The neuropeptide *Rhopr*CCHamide2 inhibits serotonin-stimulated transcellular Na^+ transport across the anterior midgut of the vector of Chagas disease *Rhodnius prolixus*.

Authors:
Natalia Capriotti[1], Paula Gioino[2], Sheila Ons[1] and Juan P. Ianowski[2]

Author affiliation:

1. Laboratorio de Neurobiología de Insectos, Centro Regional de Estudios Genómicos. Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Bvd 120 1459 (1900) La Plata, Buenos Aires, Argentina.
2. Department of Anatomy, Physiology, and Pharmacology; University of Saskatchewan, Saskatoon, SK, Canada S7N 5E5.

Corresponding author:
Juan P. Ianowski

Department of Anatomy, Physiology, and Pharmacology
Health Science Building, room 2D30.4
University of Saskatchewan
107 Wiggins Rd.
Saskatoon, Sk, S7N 5E5
Canada

juan.ianowski@usask.ca

Abstract

Rhodnius prolixus is a blood-feeding insect vector of *Tripanosoma cruzi*, a protozoan parasite that causes Chagas' disease. During each blood meal the animals ingest large volumes of blood, that may be up to 12 times the unfed body mass. These blood meals impose a significant osmotic stress for the animals due to the hyposmotic condition of the ingested blood compared to the insect's haemolymph. Thus, the insect undergoes a massive postprandial diuresis that allows for the excretion of the plasma fraction of the blood in less than two hours. Diuresis is performed by the excretory system, consisting of the Malpighian tubules and gut, under the control of diuretic and antidiuretic factors. We investigated the ion transport machinery triggered by stimulation with the diuretic factor serotonin in the anterior midgut (i.e. crop) and the effect of the diuretic modulator *Rhopr*CCHamide2. Ussing chamber assays revealed that serotonin-stimulated increase in transepithelial short circuit current (Isc) was more sensitive to the blockage with amiloride than EIPA, suggesting the involvement of Na^+ channels. Incubation in Na^+-free, but not Cl^--free saline, blocked the effect of serotonin on Isc. Moreover, treatment with NKCC and NCC blockers had no effect on fluid secretion but was blocked by amiloride. Blockage of Na^+/K^+-ATPase with ouabain inhibit Isc but the H^+-ATPase inhibitor bafilomycin had no effect. The neuropeptide *Rhopr*CCHamide2 diminished serotonin-stimulated Isc across the crop. The results suggest that Na^+ undergoes active transport via an apical amiloride-sensitive Na^+ channels and a basolateral ouabain-sensitive Na^+/K^+-ATPase while Cl^- is transported through passive paracellular pathway.

Introduction

Rhodnius prolixus is a hematophagous insect vector of the parasite *Trypanosoma cruzi* which causes Chagas' disease that affects 7 million people (www.who.int, Rassi et al., 2010). The parasite is transmitted to the human host through the excreta deposited by triatomine insects on the host's skin. Given the relevance of post prandial diuresis for triatomines bugs survival and the convenience of *R. prolixus* as a model for physiological studies (Ons, 2017), there has been significant interest in understanding the mechanism of excretion in this species.

R. prolixus ingest blood meals that can exceed ten times its unfed mass (Buxton, 1930). These large blood meals impose reduced mobility and a significant osmotic stress to the animals due to the hypoosmotic condition of the ingested blood compared to the insect's haemolymph (O'Donnell et al., 2003). Thus, the animal must trigger a rapid post-prandial diuresis to excrete the excess Na^+, Cl^- and water ingested with the blood meal. Approximately 50% of the volume of the blood meal is excreted within two hours after the meal, thanks to the coordinated action of the anterior midgut (also known as crop) and Malpighian tubules (Coast, 2009; O'Donnell et al., 2003; Maddrell, 2009; Maddrell et al., 1993).

Once ingested, the blood meal is stored in the anterior midgut; during the postprandial diuresis the excess NaCl and water ingested with the blood meal must be excreted. Isotonic NaCl solution is transported from the lumen of the anterior midgut into the haemolymph to be excreted by the Malpighian (renal) tubules (Farmer et al., 1981; Barrett et al., 1982; Billingsley and Dowe, 1986, 1989; Billingsley 1988). The activity of the anterior midgut and the Malpighian tubules is regulated by diuretic hormones, including serotonin (5-hydroxytryptamine, 5-HT, Orchard 2006), corticotropin releasing factor-like diuretic hormones (CRF-DH) (Te Brugge et al., 2009; Te Brugge et al., 2011), and Zoone-DH (Te Brugge et al 2005). The termination of the diuretic process seems to involve at least one antidiuretic hormone, *Rhopr*CAPA2 (Ianowski et al 2010, Paluzzi and Orchard, 2006; Paluzzi et al., 2008). Other neuropeptides that have been associated with diuresis in *R. prolixus* are calcitonin-like diuretic hormone (CT-DH) (Te Brugge et al., 2009; Te Brugge et al., 2005; Zandawala et al., 2011; Zandawala et al., 2015), and allatotropin (Villalobos-Sambucaro et al., 2015). Recently we have described the unique dual function of the neuropeptide *Rhopr*CCHamide2, which increase secretion by Malpighian tubules while inhibiting absorption across the anterior midgut after stimulation with serotonin (Capriotti et al., 2019). However, the mechanism of *Rhopr*CCHamide2 on the anterior midgut of *R. prolixus* and how they affect ion transport is not fully understood.

Farmer et al. (1981) were the first to study ion transport by the anterior midgut in *R. prolixus.* They concluded that during diuresis the anterior midgut transports fluid consisting of mostly Na^+ and Cl^-, which is driven by active transport of Na^+. The rate of fluid transport is strongly dependent on the concentration of Na^+ in the luminal fluid. However, the ion transport mechanisms involved, e.g. ion channels and transporters, are mostly unknown. We study the ion transport mechanisms involved in fluid transport by the anterior midgut after serotonin stimulation and the effect of *Rhopr*CCHamide2 (Capriotti et al., 2019). The results indicate that fluid secretion by the anterior midgut involves transcellular transport of Na^+ through an apical amiloride-sensitive Na^+ channel and a basolateral Na^+/K^+ ATPase. The results also suggest that Cl^- transport may be passive and does not seem to involve Na^+-dependent Cl^- transporters. Active Na^+ transport in inhibited, but not fully blocked, by treatment with *Rhopr*CCHamide2.

Materials and methods

Animals:

R. prolixus Stål were obtained from a colony maintained at the Department of Anatomy Physiology and Pharmacology, University of Saskatchewan, at 60% relative humidity in incubators at 25°C and routinely fed on defibrinated rabbits' blood (Cedarlane, Burlington, ON, Canada). Dissections and experiments were carried out at room temperature (20-25°C). Insects were dissected with the aid of a microscope under saline solution that contained (in $mmol \cdot l^{-1}$): NaCl 129, KCl 8.6, $MgCl_2$ 8.5, $CaCl_2$ 2, Glucose 20, $NaHCO_3$ 10.2, NaH_2PO_4 4.3 and HEPES 8.6 with a pH = 7. For Na^+-free saline NaCl was replaced with *N*-methyl-D-glucamine (NMDG), $NaHCO_3$ with $KHCO_3$ and NaH_2PO_4 with KH_2PO_4. For Cl^--free saline NaCl, KCl, $CaCl_2$ and $MgCl_2$ were replaced with gluconate. All chemicals were obtained from Sigma.

Midgut fluid transport assays:

Fluid transport experiments were performed with the anterior midguts dissected from fifth instars 1 to 4 weeks after ecdysis. The anterior midgut 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% to identify leakage) was injected into the lumen of the anterior midgut 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 (Te Brugge et al., 2009).

Ussing chamber experiments:

Anterior midguts were dissected from fifth instar *R. prolixus* 1 to 4 weeks after ecdysis for Ussing chamber experiments (Physiologic Instruments, San Diego, CA, USA). The anterior midgut was cut longitudinally, the apical and basolateral side identified and recorded, and clamped between a pair of Ussing chambers with circular 0.8 mm diameter opening and a volume of 1000 µl on each side containing identical saline solutions as described above (EasyMount Ussing Chamber System, Physiologic Instruments, San Diego, CA, USA). The chamber was maintained at room temperature and apical and basolateral compartments were bubbled with 95% O2 - 5% CO2 gas. After mounting the tissues in Ussing chambers, an equilibration time of 15 min was allowed for stabilization of electrophysiology parameters.

The Ussing chamber operated in voltage-clamp mode with the voltage clamped at 0 mV (VCC MC2 amplifier, Physiologic Instruments, San Diego, CA, USA). The amount of current required to maintain the transepithelial voltage (V_t) at 0 mV is called short-circuit current (Isc). The Isc is defined as the charge flow per time when the tissue is short circuited (i.e., Vt is clamped to 0 mV). We also calculated the values for the transepithelial voltage (Vt) and transepithelial resistance (Rt) by clamping the voltage to

5 mV for 3s, every 30 s and recording the corresponding change in Isc. Vt and Rt were calculated according to Ohm's law (Isc = Vt/Rt).

The voltage sensing electrodes were made with Ag/AgCl pellets and the current passing electrodes were made of a Ag chloride wires, both connected to the Ussing chamber by 150 mmol l^{-1} NaCl agar bridges and data were recorded using an A/D converter and data-acquisition system (PowerLab, 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 $P<0.05$.

Reagents

Amiloride, benzamil hydrochloride hydrate (B2417), 5-N-ethyl-N-isopropyl amiloride (EIPA; A3085), ouabain, hydrochlorothiazide, bumetanide, and serotonin were purchased from Sigma (St Louis, MO, USA). Bafilomycin A1 was purchased from Cedarlane (Burlington, Ontario, Canada). Stock solutions of the drugs were prepared in DMSO so that the maximum final concentration of DMSO was <1% (v/v). Synthetic *Rhopr*CCHa2 (GGCSAFGHSCFGGH-NH2) was obtained from Genscript Corporation (Piscataway, NJ, USA) and dissolved in saline solution.

Results

The anterior midgut section of *R. prolixus* displayed a basal short circuit current (Isc) of 74.4 ± 14.8 µA cm^{-2}, a transepithelial potential (Vt) of 4.9 ± 1.2 mV basolateral side positive with respect to the lumen, and (R) 50.2 ± 6.8 **Ω** (n=10, Fig. 1). Addition of serotonin (0.1 µM) to both sides (i.e. apical and basolateral) triggered a significant increase in Isc to 279.6 ± 42.6 µA cm^{-2}, Vt increased to 10.3 ± 1.2 mV, and R decreased to 43.4 ± 6.4 **Ω** (Fig. 1, $p<0.05$, ANOVA, Tukey's multiple comparison), consistent with serotonin stimulation of transepithelial ion transport.

Serotonin-stimulated transport does not involve Na^+:K^+:2Cl^- or Na^+:Cl^- cotransporter

We tested the potential role of the Na^+ and Cl^- cotransporters in serotonin-stimulated transepithelial ion flux by incubating the preparation with pharmacological blockers. Incubating serotonin-stimulated anterior midgut preparations with the Na^+:K^+:2Cl^- cotransporter (NKCC) blocker bumetanide (100 µM added to both apical and basolateral sides) had no effect on Isc, Vt, or R displayed by the tissues 10 min after treatment (Fig. 1). Another potential path for Cl^--linked Na^+ flux could be a Na^+:Cl^- cotransporter (NCC). Even though the existence of NCC in insects is not well established (Hartman et al., 2013), we decided to test a common NCC blocker used in vertebrates, hydrochlorothiazide. Adding hydrochlorothiazide (100 µM) to both apical and basolateral sides had no effect on Isc, Vt or R in serotonin-stimulated tissues 10 min after treatment (Fig. 2). These results suggest that, under short-circuit conditions, serotonin-stimulated transepithelial ion flux across the anterior midgut of *R. prolixus* does not involve NCC or NKCC.

To further test the potential role of NKCC or NCC on serotonin-stimulated fluid transport across the anterior midgut of *R. prolixus* we investigated the effect of bumetanide and thiazide in open-circuit condition (i.e. where the transepithelial voltage is not clamped at 0 V) using a secretion assay. Unstimulated preparations (n=28) did not transport fluid, however treatment with serotonin increased fluid transport from the lumen to the bath (0.1 ± 0.02 µl min^{-1}, n=15, Fig. 3). Co-incubation with hydrochlorothiazide (n=12) or bumetanide (n=13) for 30 min did not significantly reduce the effect of serotonin. However, co-incubation with amiloride (100 µM, n=8) significantly reduced serotonin-triggered fluid reabsorption (Fig. 3, $p<0.05$, Kruskal-Wallis test, Dunn's Multiple Comparison Test). Thus, the results suggest that amiloride-sensitive Na^+ channels or Na^+/H^+ exchangers, rather than NKCC or NCC, may be involved in serotonin-stimulated ion transport across the anterior midgut of *R. prolixus.*

Active Na^+ and passive Cl^- transport

We tested the contribution of Na^+ channels and Na^+/H^+ exchangers by measuring the effects of amiloride, 5-N-ethyl-N-isopropyl amiloride (EIPA), and benzamil in Ussing chamber assays (Giannakou and Dow, 2001; Petzel, 2000). Adding amiloride (10 µM) on both the apical and basolateral sides of serotonin-stimulated preparations significantly reduced Isc and Vt to values similar to the baseline condition (Fig. 4, n=18, $p<0.05$, Kruskal-Wallis test, Dunn's multiple comparisons test). In contrast, the effect of amiloride on R was not statistically significant (Fig. 4F). Treatment with EIPA (Fig. 5) and benzamil (Fig. S1) on both the apical and basolateral sides resulted in a reduction in Isc but at higher concentrations (100µM) than amiloride (n=6, $p<0.05$, ANOVA, Tukey's multiple comparisons test). Interestingly, Vte was more sensitive to both EIPA and benzamil, which caused a significant reduction in Vte at a concentration of 10 µM. These results suggest that Na^+ transport may occur through a channel rather than a Na^+/H^+ exchanger, which are highly sensitive to EIPA (Giannakou and Dow, 2001; Petzel, 2000).

We tested the role of the Na^+/K^+ ATPase by treating the preparations with the blocker, ouabain (100 µM). Serotonin-stimulated preparations responded to the addition of ouabain on the tissue by decreasing Isc and Vt (Fig. 6, n=8, $p<0.05$, repeated-measures ANOVA, Tukey-Kramer multiple comparison test). There was no difference in the tissue's R (Fig. 6F). Since H^+-ATPase plays a role in ion transport in mosquito gut (Pacey and O'Donnell, 2014), we tested the effect of treating our preparation with the blocker bafilomycin (10µM). Blocking H^+-ATPase had not effect on Isc, Vt or R (Fig. S2). Thus, the results are consistent with a path for Na^+ flux that is independent of NKCC or NCC and may be mediated by an apical Na^+ channel and basolateral Na^+/K^+ ATPase.

To further test the roles of Cl^- and Na^+ in fluid transport across the anterior midgut of *R. prolixus* we studied the effect of removing all the Cl^- or Na^+ from the bathing solution. Preparations incubated in Cl^--free saline responded to serotonin stimulation with an increase in Isc and Vt that resembled that of tissues bathed in control solution, and was blocked by amiloride (n=13, Fig. 7, $p<0.05$, repeated-measures ANOVA, Tukey-Kramer multiple comparison test). In contrast, preparations bathed in Na^+-free saline failed to respond to serotonin or amiloride (n=11, Fig. 8, $p>0.05$, repeated-measures ANOVA, Tukey-Kramer multiple comparison test). These results suggest that serotonin-stimulated Na^+ transport is active, transcellular, and independent of Cl^--linked Na^+ transporters, while Cl^- seem to be passive, e.g. through paracellular pathways.

Finally, we tested the effect of *Rhopr*CCHamide2 on serotonin-stimulated preparations. The addition of *Rhopr*CCHamide2 (1 µM) significantly decreased Isc and Vt but had no effect on R (n=10, Fig. 9, p<0.05, Repeated measures ANOVA, Tukey's multiple comparisons test). Addition of ouabain further decreased transport across the epithelia (Fig. 9). Similarly, amiloride caused a significant decrease in ion transport on *Rhopr*CCHamide2-treated preparations. Treatment with *RhoprC*CHamide2 reduced Isc by 50% (Fig. S3). Adding amiloride further blocked Isc by 50%, thus fully blocking the effect of 5-HT. Ouabain had a larger effect, completely blocking the Isc to 0 µA cm^{-2}. These results indicate *Rhopr*CCHamide2 reduces but does not fully block active Na^+ transport in serotonin-stimulated preparations.

Discussion

Our results are consistent with the hypothesis that serotonin stimulation triggers active Na^+ transport and passive Cl^- flow across the anterior midgut of *R. prolixus*, which is partially blocked by *Rhopr*CCHamide2.

Active Na^+ and passive Cl^- flux across the anterior midgut after serotonin stimulation was proposed by Farmer et al (1981). Here we directly test this hypothesis by studying ion transport under short-circuit conditions in a Ussing chamber assay. The mechanism of active transepithelial Na^+ flux triggered by serotonin seems to be independent of Cl^--linked Na^+ transporters. Ussing chamber assays show that in Na^+-free conditions there is no active transport, while in Cl^--free ion transport persists, indicating that 5-HT must trigger Cl^--independent active Na^+ transepithelial transport. In addition, blockers of the Cl^--linked Na^+ transporters NKCC and NCC, bumetanide and hydroclorothiazide, had no significant effect on Isc or fluid secretion assays. Moreover, secretion assays show that blocking Cl^--independent Na^+ transporters with amiloride inhibits 5-HT-stimulated fluid transport. Taken together these results support the hypothesis that 5-HT-stimulated Na^+ transport across the anterior midgut of *R. prolixus* is active and not mediated by Cl^--linked Na^+ transporters.

Na^+ entry across the apical membrane may be mediated by a Na^+ channel rather than a Na^+/H^+ exchanger (NHE). Our results show that 5-HT-triggered Isc is more sensitive to amiloride than EIPA and benzamil. In *Drosophila melanogaster* and *Aedes aegypti* Malpighian tubules, NHE-mediated ion transport has a sensitivity to EIPA (IC_{50} 7 µM) that is an order of magnitude larger than that for amiloride (IC_{50} 80 µM) or benzamil (IC_{50} 70 µM, Giannakou and Dow, 2001; Petzel, 2000). Thus, based on the patter of sensitivity to EIPA, amiloride, and benzamil our data suggest that Na^+ flux across anterior midgut of *R. prolixus* is likely mediated by an amiloride-sensitive Na^+-channel (Giannakou and Dow, 2001; Petzel, 2000). Finally, Na^+ flux was blocked by treatment with the Na^+/K^+ ATPase blocker ouabain, suggesting a contribution of the Na^+/K^+ ATPase as proposed by Farmer et al (1981; Barrett 1982).

Based on our results and the literature, the simplest working model that could explain the ion transport characteristics displayed by the anterior midgut after of serotonin stimulation would require active transcellular Na^+ flux and passive paracellular Cl^- flow to produce isotonic NaCl solution (Farmer et al., 1981, Fig. 10). We propose that Na^+ transport is mediated by an apical amiloride-sensitive Na^+ channel driven by the ouabain-sensitive basolateral Na^+/K^+ ATPase. Cl^- flow would be passive and driven by serotonin-stimulated haemolymph-side positive transepithelial potential observed in our Ussing chamber assay (Farmer et al., 1981). The simplest Cl^- pathway would be paracellular, however, transcellular passive flow cannot be ruled out. This model would explain the fact that fluid transport across this epithelium is directly proportional to the concentration of Na^+ in the lumen of the gut (Farmer et al., 1981), since increasing the Na^+ concentration in the lumen would result in a more favorable electrochemical gradient for Na^+ flux and a larger transepithelial potential favoring paracellular Cl^- flux.

The working model proposed for *R. prolixus* anterior midgut is simpler than that proposed in another blood feeder, the mosquito *Aedes aegypti*. Ion transport across the gut of adult *A. aegypti* involves Na^+/K^+ ATPase-energize Na^+ and K^+ transepithelial transport (Pacey and O’Donnell, 2014). However, there is also evidence of an apical H^+ ATPase that dives H^+-linked amino acid transport, basolateral Na^+/H^+ exchange (Pacey and O’Donnell, 2014), as well as HCO_3^-/Cl^- exchange involved in pH regulation in larval stages (Onken et al., 2004a and b; Filippov et al., 2003). Similarly, the resorptive transport across the gut of locust, one of the best studied tissues in insect, is also quite complex. It involves a basolateral Na^+/K^+ ATPase and apical electrogenic Cl^- ATPase and H^+ ATPase, as well as a large number of channels (Hanrahan et al., 1986; Robertson et al., 2014), cotransporters, and exchangers (Phillips and Audsle,1995; Audsley et al., 1992, 1994, 2013). Our results show that blocking H^+ ATPase with bafilomycin has no effect on the anterior midgut *R. prolixus* in Ussing chamber assays, suggesting that H^+ ATPase may not play a significant role in transcellular NaCl transport in this preparation.

Our results also show that *Rhopr*CCHamide2 downregulate Isc in Ussing chamber assays. *Rhopr*CCHamide2 inhibits active transport of Na^+, presumably by inhibiting apical Na^+ channels and/or basolateral Na^+/K^+ ATPase, which would result in a downregulation of transepithelial fluid flux. *Rhopr*CCHamide2 would also reduce of the driving force for passive Cl^- transport since it would reduce transepithelial potential. Interestingly, ion transport across anterior midgut is reduced, but not completely blocked by *Rhopr*CCHamide2, consistent with our previous results (Capriotti et al 2019). The effect of *Rhopr*CCHamide2 on ion flux observed here seems to be moderate compared with the activity of the anti-diuretic hormone *Rhopr*CAPA2 which completely blocks the effect of serotonin on the anterior midgut (Ianowski et al., 2010). Highlighting that among the neuropeptides controlling diuresis in *R. prolixus* identified to date, *Rhopr*CCHamide2 plays a unique dual role, an anti-diuretic factor on the anterior midgut and a diuretic factor on the Malpighian tubules where it stimulates urine formation. These subtle and dual modulatory effects could contribute to a fine tuning of changes in volume and ion composition of haemolyph during postprandial diuresis.

Acknowledgments

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to JPI; the Agencia Nacional de Promoción Científica y Tecnológica (Argentina, PICT2018-00862) to SO and JPI; and a Travelling Fellowship from 'The Company of Biologists', Journal of Experimental Biology to NC. SO and NC are researchers of Consejo Nacional de Investigaciones Científicas y Técnicas.

Competing interests

The authors declare no competing or financial interests.

References

Audsley, N., Mclntosh, C., Phillips, J.E., Schooley, D.A., and Coast, G.M. (1994) Neuropeptide regulation of ion and fluid reabsorption in the insect excretory system. In: Davey KG, Peter RE, Tobe SS, eds. Perspectives in Comparative Endocrinology, National Research Council of Canada, Ottawa 74-80.

Audsley, N., Mclntosh, C., and Phillips, J.E. (1992) Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J Exp Biol* 173:261-274.

Audsley, N., Jensen, D. and Schooley, D. A. (2013) Signal transduction for *Schistocerca gregaria* ion transport peptide is mediated via both cyclic AMP and cyclic GMP. *Peptides* 41, 74-80.

Barrett, M.F. (1982) Absorption of fluid from the anterior midgut in *Rhodnius. J. Insect Physiol*. 28(4): 335-341,

Billingsley, P.F. and Downe, A.E.R. (1986) Nondigestive cell types in the midgut epithelium of *Rhodnius prolixus* (Hemiptera: Reduviidae). *J. Med. Entomol*. 23 (2): 212-216.

Billingsley, P.F. and Downe, A.E.R. (1989) The effects of artificial diets on the anterior intestinal cell ultrastructure of *Rhodnius prolixus* (Hemiptera : Reduviidae). *Int. J. Parasitol.* 19(3): 291-299.

Billingsley, P.F. (1988) Morphometric analysis of *Rhodnius prolixus* Stål (hemiptera:reduviidae) midgut cells during blood digestion. *Tissue & Cell.* 20 (2) 291-301.

Buxton, P.A. (1930) The biology of the blood-sucking bug, *Rhodnius prolixus. Trans. R. Ent. Soc., Lond*. 78, 227-236, 1930.

Capriotti, N., Ianowski, J.P., Gioino, P., Ons, S. (2019) The neuropeptide CCHamide2 regulates diuresis in the Chagas disease vector *Rhodnius prolixus. J. Exp. Biol.* 222(Pt 10):jeb203000.

Chen, Z., Wang, O., and Wang, Z. (2010) The Amiloride-Sensitive Epithelial Na^+ Channel PPK28 Is Essential for Drosophila Gustatory Water Reception. *J Neurosci*. 30(18): 6247–6252.

Coast, G.M. (2009) Neuroendocrine control of ionic homeostasis in blood-sucking insects. *J. Exp. Biol.* 212, 378-386

Farmer, J., Maddrell, S.H.P., and Spring, J.H. (1981) Absorption of fluid by the idgut of *Rhodnius*. *J. Exp. Biol* 94:301-316.

Filippov, V., Aimanova, K., and Gill, S.S. (2003) Expression of an Aedes aegypti cation-chloride cotransporter and its Drosophila homologues. *Insect Molecular Biology*. 12 (4), 319–331.

Giannakou, M.E. and Dow, J.A.T. (2001) Characterization of the *Drosophila melanogaster* alkali-metal/proton exchanger (NHE) gene family. *J. Exp. Biol*. 204, 3703–3716.

Hanrahan, J.W., Wills, N.K., Phillips, J.E., and Lewis, A.S. (1986) Basolateral K channels in an insect epithelium. Channel density, conductance, and block by barium. *J. Gen. Physiol*. 87(3): 443–466.

Hartmann, A., Tesch, D., Nothwang, H.G., and Bininda-Emonds, O.R.P. (2014) Evolution of the cation chloride cotransporter Family: Ancient origins, gene losses, and subfunctionalization through duplication. Mol. Biol. Evol.31(2):434–447.

Ianowski, J.P., Paluzzi, J.P., Te Brugge, V.A., and Orchard, I. (2010) The antidiuretic neurohormone *Rhopr*CAPA-2 downregulates fluid transport across the anterior midgut in the blood-feeding insect *Rhodnius prolixus. Am J Physiol Regul Integr Comp Physiol*. 298(3):R548-57.

Maddrell, S. (2009) Insect homeostasis: past and future. *J. Exp. Biol.* 212, 446-451

Maddrell, S.H.P., O'Donnell, M.J., and Caffrey, R. (1993) The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus. J Exp Biol* 177:273-285.

O'Donnell, M.J., Dow, J.A., Huesmann, G.R., Tublitz, N.J., and Maddrell, S.H. (1996) Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* 199: 1163-1175.

O'Donnell, M.J., Ianowski, J.P., Linton, S.M., and Rheault, M.R. (2003) Inorganic and organic anion transport by insect renal epithelia. *Biochim Biophys Acta*. 1618(2):194-206.

Onken, H., Moffett, S.B., and Moffett, D.F. (2004a) The transepithelial voltage of the isolated anterior stomach of mosquito larvae (*Aedes aegypti*): pharmacological characterization of the serotonin-stimulated cells. *J. Exp. Biol.* 207, 1779-1787.

Onken, H., Moffett, S.B., and Moffett, D.F. (2004b) The anterior stomach of larval mosquitoes (*Aedes aegypti*): effects of neuropeptides on transepithelial ion transport and muscular motility. *J Exp Biol* 207:3731-3739.

Ons, S. (2017). Neuropeptides in the regulation of *Rhodnius prolixus* physiology. *J. Insect Physiol.* 97, 77-92.

Orchard, I. (2006) Serotonin: a coordinator of feeding-related physiological events in the blood-gorging bug, *Rhodnius prolixus. Comp Biochem Physiol A Mol Integr Physiol* 144:316-24.

Pacey, E.K., and O'Donnell, M.J. (2014) Transport of H^+, Na^+ and K^+ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*). *J. Insect Physiol.* 61 42–50.

Paluzzi, J.P., and Orchard, I. (2006) Distribution, activity and evidence for the release of an anti-diuretic peptide in the kissing bug *Rhodnius prolixus*. *J Exp Biol* 209:907-15.

Paluzzi, J.P., Russell, W.K., Nachman, R.J., and Orchard, I. (2008) Isolation, cloning and expression mapping of a gene encoding an anti-diuretic hormone and other CAPA-related peptides in the disease vector, *Rhodnius prolixus*. *Endocrinology* 149:4638–4646.

Petzel, D.H. (2000) Na1/H1 exchange in mosquito Malpighian tubules. *Am J Physiol Regulatory Integrative Comp Physiol.* 279: R1996–R2003

Phillips, J.E., and Audsle, N. (1995) Neuropeptide Control of Ion and Fluid Transport Across Locust Hindgut. *Amer. Zool.* 35:503-514.

Rassi, A. Jr., Rassi, A., and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* 375, 1388-1402.

Robertson, L., Donini, A., and Lange, A.B. (2014) K^+ absorption by locust gut and inhibition of ileal K^+ and water transport by FGLamide allatostatins. *J. Exp. Biol.* 217, 3377-3385.

Te Brugge, V., Ianowski, J.P., and Orchard, I. (2009) Biological activity of diuretic factors on the anterior midgut of the blood-feeding bug, *Rhodnius prolix*us. *Gen Comp Endocrinol* (in press, Gen Comp Endocrinology), 2009.

Te Brugge, V. A., Lombardi, V. C., Schooley, D. A. and Orchard, I. (2005). Presence and activity of a Dippu-DH31-like peptide in the blood-feeding bug, *Rhodnius prolixus. Peptides* 26, 29-42.

Te Brugge, V., Paluzzi, J. P., Schooley, D. A. and Orchard, I. (2011). Identification of the elusive peptidergic diuretic hormone in the blood-feeding bug *Rhodnius prolixus*: a CRF-related peptide. *J. Exp. Biol.* 214, 371-381.

Villalobos-Sambucaro, M. J., Lorenzo-Figueiras, A. N., Riccillo, F. L., Diambra, L. A., Noriega, F. G. and Ronderos, J. R. (2015). Allatotropin modulates myostimulatory and cardioacceleratory activities in *Rhodnius prolixus* (Stål). *PLoS ONE* 10, e0124131.

Zandawala, M., Paluzzi, J.-P. and Orchard, I. (2011). Isolation and characterization of the cDNA encoding DH(31) in the kissing bug, *Rhodnius prolixus. Mol. Cell. Endocrinol*. 331, 79-88.

Zandawala, M., Poulos, C. and Orchard, I. (2015). Structure-activity relationships of two *Rhodnius prolixus* calcitonin-like diuretic hormone analogs. *Peptides* 68, 211-213.

Figures

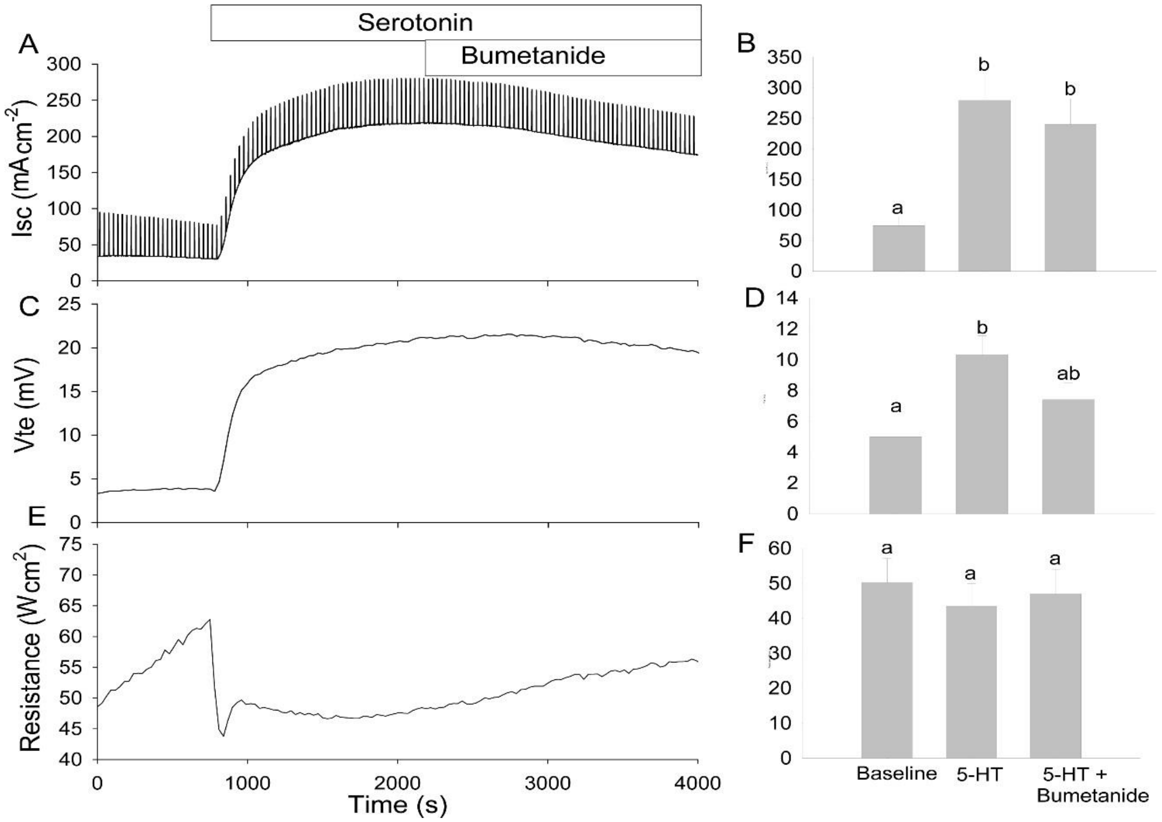

Figure 1: Effect of bumetanide on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of 100 nmol/l serotonin (5-HT) increased transepithelial transport that was not affected by treatment with bumetanide (100 µM). The upward deflections on Isc observed in A are caused by the passage of 5 mV pulses across the epithelia. The size of these deflections is proportional to the transepithelial resistance and were used to calculate the resistance and Vt (see materials and methods). 5-HT and bumetanide were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, n = 10, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

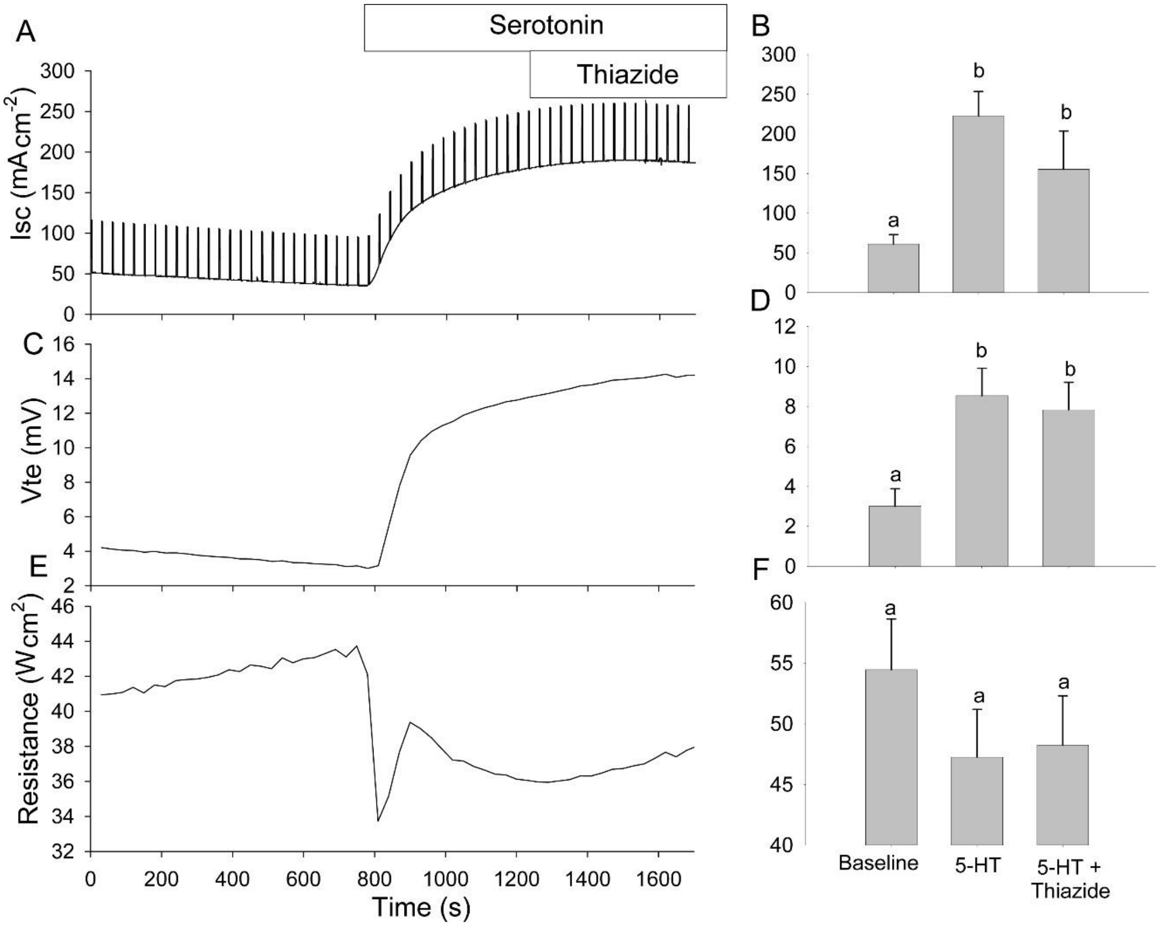

Figure 2: Effect of hydrochlorothiazide on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT that was not affected by addition of hydroclorothiazide (100 µM). Serotonin and hydroclorothiazide (Thiazide) were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, n = 16, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

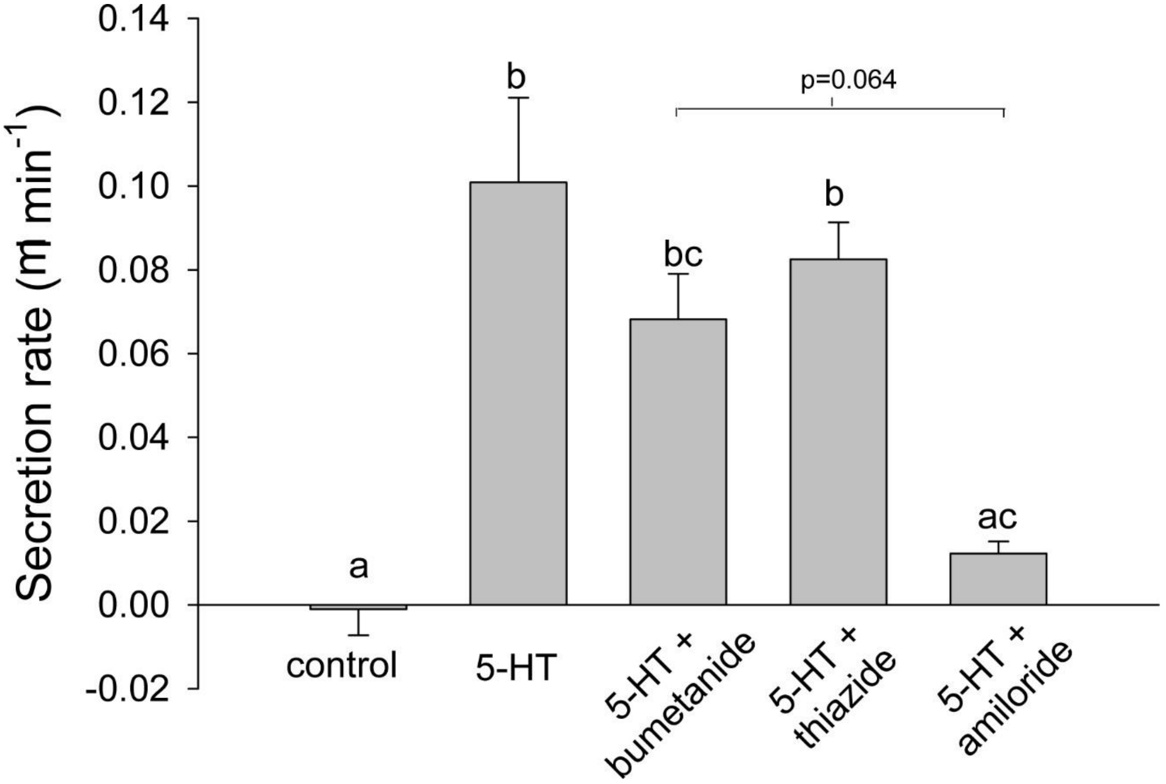

Figure 3: Effect of bumetanide, hydrochlorothiazide, and amiloride on serotonin-stimulated fluid transport rate. Anterior midguts from fifth-instar *R. prolixus* were incubated with saline (control, n = 28), 100 nM serotonin (5-HT; n = 15), 5-HT+bumetanide (100 µM, n = 13), 5-HT+ hydrochlorothiazide (100 µM, n = 12), or amiloride (100 µM, n=8). Columns marked with different letters are significantly different (p<0.05, Kruskal-Wallis test, Dunn's Multiple Comparison Test).

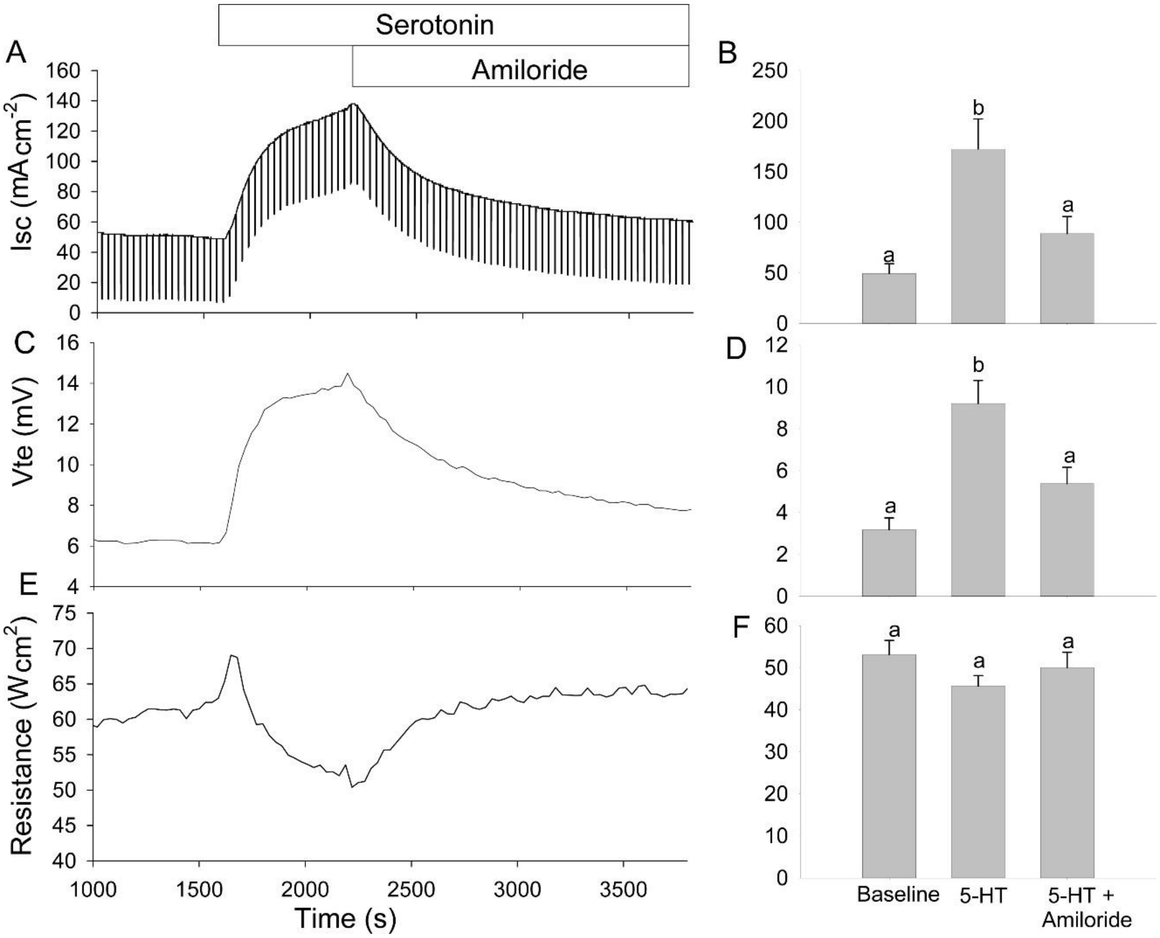

Figure 4: Effect of amiloride on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of amiloride (10µM) blocked the effect of 5-HT (n = 18). Serotonin and amiloride were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, *P* < 0.05).

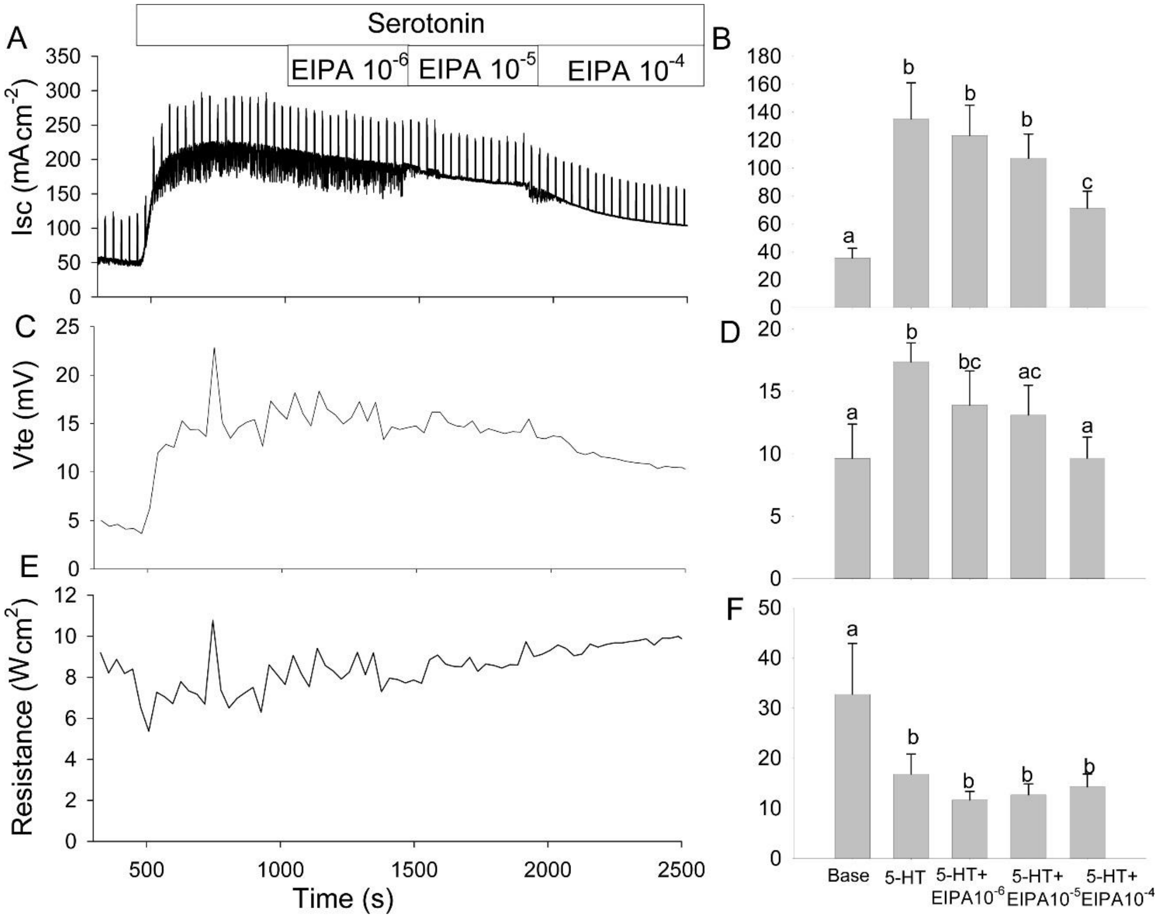

Figure 5: Effect of EIPA on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of 5-N-ethyl-N-isopropyl amiloride (EIPA) (1, 10 and 100 µM) blocked the effect of 5-HT (n = 6). Serotonin and EIPA were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

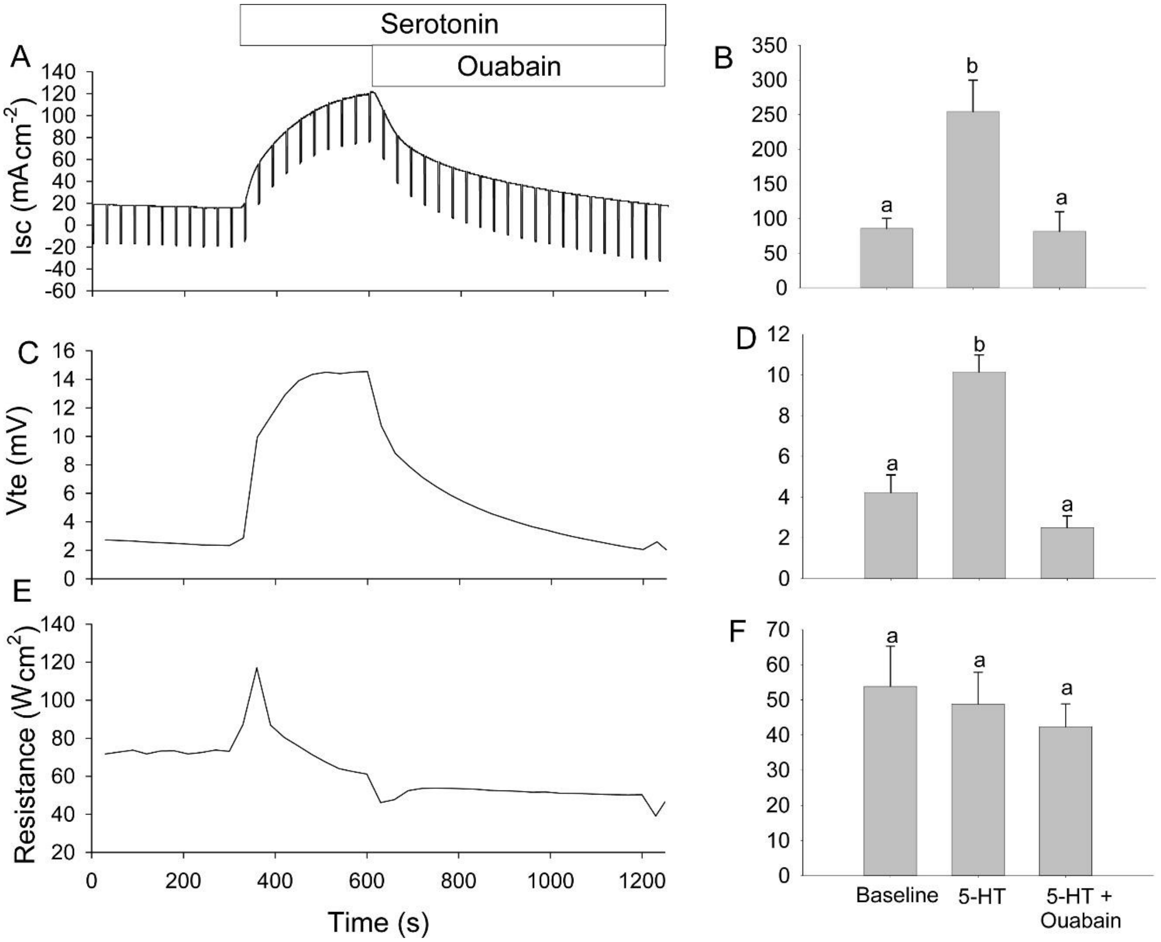

Figure 6: Effect of ouabain on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of ouabain (100µM) blocked the effect of 5-HT (n = 8). Serotonin and ouabain were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, *P* < 0.05).

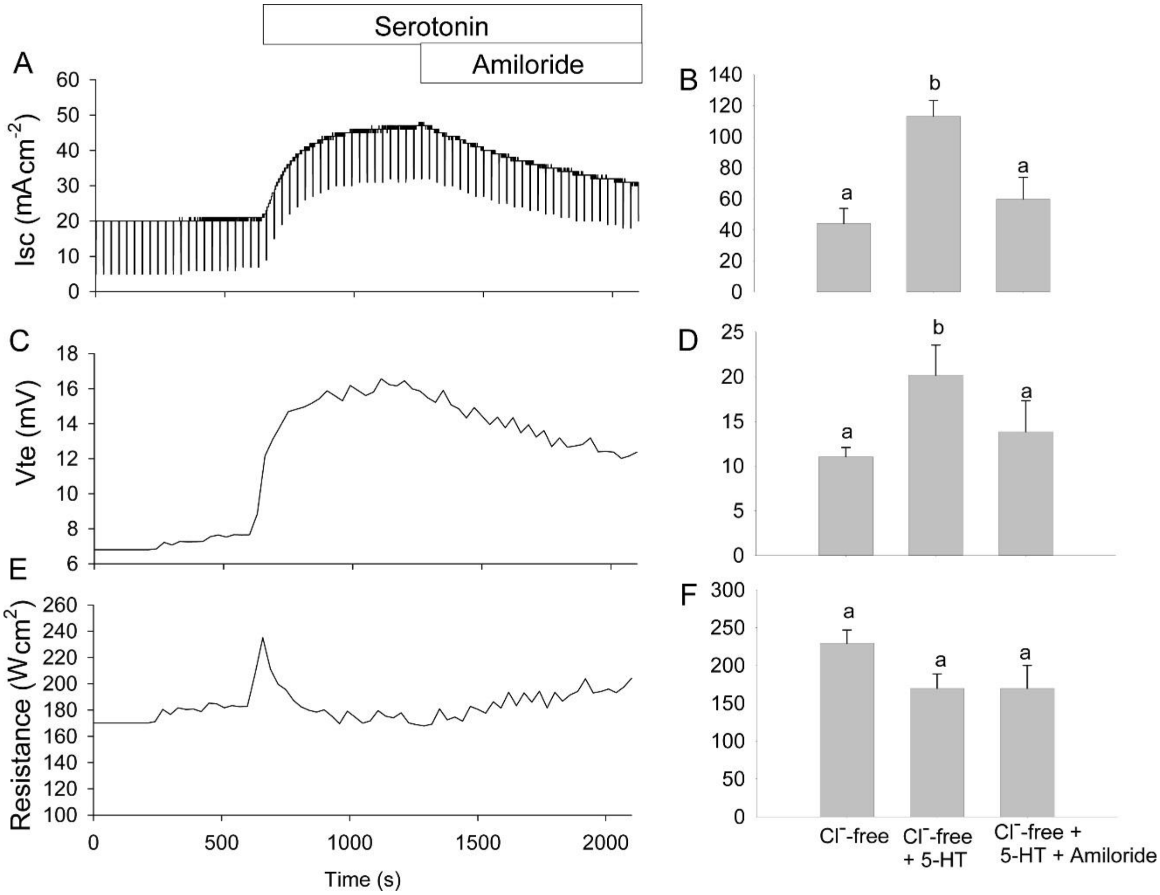

Figure 7: Effect of Cl$^-$-free bath on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus* (n = 13). Serotonin and amiloride were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

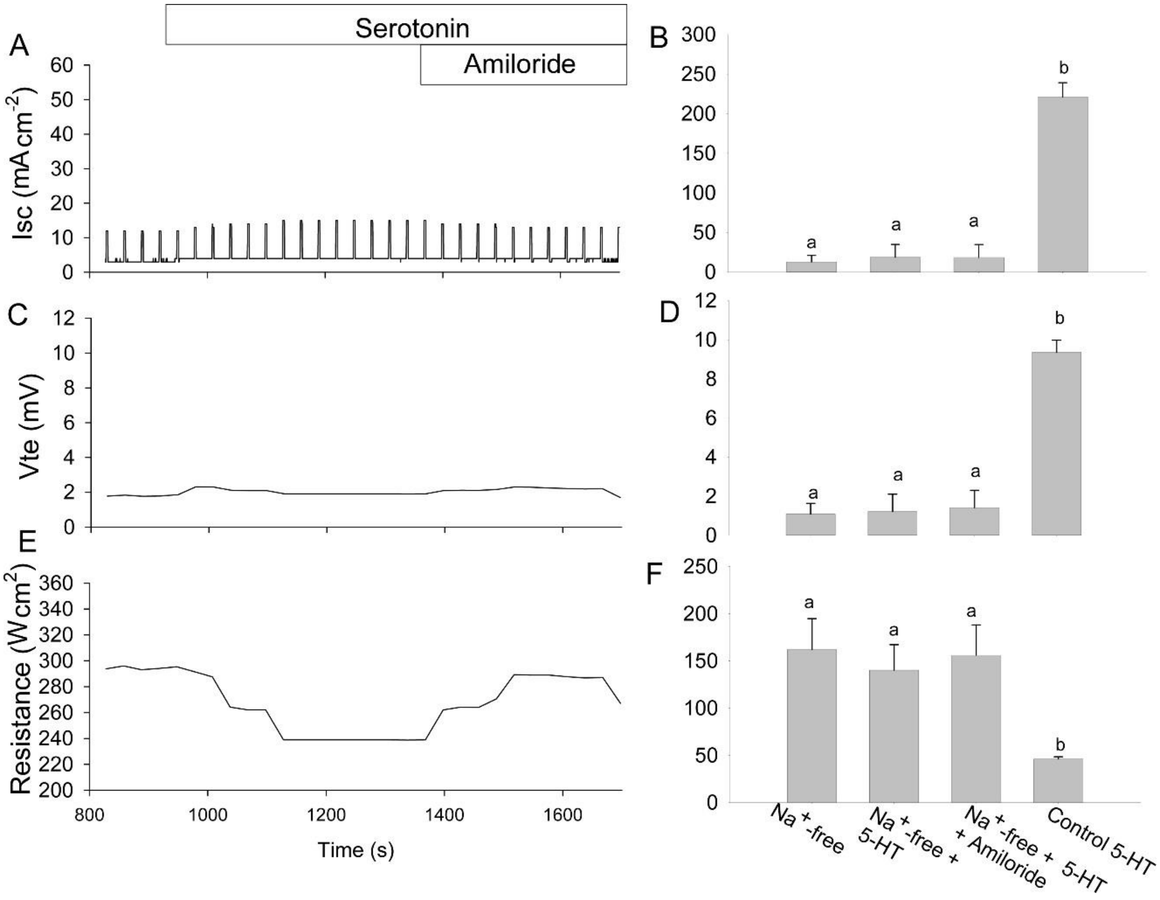

Figure 8: Effect of Na$^+$-free bath on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus* (n = 11). Serotonin and amiloride were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

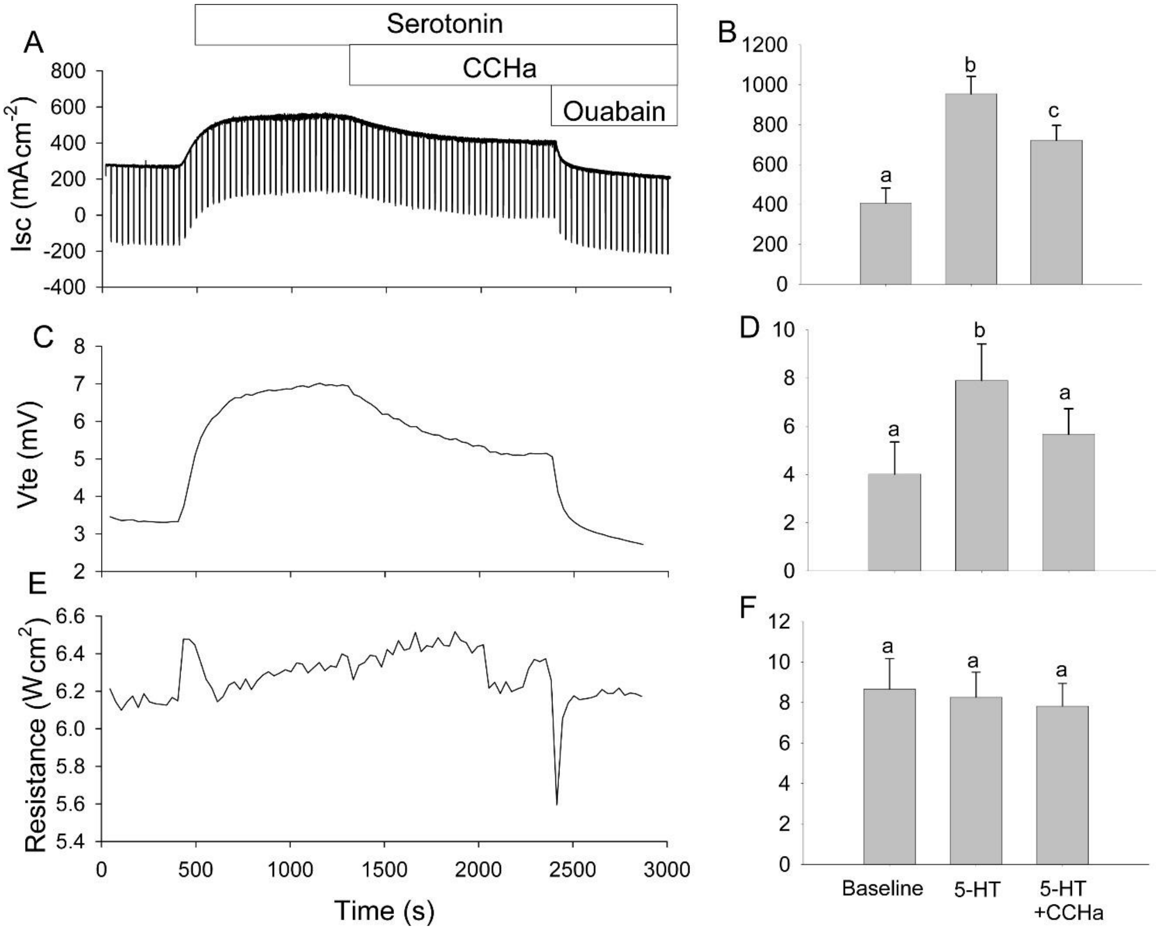

Figure 9: Effect of *Rhopr*CCHamide2 on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of 5-HT on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of *Rhopr*CCHamide2 (1µM) reduced the Isc (n = 10) which was further inhibited by ouabain. Serotonin, *Rhopr*CCHamide2, and amiloride were added during the times indicated by the horizontal bars in A. The group treated with ouabain was not included in the statistical analysis in figure B, D, and F. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

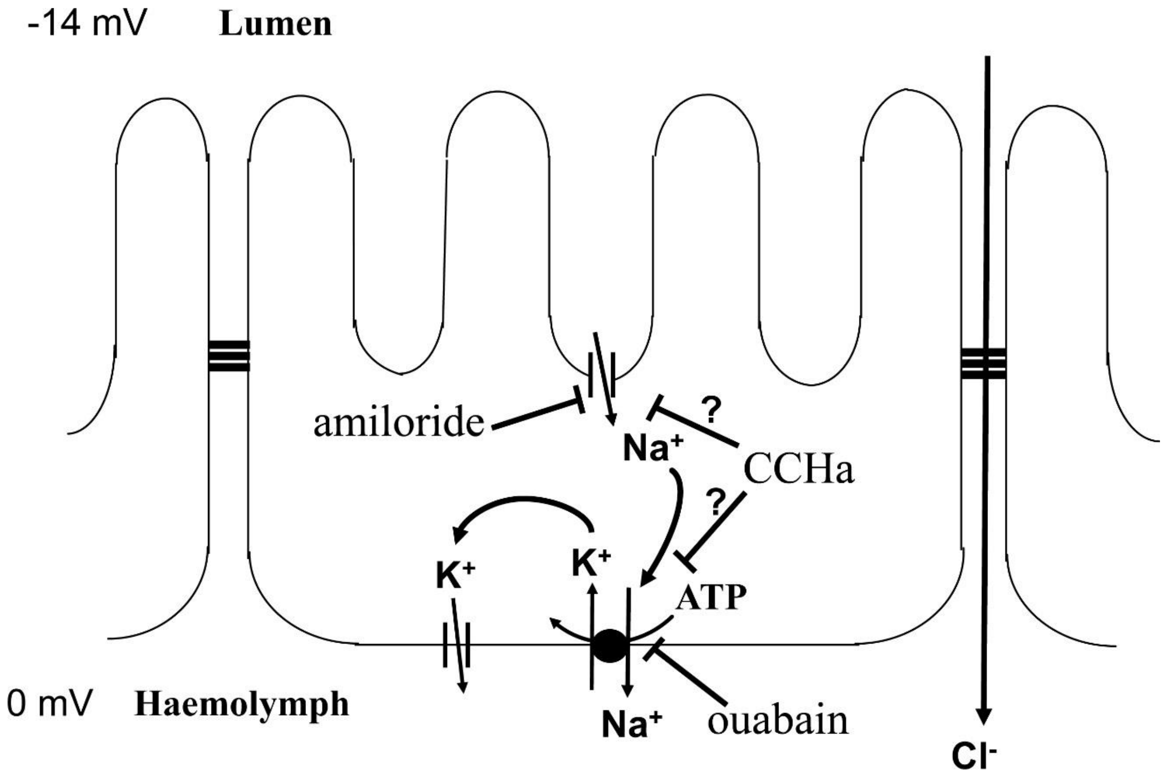

Figure 10: Proposed working model of serotonin-stimulated ion transport across the anterior midgut of *R. prolixus*. The model incorporates our results and the published work by Farmer et al., 1981. The model includes the inhibitory effects of ouabain, amiloride, and the potential effect of CCHa.

Supplemental Figure 1

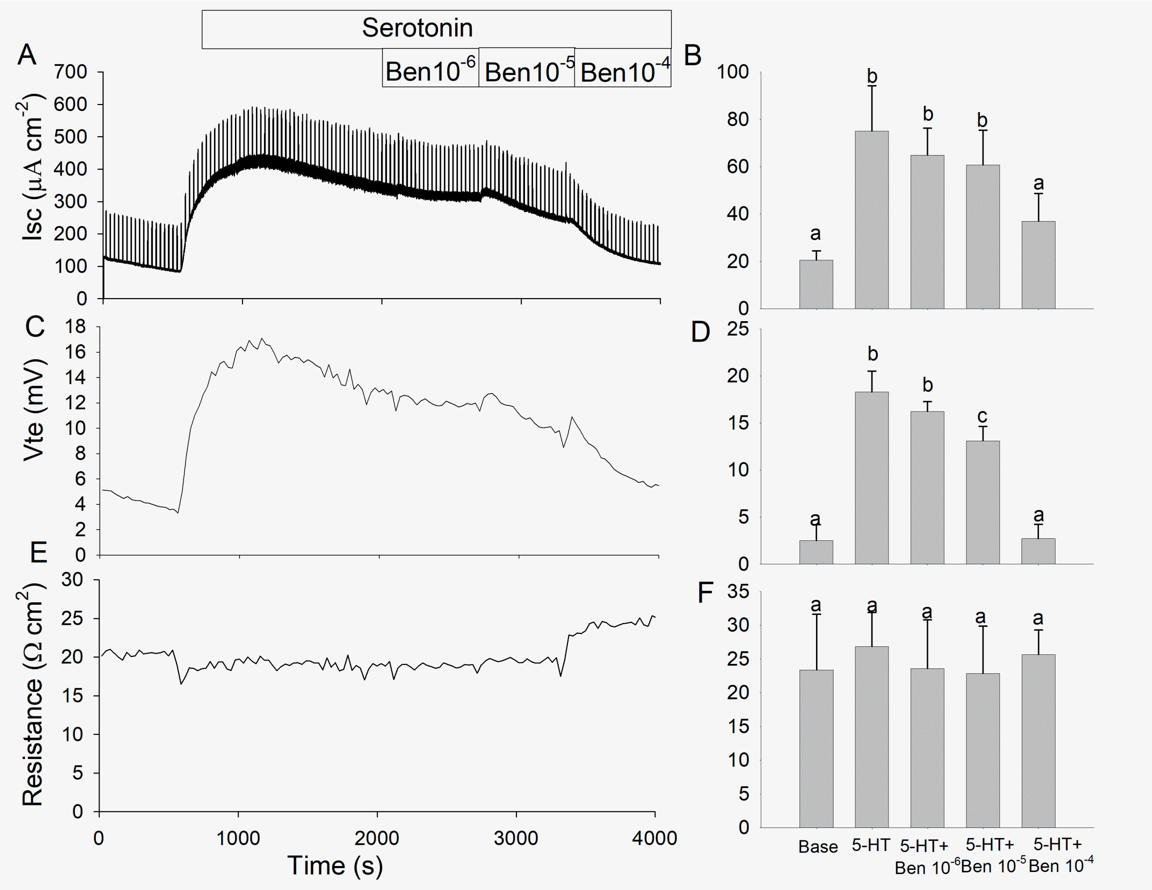

Figure S1. Effect of benzamil on serotonin-stimulated ion transport. A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of benzamil (Ben, 1, 10 and 100 µM) blocked the effect of 5-HT (n = 6). Serotonin and benzamil were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

Supplemental Figure 2

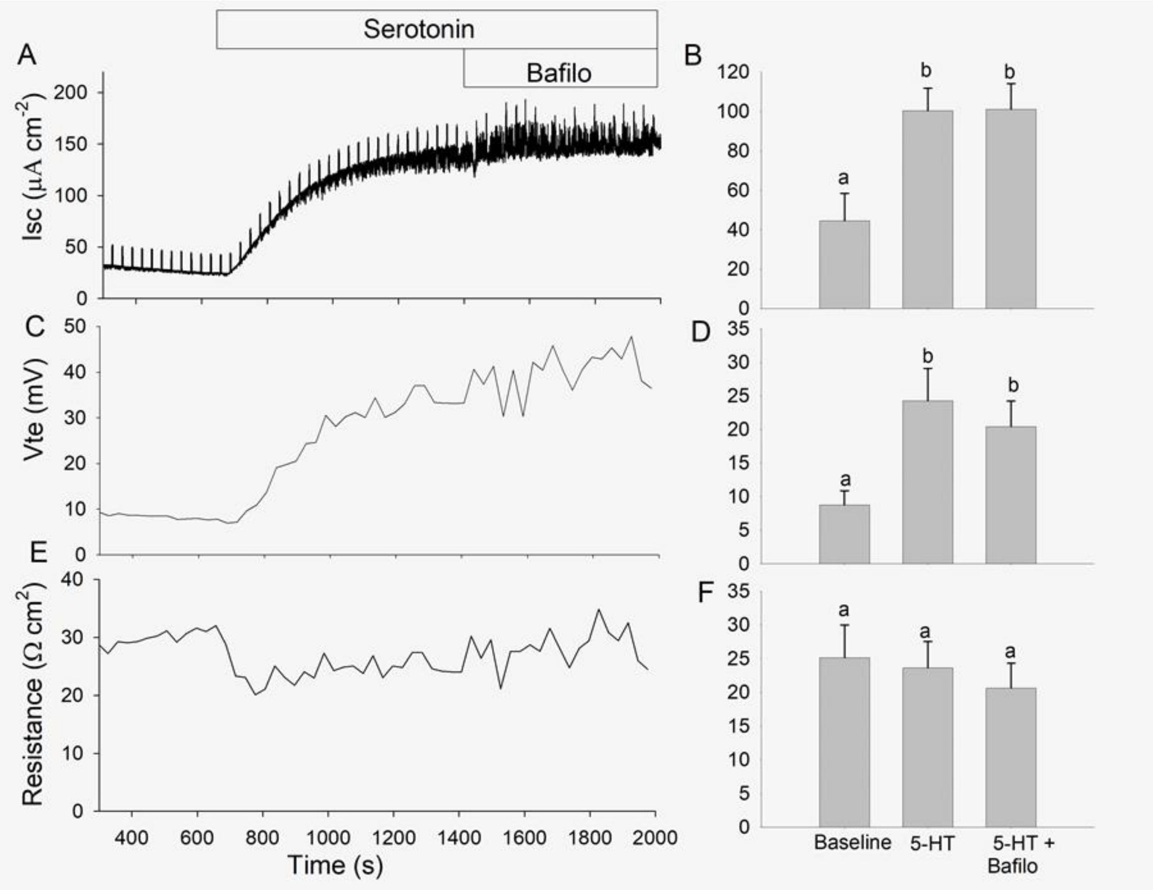

Figure S2. Effect of bafilomycin on serotonin-stimulated ion transport.

A and B: short-circuit current (Isc). C and D: Transepithelial voltage (Vt). And E and F resistance (R) across the anterior midgut from fifth-instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in Isc and VT. Addition of bafilomycin (Bafilo, 100µM) blocked the effect of 5-HT (n = 6). Serotonin and bafilomycin were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means ± SE, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, $P < 0.05$).

Supplemental Figure 3

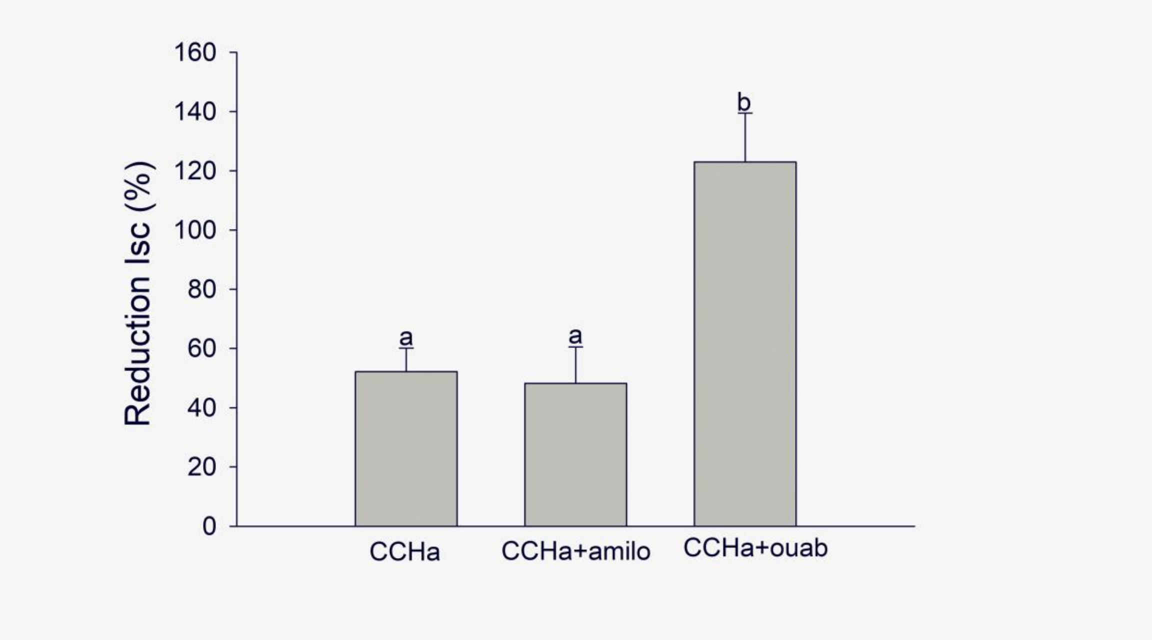

Figure S3. Percentage of peak serotonin-stimulate Isc in response to *Rhopr*CCHamide2, amiloride, and ouabain. Effect of *Rhopr*CCHamide2 (1µM, CCHa, n = 10), *Rhopr*CCHamide2 + amiloride (100 µM, CCHa+amilo, n = 5), and *Rhopr*CCHamide2 + ouabain (100µM, CCHa+ouab, n = 5). Columns marked with different letters are significantly different (means ± SE, non-parametric Kruskal-Wallis test, Dunn's multiple comparisons test, $P < 0.05$).