The antidiuretic neurohormone RhoprCAPA-2 down regulates fluid transport across the anterior midgut in the blood-feeding insect *Rhodnius prolixus*.

**Abbreviated title:** RhoprCAPA-2 blocks transport by the midgut

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Abstract

Osmotic balance in insects is regulated by the excretory system, consisting of Malpighian tubules and the gut under the control of diuretic and antidiuretic factors. Terrestrial insects must conserve water and antidiuresis is the norm, only interrupted by brief diuretic periods. Surprisingly, little is known about antidiuresis in insects. Two antidiuretic strategies have been described. The first antidiuretic mechanism involves the reabsorption of fluid from the primary urine in the hindgut. More recently, a second antidiuretic strategy was reported, consisting of inhibition of primary urine formation by the Malpighian tubules. Recently, we isolated, characterized and cloned the gene encoding for the antidiuretic neurohormone acting on the Malpighian tubules of *Rhodnius prolixus*, referred to as RhoprCAPA-2. Here we describe a third, novel mechanism central to the antidiuretic strategy of *Rhodnius prolixus*, the inhibition of ion and fluid transport across the anterior midgut by RhoprCAPA-2. Our results show that RhoprCAPA-2 (1µmol l⁻¹) reduces serotonin stimulated fluid transport from 83±11 to 12±12 nl min⁻¹ and equivalent short circuit current from 20±4 to 5±0.7 µA cm⁻² in diuretic hormone-stimulated anterior midgut. RhoprCAPA-2 appears to function independently of intracellular cGMP or Ca²⁺ in the midgut. Thus, the antidiuretic neurohormone RhoprCAPA-2 has multiple target tissues and we hypothesize that RhoprCAPA-2 functions to coordinate the transport activity of the anterior midgut and Malpighian tubules so that the rate of fluid transport into the haemolymph by the anterior midgut matches the transport rate of Malpighian tubules to maintain the volume and ion composition of haemolymph.
Introduction

Most insects regulate their blood (haemolymph) composition and volume within a narrow range even when exposed to extreme or variable environmental conditions (50). The composition and volume of the haemolymph is regulated by the action of the excretory system which consists of the Malpighian (renal) tubules and the gut by active solute secretion and the consequent flow of osmotically-obliged water (39, 12, 13, 44, 25).

Ion and fluid transport by excretory organs is controlled by diuretic and antidiuretic factors acting on the Malpighian tubules and gut epithelia. Diuretic factors include the biogenic amines tyramine (4, 34) and serotonin (46), and a number of families of peptides, including corticotropin-releasing factor-related peptide (CRF-related peptide, 29), insect kinins (23, 22, 21, 7), calcitonin-like peptides (17) and the cardioacceleratory peptide 2b/CAPA family of peptides that have diuretic effects in dipterans (9, 52, for review see 8). Most diuretic factors increase urine production by stimulating the Malpighian tubules, but in *Rhodnius prolixus* they also stimulate the midgut (15), such that fluid flows across the midgut and immediately into the Malpighian tubules. The primary urine is then modified downstream either by the lower portion of the Malpighian tubules or the hindgut (8). Thus, in the blood-feeding insect *Rhodnius prolixus* during postprandial diuresis, the diuretic hormone serotonin and diuretic peptides stimulate salt and water absorption across the anterior midgut into the haemolymph and also secretion of salt and water by the Malpighian tubules.

Much less is known about antidiuresis in spite of the fact that terrestrial insects need to conserve water and, thus, antidiuresis is the norm only interrupted by periods of
diuresis associated with increased dietary intake of water, increased metabolic water production or a need to reduce the haemolymph volume prior to flight (2). Two antidiuretic strategies have been described in insects. The first antidiuretic mechanism was described on the hindgut of the desert locust *Schistocerca gregaria* where two hormones, ion transport peptide (ITP, 1, 2) and Cl⁻ transport stimulating hormone (CTSH, 51), elevate intracellular cyclic AMP (cAMP) in the ileum and rectum, respectively, and stimulate reabsorption of fluid from the primary urine produced by the Malpighian tubules (2). Similarly, neuroparsins have been proposed to stimulate reabsorption of fluid by the rectum of the migratory locust, *Locusta migratoria*, through a Ca²⁺-dependent pathway (18, 19), however controversy still remains on the function of neuroparsins (27).

More recently a second antidiuretic strategy was described by O’Donnell and coworkers (54, 53). They demonstrated that the cardioacceleratory peptide 2b isolated from the tobacco hornworm, *Manduca sexta* (ManseCAP2b, also known as Mas-CAPA-1, 38) and shown to have diuretic effects in dipterans (52), inhibits primary urine production by the Malpighian tubules of the blood-feeding hemipteran *R. prolixus* (54, 53). Recently, we isolated, characterized and cloned the gene encoding for the endogenous *R. prolixus* CAP2b, termed RhoprCAPA-2 (48). RhoprCAPA-2 is expressed by neurosecretory cells and is believed to be released at the time of termination of diuresis thereby inhibiting primary urine production by Malpighian tubules (47).

Here we describe a novel aspect of the antidiuretic strategy of *Rhodnius prolixus*. The antidiuretic neurohormone RhoprCAPA-2 inhibits fluid transport across the anterior midgut of *R. prolixus*. These results, taken together with our recent work on the
Malpighian tubules (47, 48), reveal that RhoprCAPA-2 has multiple target tissues during the termination of the rapid diuresis following a blood meal.

**Materials and methods**

*Animals:*

Adults and fifth-instar *R. prolixus* Stål were reared at the Department of Biology, University of Toronto Mississauga at 60% relative humidity in incubators at 25°C and routinely fed on defibrinated rabbits’ blood. Dissections and experiments were carried out at room temperature (20–25°C). Insects were dissected with the aid of a dissecting microscope under saline that contained (in mmol·l⁻¹): NaCl 129, KCl 8.6, MgCl₂ 8.5, CaCl₂ 2, Glucose 20, NaHCO₃ 10.2, NaH₂PO₄ 4.3 and HEPES 8.6 with a pH = 7. For Ca²⁺-free saline CaCl₂ was replaced with 2 mmol l⁻¹ MgCl₂ or N-methyl-D-glucamine (NMDG). All chemicals were obtained from Sigma, except for RhoprCAPA-2 which was synthesized by Dr. R. Nachman (EGGFISFPRV-NH₂, see 48).

*Absorption assays:*

Fluid transport experiments were performed with the anterior midguts dissected from fifth instars and adults of either sex. The anterior midgut, also referred to as the crop in *R. prolixus*, was exposed by removing the terga of the abdomen, dissected and transferred to a dish under saline. The posterior end of the anterior midgut, at the juncture with the posterior midgut, was ligated with silk thread. Saline (50 µl) containing methylene blue (~0.01%) was injected into the lumen of the anterior midgut (to check for leakage) and the anterior end was then ligated, creating a sac-like preparation. The anterior midgut preparation was blotted and weighed in a microbalance and then incubated in saline
containing different experimental conditions. After 30 min incubation the weight of the anterior midgut was measured for a second time and the difference in the weight was used to calculate the volume of saline transported by the anterior midgut (57).

*Anterior midgut cyclic GMP and cyclic AMP radioimmunoassay:*

Radioimmunoassays were performed as previously described (47). Anterior midguts were dissected from adults from either sex under saline and any remaining blood from the meal was flushed with saline. The anterior midguts were then transferred to a microcentrifuge tube containing saline, 50·nmol·l⁻¹ serotonin alone or 50·nmol·l⁻¹ serotonin combined with 0.1 µmol l⁻¹ RhoprCAPA-2 in a total volume of 100·µl. Anterior midguts were incubated for 10·min and the experiment terminated by adding 500·µl of boiling 50·mmol·l⁻¹ sodium acetate (pH·6.2). The incubation tubes were then immediately placed in a boiling water bath for 5·min and then stored at −20°C. To prepare the samples for the assay, tubes were thawed, sonicated briefly on ice and centrifuged at 4°C for 10·min at 8800·g. The supernatant was then collected and assayed using a cyclic GMP (cGMP) or cyclic AMP (cAMP) RIA kit (PerkinElmer/NEN, Boston, MA, USA). Assays were performed according to the manufacturer’s instructions except for some minor changes in volumes and ratios of reagents.

*Ussing chamber experiments:*

Anterior midguts were dissected from adult *R. prolixus* 1 to 4 weeks after ecdysis. The anterior midgut was cut longitudinally and clamped between a pair of Ussing chambers (circular with 4 mm diameter and a volume of 500 µl on each side) whilst viewing under a dissecting microscope. The chamber was maintained at room temperature and apical and basolateral compartments were air bubbled.
The Ussing chamber can be operated in open-circuit, current-clamp or voltage-clamp modes. One of the most common modes of operation is clamping the voltage at 0 mV. The amount of current required to maintain the Vt at 0 mV is called short-circuit current (Isc) and is an accurate reflection of the absorptive or secretory capacity of the tissue. The short-circuit current (Isc) is defined as the charge flow per time when the tissue is short-circuit (i.e. transepithelial voltage, Vt, is clamped to 0 mV). The drawback from this mode of operation is that the clamp voltage imposed on the two sides of a living cell forces electrolyte through the cell and the cell might need to activate housekeeping systems that could deplete the cell of energy reserves and ultimately damage the tissue. However, Isc is also given by the equation Isc = Vt/Rt, where Rt is transepithelial resistance. From this simple equation, it is apparent that Isc can also be calculated under open-circuit conditions (i.e. without constantly clamping the epithelia to 0 V) when Rt and Vt are known. This value is often referred to in the literature as “virtual Isc” or “equivalent Isc” (eIsc) and has been extensively used in the past (36, 31, 33, 59, 3). To minimize the risk of damaging the tissue the experiments were performed under open circuit conditions.

The values for the transepithelial voltage (Vt) were referenced to the lumen side of the epithelium. Transepithelial resistance (Rt) was determined by applying short current pulses (current-clamp at 5 µA for 300 ms, every 6 s) and recording the corresponding changes in Vt (see Fig. 2C). The equivalent short circuit current (eIsc) was calculated according to Ohm’s law from Vt and Rt (eIsc = Vt/Rt). After mounting the tissues in Ussing chambers, an equilibration time of 15 min was allowed for stabilization of basal Vt and Rt. The Vt was monitored using calomel electrodes connected to the
Ussing chamber by 150 mmol l\(^{-1}\) NaCl agar bridges and monitored using a World Precision Instruments EVC400 amplifier (WPI, Sarasota, USA) and data were recorded using an A/D converter (PowerLab, ADInstruments, Colorado Springs, USA) and data-acquisition system (Chart 4, ADInstruments, Colorado Springs, USA).

**Statistics**

Results are expressed as mean ± SEM. Significance of differences between means was determined using unpaired or paired parametric or non-parametric tests as appropriate. Data were considered statistically different when \(P<0.05\).

**Results**

The anterior midguts from adult (Fig. 1A) and fifth instar (Fig. 1B) *R. prolixus* responded to serotonin (5-hydroxytryptamine, 5-HT, 0.1 \(\mu\)mol l\(^{-1}\)) by increasing the rate of fluid transport to 83 ± 11 (n=10) and 74 ± 7 nl min\(^{-1}\) (n=24) respectively. The serotonin-stimulated fluid transport was inhibited by RhoprCAPA-2 in a dose-dependent manner (Fig. 1). Similarly, treatment with serotonin increased \(eIsc\) and \(Vt\) and reduced resistance of the epithelium (Fig. 2). Addition of serotonin (5-HT) increased \(eIsc\) from 23 ± 4 \(\mu\)A cm\(^{-2}\) to a peak of 126 ± 20 \(\mu\)A cm\(^{-2}\) and a stable plateau of 70 ± 16 \(\mu\)A cm\(^{-2}\) (Fig. 2A and 2B, n=13, repeated measurements ANOVA, Tukey-Kramer \(p<0.05\)). \(Vt\) increased from 6 ± 1 mV to a peak value of 20 ± 2 mV and a plateau of 10 ± 1 mV basolateral side positive with respect to the lumen (Fig. 2C and 2D, n=13, repeated measurements ANOVA, Tukey-Kramer \(p<0.05\)). The resistance decreased from 286 ± 25 \(\Omega\) cm\(^{2}\) to lowest value of 158 ± 14 \(\Omega\) cm\(^{2}\) and finally reaching a plateau of 183 ± 21 \(\Omega\) cm\(^{2}\) (Fig. 2E and 2F, n=13, repeated measurements ANOVA, Tukey-Kramer \(p<0.05\)), consistent with
previous reports (30). Addition of RhoprCAPA-2 (1 µmol l⁻¹) blocked the effects of serotonin (Fig. 2). RhoprCAPA-2 reduced both serotonin-stimulated $E_{Sc}$ and $V_{t}$ to $29 \pm 3$ µA cm⁻² and $4 \pm 0.4$ mV, respectively while increasing resistance to $258 \pm 24$ Ω cm². These results indicate that serotonin-stimulated fluid transport by the anterior midgut is abolished by the antidiuretic neurohormone RhoprCAPA-2.

In the Malpighian tubules of *R. prolixus*, the CAPA peptide from *Manduca sexta*, ManseCAP2b, triggers antidiuresis through an intracellular second messenger pathway that may involve cGMP (54, 53). Similarly, treatment with the endogenous peptide, RhoprCAPA-2, increases cGMP in serotonin-stimulated Malpighian tubules (47, 48). This increment in cGMP correlates with a reduction of intracellular cAMP levels (54, 53). cGMP is involved in the effect of *Tenebrio molitor* antidiuretic Tenmo ADFa on Malpighian tubules of *T. molitor* and mosquito (14, 41). We decided, then, to study the effect of RhoprCAPA-2 (1 µmol l⁻¹) on intracellular cAMP and cGMP levels in anterior midgut from adult *R. prolixus*. Radioimmunoassay results showed that stimulation with serotonin (0.1 µmol l⁻¹) increases intracellular cAMP (Fig. 3A, n=10, ANOVA, Tukey-Kramer p<0.05). This effect was blocked when anterior midguts were simultaneously treated with serotonin (0.1 µmol l⁻¹) and RhoprCAPA-2 (1 µmol l⁻¹, Fig. 3A, n=10). However, RhoprCAPA-2 alone had no effect on intracellular cAMP levels (Fig. 3A, n=10). Treatment of isolated anterior midguts with the membrane-permeable cAMP analog 8-Br-cAMP (1 mmol l⁻¹) stimulated fluid transport to $192 \pm 60$ nl min⁻¹ (Fig. 3B, n=6 to 15, ANOVA, Tukey-Kramer p<0.05). The effect of 8-Br-cAMP on fluid transport of isolated anterior midguts was blocked by incubation with RhoprCAPA-2 (1 µmol l⁻¹, Fig. 3B).
The effect of 8-Br-cAMP was also detected in Ussing chamber preparations (Fig. 4, n=11, repeated measurements ANOVA, Tukey-Kramer p<0.05). The elsc increased from 46 ± 8 to a stable value of 142 ± 22 µA cm\(^{-2}\) after addition of 8-Br-cAMP (1 mmol l\(^{-1}\), Fig. 4A and 4B). The addition of RhoprCAPA-2 (1 µmol l\(^{-1}\)) reduced elsc to 75 ± 14 µA cm\(^{-2}\) (Fig. 4A and 4B). Similarly, Vt was reduced by addition of RhoprCAPA-2 (Fig. 4C and 4D). In contrast, the resistance suffered a small increase but did not fully recover after RhoprCAPA-2 treatment (Fig. 4E and 4F).

Radioimmunoassays also showed that the intracellular cGMP level is significantly lower in anterior midguts treated with serotonin (0.1 µmol l\(^{-1}\)) than those treated with serotonin (0.1 µmol l\(^{-1}\)) plus RhoprCAPA-2 (1 µmol l\(^{-1}\), Fig. 5A, n=10, Kruskal-Wallis Nonparametric ANOVA, Dunn's p<0.05). However, treatment with RhoprCAPA-2 alone does not result in a significant increment in intracellular cGMP levels. However, treatment with RhoprCAPA-2 (1 µmol l\(^{-1}\)) in the absence of serotonin produced a small but significant decrease in elsc from 56 ± 11 to 29 ± 3 µA cm\(^{-2}\) (Fig 5B and 5C, n=6, repeated measurements ANOVA, Tukey-Kramer, p<0.05).

In order to further analyze the role of cGMP we treated anterior midgut preparations with the membrane-permeable analogue of cGMP, 8-Br-cGMP (1 mmol l\(^{-1}\)). The data show that 8-Br-cGMP increased fluid secretion rate in unstimulated preparation and did not block serotonin-stimulated fluid secretion (Fig. 5D, n=6 to 15, ANOVA, Tukey-Kramer, p<0.05). 8-Br-cGMP also had a significant effect on the elsc, Vt and resistance of the anterior midgut. The addition of 8-Br-cGMP to serotonin-stimulated anterior midgut preparations did not block ion transport but rather stimulated it as indicated by the increase in elsc and Vt and the decrease in resistance (Fig 6A and B,
n=8, repeated measurements ANOVA, Tukey-Kramer p<0.05). Treatment with RhoprCAPA-2 (1 µmol l⁻¹) reduced else and Vt of anterior midguts stimulated with serotonin (50 nmol l⁻¹) and 8-Br-cGMP (1 mmol l⁻¹) from 132 ± 23 to 57 ± 11 µA cm⁻² and 26 ± 5 to 17 ± 3 mV, respectively (Fig. 6A and B, n=8).

The addition of 8-Br-cGMP, in the absence of serotonin, produced an increase in transepithelial transport by the anterior midgut (Fig 6C and D). Both else and Vt increased in response to 8-Br-cGMP (1 mmol l⁻¹) treatment from 41 ± 5 to 181 ± 47 µA cm⁻² and 17 ± 1 to 20 ± 2 mV respectively (Fig. 6C and D, n = 7, repeated measurement, ANOVA, Tukey-Kramer p<0.05). The effect of 8-Br-cGMP was blocked by treatment with RhoprCAPA-2 (1 µmol l⁻¹) (Fig. 6C and D, n=7). Even a lower dose of 8-Br-cGMP (50 µmol l⁻¹) increased transepithelial transport as indicated by the increase in else and Vt and the reduction in resistance (Fig. 6E and F, n=6, repeated measurements ANOVA, Tukey-Kramer p<0.05).

Since cGMP does not seem to be involved in the RhoprCAPA-2 intracellular second messenger pathway, we decided to test whether Ca²⁺ may play a role. The effect of the CAPA peptide in principal cells of D. melanogaster Malpighian tubules is mediated by intracellular Ca²⁺ that leads, downstream, to elevation of cGMP in the cytoplasm (55). In addition, elevation of intracellular Ca²⁺ concentration results in increased mitochondrial membrane polarization and elevated cellular ATP levels (58). Moreover, the effect of the CAPA peptide (CAP2b) on Drosophila Malpighian tubules is absolutely dependent on the presence of extracellular Ca²⁺ (55).

Thus, we tested the effect of serotonin and RhoprCAPA-2 on anterior midgut preparations in Ca²⁺-free saline or Ca²⁺-free saline containing the Ca²⁺ chelators ethylene
glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) or the membrane permeable bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid acetoxyethyl ester (BAPTA AM). The results show that serotonin stimulation of ion transport was not affected by Ca\textsuperscript{2+}-free saline (Fig. 7A). BAPTA AM was dissolved in a dimethyl sulfoxide (DMSO) solution that resulted in a final DMSO concentration in the Ussing chamber of 0.15%. DMSO at this concentration had no effect on the eIsc, Vt or R of the preparation or on the effect of serotonin and RhoprCAPA-2 (data not shown).

Serotonin stimulates eIsc in anterior midgut preparations in Ca\textsuperscript{2+}-free saline (Fig. 7A, n=9, repeated measurements ANOVA, Tukey-Kramer p<0.05). Addition of RhoprCAPA-2 (1 µmol l\textsuperscript{-1}) reduced eIsc of anterior midguts stimulated with serotonin (50 nmol l\textsuperscript{-1}) from 61 ± 10 to 27 ± 5 µA cm\textsuperscript{-2} (Fig. 7A, n=9). In order to minimize the possibility of Ca\textsuperscript{2+} contamination of our Ca\textsuperscript{2+}-free solution, we added the Ca\textsuperscript{2+} chelator EGTA. The preparation responded to serotonin by increasing the eIsc (Fig 7B, n=9, repeated measurements ANOVA, Tukey-Kramer p<0.05). Treatment with RhoprCAPA-2 reduced the serotonin-stimulated eIsc from 52 ± 5 to 25 ± 3 µA cm\textsuperscript{-2} (Fig. 7B, n=9).

These results suggest that RhoprCAPA-2 effect on ion transport across the anterior midgut is independent of extracellular Ca\textsuperscript{2+}. In order to test the possible role of intracellular Ca\textsuperscript{2+} stores in RhoprCAPA-2 antagonistic effect on serotonin-stimulated secretion, we tested the effect of the membrane permeable chelator BAPTA AM. The preparation was tested in a solution containing Ca\textsuperscript{2+}-free saline, 1 mmol l\textsuperscript{-1} EGTA and 50 µmol l\textsuperscript{-1} BAPTA AM in 0.15% DMSO. Stimulation with serotonin (50 nmol l\textsuperscript{-1}) increased eIsc from 19 ± 3 to a peak of 120 ± 25 µA cm\textsuperscript{-2} that plateaus at 98 ±5 µA cm\textsuperscript{-2} (Fig 7C, n=4, repeated measurements ANOVA, Tukey-Kramer p<0.05). Treatment with
RhoprCAPA-2 (1 µmol l⁻¹) in the presence of BAPTA AM reduced the serotonin-stimulated eIsc from 98 ± 5 to 25 ± 5 µA cm⁻² (Fig. 7C, n=4). Similarly, the inhibition of intracellular Ca²⁺ availability with 1 nmol l⁻¹ TMB-8 (8-(N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride), that blocks intracellular Ca²⁺ mobilization (56, 16, 6), had no effect on the modulation of serotonin-stimulated secretion by RhoprCAPA-2 (Fig. 7D). The anterior midguts from adult *R. prolixus* treated with Ca²⁺-free saline and 1 mmol l⁻¹ EGTA responded to 5-HT (0.1 µmol l⁻¹) by increasing the rate of fluid transport to 86 ± 18 nl min⁻¹ (n=16) and was inhibited by 1 µmol l⁻¹ RhoprCAPA-2 (Fig. 7D). Most importantly, treatment of the preparation with 1 nmol l⁻¹ TMB-8 did not prevent the effect of RhoprCAPA-2, suggesting that Ca²⁺ has no role in the effect of RhoprCAPA-2 (Fig. 7D). These results suggest that Ca²⁺, either from intracellular stores or extracellular sources, is not involved in the RhoprCAPA-2 effect on ion transport across the anterior midgut.

**Discussion**

The novel key finding we report here is the blockage of serotonin-stimulated fluid transport across the anterior midgut by the antidiuretic neurohormone, RhoprCAPA-2. Taken together with our recent work on the Malpighian tubules (47, 48), these results reveal that the antidiuretic state triggered by RhoprCAPA-2 is a complex process that involves multiple target-tissues (*i.e.* Malpighian tubules and the anterior midgut) and perhaps different intracellular second messenger pathways.

*The effect of RhoprCAPA-2*
The effect of CAPA peptides (CAP2b) on fluid transport has been best studied in the Malpighian tubules of *Drosophila melanogaster*. In this insect CAP2b-related peptides bind to a G protein-coupled receptor (49, 26), which in turn activates the phospholipase C (PLC) pathway, leading to increased levels of inositol trisphosphate (IP3) and Ca$^{2+}$ release from intracellular stores (5). The resultant rise in intracellular Ca$^{2+}$ activates a Ca$^{2+}$-calmodulin (CAM)-sensitive nitric oxide synthase (NOS) increasing production of nitric oxide (NO, 12, 30). NO in turn activates a soluble guanylate cyclase to increase production of cyclic GMP and activate cGMP-dependent protein kinases which stimulates ion transport, and hence fluid secretion, by activating an apical V-H$^+$-ATPase and, possibly, ion channels (13, 30, 37). The effect of RhoprCAPA-2 in *R. prolixus* Malpighian tubules may also be mediated by cGMP, and independent of NO, (54, 53). Similar effects of cGMP have been described in *Tenebrio molitor* and mosquito Malpighian tubules treated with Tenmo ADFa (14, 41). Thus, it seems that the CAPA peptides stimulate similar pathways in tubules in both species but with opposite final results, in *D. melanogaster* stimulating transport while in *R. prolixus* blocking transport. Our data suggest a different scenario in the anterior midgut of *R. prolixus*.

In the anterior midgut treated with serotonin plus RhoprCAPA-2, the intracellular cGMP levels are significantly higher than that in anterior midguts stimulated with serotonin only. These results are consistent with those in *R. prolixus* tubules (54, 53, 47, 48). However, treatment of anterior midguts with RhoprCAPA-2 alone, without serotonin, does not produce a detectable change in intracellular cAMP or cGMP. Nevertheless, in these conditions, RhoprCAPA-2 produces a small but significant decrement in ISc, suggesting that the RhoprCAPA-2 effect might be independent of
intracellular cGMP. These conclusions are supported by the effect of direct application of the membrane-permeable cGMP analogue, 8-Br-cGMP. The concentration of 8-Br-cGMP used in these experiments (1 mmol l\(^{-1}\)) is likely to saturate the cGMP pathway, thus, it could not be increased any further by RhoprCAPA-2. Since RhoprCAPA-2 is very effective in down regulating cGMP-stimulated and cGMP plus serotonin-stimulated transport, the results suggest that RhoprCAPA-2 utilizes a cGMP-independent second messenger pathway in the anterior midgut. It is also interesting that treatment with 8-Br-cGMP at low concentration (50 µmol l\(^{-1}\)) stimulates elsc, suggesting that it could increase fluid transport. The effect of cGMP on the anterior midgut is similar to that reported in *D. melanogaster* tubules where cGMP stimulates fluid transport even at low micromolar concentrations (9, 52). Thus, the results suggest that cGMP is a stimulatory signal, rather than inhibitory, in anterior midgut of *R. prolixus* as observed in *D. melanogaster* Malpighian tubules. These results suggest that cGMP is not the second messenger pathway triggered by RhoprCAPA-2 in the anterior midgut of *R. prolixus*, in contrast with the Malpighian tubules of *R. prolixus* and *D. melanogaster* (9, 52, 54, 53). Similarly, Ca\(^{2+}\) does not seem to be the intracellular messenger triggered by RhoprCAPA-2 in *Rhodnius prolixus* anterior midgut. Our results show that RhoprCAPA-2 blocks serotonin-stimulated elsc in preparations exposed to Ca\(^{2+}\)-free saline. Moreover, the Ca\(^{2+}\) chelators EGTA and BAPTA AM fail to block the RhoprCAPA-2 effect. In addition, the antagonistic effect that RhoprCAPA-2 has on serotonin-stimulated fluid secretion rate was not affected by treatment with TMB-8, that inhibits availability if intracellular Ca\(^{2+}\). Thus, we conclude that in *Rhodnius prolixus* anterior midgut, the effect of the CAPA peptides is independent of Ca\(^{2+}\). These results contrast with those reported in *Drosophila*
Ianowski et al. RhoprCAPA-2 blocks transport by the midgut

Malpighian tubules where the effect of CAPA peptides requires extracellular Ca\(^{2+}\) (55). More research is needed to understand the intracellular second messenger pathways stimulated by RhoprCAPA-2.

RhoprCAPA-2 has been proposed to block fluid transport by the Malpighian tubules of *R. prolixus* by increasing the activity of phosphodiesterases and reducing the levels of cAMP, the intracellular second messenger of the diuretic factor serotonin (54, 53, 48). Thus, the inhibitory effect of ManseCAP2b on ion transport by *R. prolixus* Malpighian tubules is abrogated when the cAMP pathway is saturated by treatment with high concentrations of cAMP (2 mmol l\(^{-1}\), 54). In contrast, treatment of anterior midgut of *R. prolixus* with RhoprCAPA-2 does not result in a reduction in intracellular cAMP. Moreover, RhoprCAPA-2 blocked fluid transport and else even when the anterior midgut was stimulated with high levels of 8-Br-cAMP (1 mmol l\(^{-1}\)) that would likely saturate the cAMP intracellular second messenger pathway due to the high concentration and the 8-Br-cAMP resistance to hydrolysis. Thus, it is unlikely that the inhibitory effect of RhoprCAPA-2 on *R. prolixus* anterior midgut results from cAMP hydrolysis. An alternative explanation could be that RhoprCAPA-2 may block an ion transport system involved in transepithelial transport across the anterior midgut. The *D. melanogaster* CAPA peptides have been shown to modulate the activity of the V-H\(^{+}\)-ATPase in Malpighian tubules (11). Thus, it is possible that RhoprCAPA-2 may down regulate the activity of an ion transport system in the anterior midgut of *R. prolixus*. Unfortunately, we have a very crude understanding of the transport machinery involved in fluid transport by the anterior midgut of *R. prolixus*. Therefore, at this point it is very difficult to hypothesize on the possible effects of RhoprCAPA-2 on the ion transport systems.
The hypothesis that RhoprCAPA-2 may regulate the activity of ion transport systems is consistent with previous work showing that endocrine factors, involved in digestion and pH and K⁺ regulation, regulate ion transport system activity in the midgut of lepidopterans and dipterans. In lepidopteran posterior midgut, two families of peptides, allatotropins and extended FLRFamides, down regulate active ion transport by goblet cells to modulate nutrient absorption and K⁺ regulation (11, 35, 42, 61, 60, 32). Similarly, the ion transport machinery involved in establishing pH gradients in the midgut of the mosquito *Aedes aegypti* larvae is down regulated by several peptides, including allatostatin, proctolin and neuropeptide F (45). Our future research will investigate the inhibitory cascade triggered by RhoprCAPA-2 to unveil the targets and contributors of this mechanism, central to the antidiuretic strategy of *R. prolixus*.

*Perspectives and physiological relevance of RhoprCAPA-2*

*Rhodnius prolixus* is a blood-sucking insect that ingests blood meals that may exceed 10 times the unfed mass. Subsequent reduction in the insect's mass, by rapid elimination of urine in the first few hours after the blood meal, concentrates the nutritive fraction of the blood meal and enhances the insect’s mobility, thereby minimizing the risk of predation. The production of urine by *R. prolixus* requires the transport of the plasma fraction of the blood stored in the anterior midgut into the haemolymph, where it is then secreted by the Malpighian tubules as primary urine. The fluid transport rates displayed by the anterior midgut and Malpighian tubules are very high, and, in the absence of regulatory mechanism, may alter the composition and volume of the haemolymph with deleterious effects. For example, fully stimulated tubules transport at a rate that may
deplete the whole body $K^+$ concentration within 1 minute (24, 40), and could reduce haemolymph volume (20). Thus, the transport activity of the anterior midgut epithelia and that of the Malpighian tubules must be coordinated so that the rate of fluid transport into the haemolymph through the anterior midgut epithelium is matched by the rate of Malpighian tubule excretion. Without this, the volume and ion composition of the haemolymph would vary widely, affecting the concentration of hormones and nutrients.

Our results indicate that the antidiuretic neurohormone RhoprCAPA-2, that down regulates secretion by the Malpighian tubules, also inhibits fluid transport across the anterior midgut of *R. prolixus*. We hypothesize that the function of RhoprCAPA-2 is to match the fluid transport across the Malpighian tubules and the anterior midgut during antidiuresis to maintain the haemolymph volume and composition within an acceptable level. This novel antidiuretic mechanism may apply to other insects that face dietary intake of excess water and solutes (10), such as insects that feed on a liquid meal of blood (44) or sap (28) or xeric insects feeding on succulent plant material (43).

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References


Ianowski et al.  RhoprCAPA-2 blocks transport by the midgut


17. **Furuya K, Milchak RJ, Schegg KM, Zhang J, Tobe SS, Coast GM, Schooley DA.**


Figure legends

Figure 1: Effect of RhoprCAPA-2 on fluid transport rate (mean ± S.E.M.) by anterior midguts from (A) adults and (B) fifth instar *R. prolixus*. The anterior midguts were incubated with saline (n=7), 0.1 µmol l⁻¹ serotonin (5-HT, n=10), or 0.1 µmol l⁻¹ serotonin (5-HT) plus RhoprCAPA-2 at different concentration (n=7 to 9). Columns marked with different letters are significantly different (ANOVA, Tukey-Kramer multiple comparison test, p<0.05).

Figure 2: Effect of RhoprCAPA-2 on serotonin (5-HT)-stimulated ion transport. (A and B) Equivalent short circuit current (eIsc), (C and D) transepithelial voltage ($V_t$) and (E and F) resistance across the anterior midgut from adult *R. prolixus*. Apical application of 50 nmol l⁻¹ serotonin (5-HT) increased transepithelial transport. This effect was blocked by treatment with 1 µmol l⁻¹ RhoprCAPA-2. The upward deflections on $V_t$ observed in C are caused by the passage of 5 µA pulses across the epithelia. The size of these deflections is proportional to the transepithelial resistance and were used to calculate the resistance and eIsc (see Materials and Methods). 5-HT and RhoprCAPA-2 were added during the times indicated by the horizontal bars in panel A. Columns marked with different letters are significantly different (mean ± S.E.M., n=13, repeated measures ANOVA, Tukey-Kramer multiple comparison test, p<0.05).

Figure 3: Role of cAMP on anterior midgut fluid transport. (A) Radioimmunoassay for cAMP for anterior midguts treated with saline (n=10), 0.1 µmol l⁻¹ serotonin (5-HT, n=10), 1 µmol l⁻¹ RhoprCAPA-2 (n=10) or 0.1 µmol l⁻¹ serotonin (5-HT) plus
1 µmol l⁻¹ RhoprCAPA-2 (n=10, mean ± S.E.M., ANOVA, Tukey-Kramer multiple comparison test, p<0.05). (B) Effect of the membrane permeable cAMP analog, 8-Br-cAMP on fluid transport rate. Anterior midguts treated with saline (n=12), 1 mmol l⁻¹ 8-Br-cAMP (n=6) or 1 mmol l⁻¹ 8-Br-cAMP plus 1 µmol l⁻¹ RhoprCAPA-2 (n=6) Columns marked with different letters are significantly different (mean ± S.E.M., ANOVA, Tukey-Kramer multiple comparison test, p<0.05).

Figure 4: Effect of RhoprCAPA-2 on 8-Br-cAMP-stimulated transport. (A and B) Equivalent short circuit current (eIsc), (C and D) transepithelial voltage (V_t) and (E and F) resistance across the anterior midgut from adult R. prolixus. Application of 1 mmol l⁻¹ 8-Br-cAMP on both the basolateral and apical sides induced a positive deflection in V_t and eIsc that was blocked by addition of 1 µmol l⁻¹ RhoprCAPA-2 on the basolateral side. 8-Br-cAMP and RhoprCAPA-2 were added during the times indicated by the horizontal bars in panel A. Columns marked with different letters are significantly different (mean ± S.E.M., n=11, repeated measures ANOVA, Tukey-Kramer multiple comparison test, p<0.05).

Figure 5: Role of cGMP on RhoprCAPA-2 treated anterior midguts. (A) Radioimmunoassay for cGMP for anterior midguts treated with saline (n=10), 0.1 µmol l⁻¹ serotonin (5-HT, n=10), 1 µmol l⁻¹ RhoprCAPA-2 (n=10) and 0.1 µmol l⁻¹ serotonin (5-HT) plus 1 µmol l⁻¹ RhoprCAPA-2 (n=10, mean ± S.E.M., Kruskal-Wallis nonparametric ANOVA, Dunn’s test, p<0.05). (B) Representative trace of the effect of 1 µmol l⁻¹ RhoprCAPA-2 on equivalent short circuit current (eIsc)
and (C) mean ± S.E.M (n=7, repeated measures ANOVA, Tukey-Kramer multiple comparison test, p<0.05). (D) Effect of the membrane permeable cGMP analog, 8-Br-cGMP on fluid transport rate. Anterior midguts treated with saline, 0.1 µmol l⁻¹ serotonin (5-HT), 1 mmol l⁻¹ 8Br-cGMP plus 0.1 µmol l⁻¹ serotonin (5-HT) or 1 mmol l⁻¹ 8Br-cGMP. Columns marked with different letters are significantly different (mean ± S.E.M., n = 6 to 15, ANOVA, Tukey-Kramer multiple comparison test, p<0.05).

Figure 6: Effect of 8-Br-cGMP, RhoprCAPA-2 and serotonin (5-HT) on equivalent short circuit current (eIsc). (A and B) Effect of 8-Br-cGMP and RhoprCAPA-2 on serotonin (5-HT)-stimulated ion transport rate (n=8). Basolateral application of 50 nmol l⁻¹ serotonin (5-HT) and bilateral addition of 1 mmol l⁻¹ 8-Br-cGMP induced an increase in eIsc that was blocked by treatment with 1 µmol l⁻¹ RhoprCAPA-2 on the basolateral side. (C and D) Effect of RhoprCAPA-2 on 8-Br-cGMP-stimulated eIsc. Bilateral addition of 1 mmol l⁻¹ 8-Br-cGMP induced an increase in eIsc that was blocked by treatment with 1 µmol l⁻¹ RhoprCAPA-2 on the basolateral side (n=7). (E and F) Effect of low concentration 8-Br-cGMP on serotonin (5-HT)-stimulated eIsc. Stimulation of the basolateral membrane with 50 nmol l⁻¹ serotonin (5-HT) induced an increase in eIsc. Bilateral addition of 50 µmol l⁻¹ 8-Br-cGMP further increased eIsc suggesting a stimulation of transport activity (n=6). Columns marked with different letters are significantly different (mean ± S.E.M., repeated measures ANOVA, Tukey-Kramer multiple comparison test, p<0.05)
Figure 7: Effect of Ca$^{2+}$-free saline, EGTA, BAPTA AM, RhoprCAPA-2 and serotonin (5-HT) on equivalent short circuit current (eIsc). (A) Effect of RhoprCAPA-2 on serotonin (5-HT)-stimulated ion transport rate by preparations exposed to Ca$^{2+}$-free saline (n=9). Basolateral application of 50 nmol l$^{-1}$ serotonin (5-HT) induced an increase in eIsc that was blocked by treatment with 1 µmol l$^{-1}$ RhoprCAPA-2 on the basolateral side. (B) Effect of RhoprCAPA-2 on serotonin (5-HT)-stimulated ion transport rate by preparations exposed to Ca$^{2+}$-free saline containing 1 mmol l$^{-1}$ EGTA (n=9). Basolateral application of 50 nmol l$^{-1}$ serotonin (5-HT) induced an increase in eIsc that was blocked by treatment with 1 µmol l$^{-1}$ RhoprCAPA-2 on the basolateral side. (C) Effect of RhoprCAPA-2 on serotonin (5-HT)-stimulated ion transport rate by preparations exposed to Ca$^{2+}$-free saline, 1 mmol l$^{-1}$ EGTA and 0.15% DMSO containing 50 µmol l$^{-1}$ BAPTA AM (n=4). Basolateral application of 50 nmol l$^{-1}$ serotonin (5-HT) induced an increase in eIsc that was blocked by treatment with 1 µmol l$^{-1}$ RhoprCAPA-2 on the basolateral side. (D) Effect of RhoprCAPA-2 on fluid transport rate (mean ± S.E.M.) by anterior midguts from adults $R$. prolixus. The anterior midguts were incubated with saline (n=6), 0.1 µmol l$^{-1}$ serotonin (5-HT, n=16), 0.1 µmol l$^{-1}$ serotonin (5-HT) plus 1 µmol l$^{-1}$ RhoprCAPA-2 (n=8) or 0.1 µmol l$^{-1}$ serotonin (5-HT) plus 1 µmol l$^{-1}$ RhoprCAPA-2 and 1nmol l$^{-1}$ TMB-8 (n=11). Columns marked with different letters are significantly different (mean ± S.E.M., ANOVA, Tukey-Kramer multiple comparison test, p<0.05).
Figure A: Graph showing electrolyte flux (e_{sc} (\mu A \text{ cm}^{-2})) over time.

Figure B: Bar graph comparing electrolyte flux (e_{sc} (\mu A \text{ cm}^{-2})) under different conditions.

Figure C: Graph showing V_{I} (mV) over time.

Figure D: Bar graph comparing V_{I} (mV) under different conditions.

Figure E: Graph showing resistance (\Omega \text{ cm}^{2}) over time.

Figure F: Bar graph comparing resistance (\Omega \text{ cm}^{2}) under different conditions.