NaCl using a plastic 1 mL syringe pulled over a flame to a fine tip (Thomas, 1978) and were then forward-filled with a Na^+ ionophore cocktail which consisted of 10% Na^+ ionophore X, 89.75% nitrophenyl octyl ether and 0.25% sodium tetraphenylborate (Messerli et al., 2008). Selectivity of these microelectrodes for Na^+ exceeds that for K^+ by a factor of $10^{2.6}$. K^+-selective microelectrodes were backfilled with 150 mM KCl and forward-filled with K^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). Selectivity of these microelectrodes for K^+ exceeds that for Na^+ by a factor of $10^{3.9}$. Reference microelectrodes were pulled as described above and were backfilled with 150 mM KCl. Microelectrodes were connected through chlorided silver wires to a high input impedance ($>10^{13}\ \Omega$) electrometer and signals were recorded using a data acquisition and analysis system running Chart 5.0 software (Powerlab, ADInstruments, Bella Vista, NSW, Australia). Concentrations of Na^+ and K^+ in droplets of secreted fluid were measured under paraffin oil by positioning both the reference and ion-selective electrodes in the sample droplet and measuring the potential change relative to that in the calibration solutions. Na^+-selective microelectrodes were calibrated in solutions containing 15 mM NaCl, 135 mM KCl and 150 mM NaCl. K^+-selective microelectrodes were calibrated in solutions containing 15 mM KCl, 135 mM NaCl and 150 mM KCl. Slopes for the Na^+ and K^+ electrodes (mean ± SEM) for a 10-fold change in ion concentration were 60.6 ± 0.3 mV (n = 17) and 53.3 ± 0.4 mV (n = 14), respectively. The ion concentration of the secreted fluid droplet was calculated as described previously (Donini et al., 2008) using the following formula: $[\text{ion}]_{sf} = C \times 10^{(\Delta V/\text{slope})}$. The ion concentration in the secreted fluid droplet is denoted by $[\text{ion}]_{sf}$; C denotes the ion concentration in one of the calibration solutions; ΔV denotes the voltage difference between the secreted fluid droplet sample and the same calibration solution; and slope is the change in voltage between the two calibration solutions having a 10-fold difference in ion concentration. Ion-selective electrodes measure ion activity; however, if it is assumed that the ion activity coefficient is the same in calibration solutions and secreted fluid droplets, data can be expressed in terms of concentration (Donini and O'Donnell, 2005; Haley and O'Donnell, 1997). Ion concentrations were monitored over 10 min intervals for the first 70 min and a final measurement was made at the end of the experiment (100 min). Fluid secretion rates were simultaneously measured over the first 70 min and normalized to maximum fluid secretion rates for individual MTs