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RESEARCH ARTICLE

The neuropeptide RhoprCCHamide2 inhibits serotonin-stimulated transcellular Na^+ transport across the anterior midgut of the vector of Chagas disease, *Rhodnius prolixus*

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ABSTRACT

Rhodnius prolixus is a blood-feeding insect vector of *Trypanosoma 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 with the insect's hemolymph. 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 anti-diuretic 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 RhoprCCHamide2. Ussing chamber assays revealed that serotonin-stimulated increase in transepithelial short-circuit current (I_{sc}) was more sensitive to the blockage with amiloride than 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA), suggesting the involvement of Na^+ channels. Incubation in Na^+-free, but not Cl^--free saline, blocked the effect of serotonin on I_{sc}. Moreover, treatment with Na^+–K^+–$2Cl^-$ cotransporter (NKCC) and Na^+–Cl^- cotransporter (NCC) blockers had no effect on fluid secretion but was blocked by amiloride. Blockage of Na^+/K^+-ATPase with ouabain inhibited I_{sc} but the H^+-ATPase inhibitor bafilomycin had no effect. The neuropeptide RhoprCCHamide2 diminished serotonin-stimulated I_{sc} across the crop. The results suggest that Na^+ undergoes active transport via an apical amiloride-sensitive Na^+ channel and a basolateral ouabain-sensitive Na^+/K^+-ATPase, while Cl^- is transported through a passive paracellular pathway.

KEY WORDS: CCHamide2, Crop, Ion transport, Osmoregulation, *Rhodnius prolixus*

INTRODUCTION

Rhodnius prolixus is a hematophagous insect vector of the parasite *Trypanosoma cruzi*, which causes Chagas disease that affects 7 million people (https://www.who.int/health-topics/chagas-disease#tab=tab_1; 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 triatomine bug 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.

Rhodnius 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 with the insect's hemolymph (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 2 h 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 post-prandial 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 hemolymph to be excreted by the Malpighian (renal) tubules (Barrett, 1982; Billingsley, 1988; Billingsley and Dowe, 1986; Billingsley and Dowe, 1989; Farmer et al., 1981). 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, 2011) and Zoone-DH (Te Brugge et al., 2005). The termination of the diuretic process seems to involve at least one anti-diuretic hormone, RhoprCAPA2 (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., 2005; Te Brugge et al., 2009; 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 RhoprCCHamide2, which increases secretion by Malpighian tubules while inhibiting absorption across the anterior midgut after stimulation with serotonin (Capriotti et al., 2019). However, the mechanism of RhoprCCHamide2 on the anterior midgut of *R. prolixus* and how it affects 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 RhoprCCHamide2 (Capriotti et al., 2019). The results indicate

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Received 23 January 2021; Accepted 31 March 2021

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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 is inhibited, but not fully blocked, by treatment with RhoprCCHamide2.

MATERIALS AND METHODS

Animals

Rhodnius prolixus Stål 1859 were obtained from a colony maintained at the Department of Anatomy, Physiology and Pharmacology, University of Saskatchewan, Saskatoon, Canada, at 60% relative humidity in incubators at 25°C, and routinely fed on defibrinated rabbit 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 (mmol l^{-1}): 129 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4 and 8.6 Hepes at 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 (St Louis, MO, USA).

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 reagents. After 30 min incubation, the weight of the anterior midgut was measured for a second time and the difference in mass was used to calculate the volume of saline transported by the anterior midgut (Te Brugge et al., 2009).

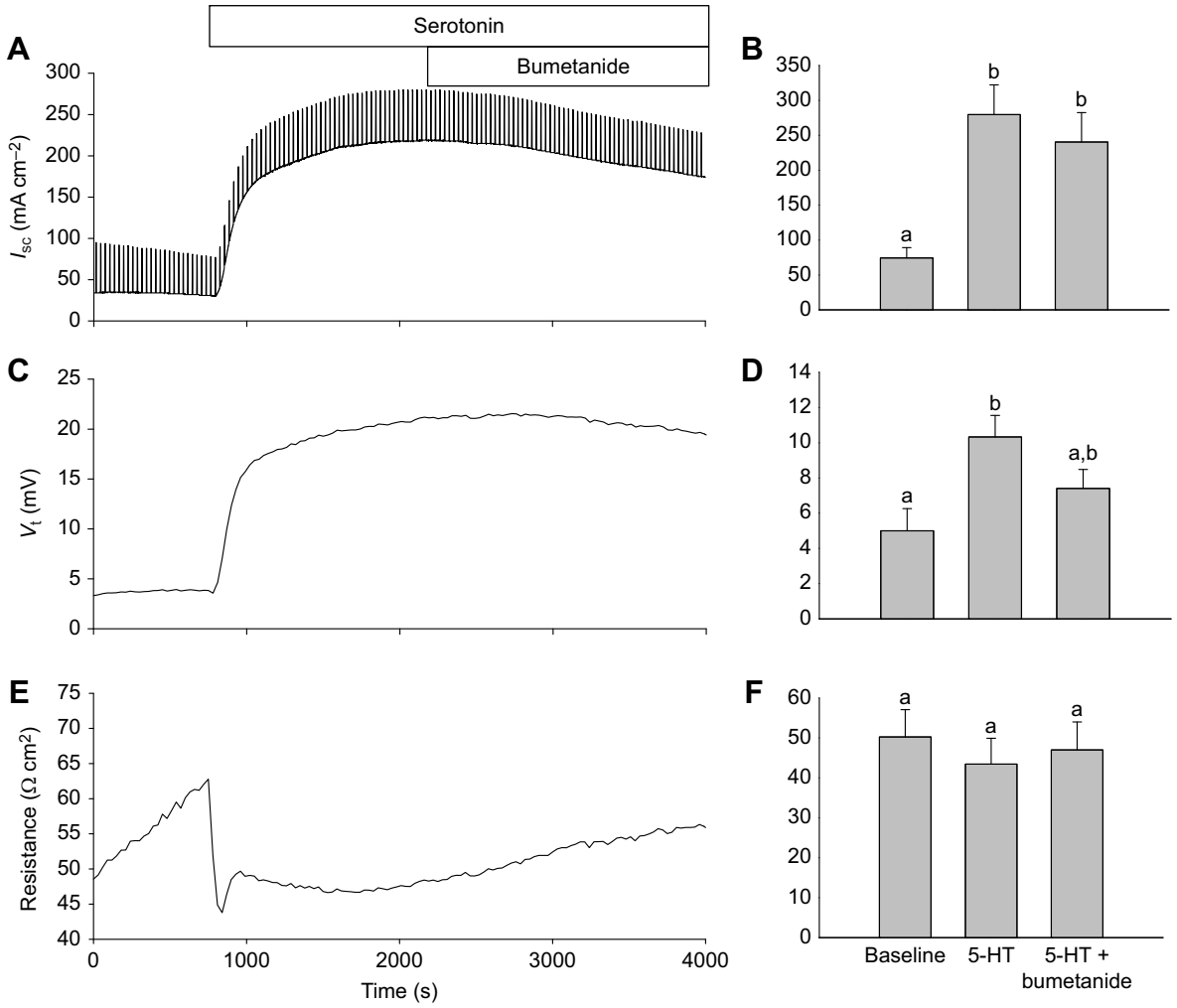

Fig. 1. Effect of bumetanide on serotonin-stimulated ion transport in *Rhodnius prolixus*. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of 100 nmol l^{-1} serotonin (5-HT) increased transepithelial transport that was not affected by treatment with bumetanide (100 µmol l^{-1}). The upward deflections on I_{sc} 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 V_t (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±s.e.m., N=10, repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

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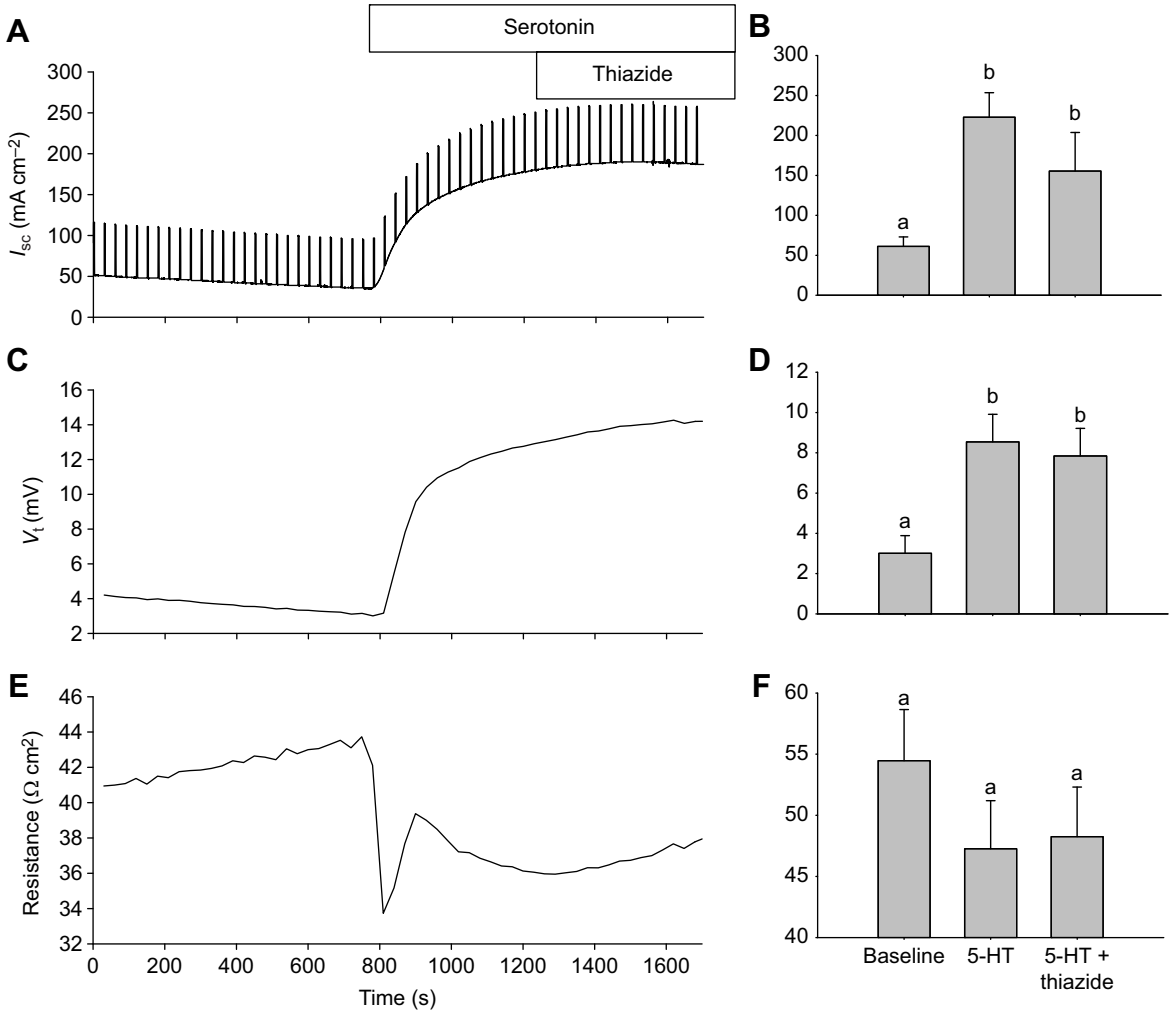

Fig. 2. Effect of hydrochlorothiazide on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in I_{sc} and V_t that was not affected by addition of hydroclorothiazide (100 µmol l^{-1}). 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±s.e.m., N=16, repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

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). The chamber was maintained at room temperature and apical and basolateral compartments were bubbled with 95% O_2 and 5% CO_2 gas. After mounting the tissues in Ussing chambers, an equilibration time of 15 min was allowed for stabilization of electrophysiological parameters.

The Ussing chamber operated in voltage-clamp mode with the voltage clamped at 0 mV (VCC MC2 amplifier; Physiologic Instruments). The amount of current required to maintain the transepithelial voltage (V_t) at 0 mV is called the short-circuit current (I_{sc}). The I_{sc} is defined as the charge flow per time when the tissue is short circuited (i.e. V_t is clamped to 0 mV). We also calculated the values for the transepithelial voltage (V_t) and transepithelial resistance (R_t) by clamping the voltage to 5 mV for 3 s, every

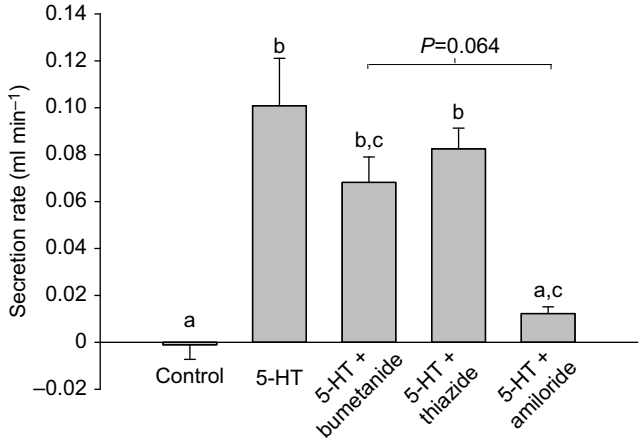

Fig. 3. Effect of bumetanide, hydrochlorothiazide and amiloride on serotonin-stimulated fluid transport rate. Anterior midguts from fifth instar *R. prolixus* were incubated with saline (control, N=28), 100 nmol l^{-1} serotonin (5-HT; N=15), 5-HT+bumetanide (100 µmol l^{-1}, N=13), 5-HT+hydrochlorothiazide (100 µmol l^{-1}, N=12) or amiloride (100 µmol l^{-1}, N=8). Columns marked with different lower case letters are significantly different (P<0.05, Kruskal–Wallis test, Dunn's multiple comparison test).

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30 s and recording the corresponding change in I_{sc}. V_t and R_t were calculated according to Ohm's law ($I_{sc}=V_t/R_t$).

The voltage-sensing electrodes were made with Ag/AgCl pellets and the current passing electrodes were made of silver chloride wires, both connected to the Ussing chamber by 150 mmol l^{-1} NaCl agar bridges; data were recorded using an A/D converter and data acquisition system (PowerLab; AD Instruments, Colorado Springs, CO, USA)

Reagents

Amiloride, benzamil hydrochloride hydrate (B2417), 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA; A3085), ouabain, hydrochlorothiazide, bumetanide and serotonin were purchased from Sigma. Bafilomycin A1 was purchased from Cedarlane. Stock solutions of the drugs were prepared in DMSO so that the maximum final concentration of DMSO was <1% (v/v). Synthetic RhoprCCHa2 (GGCSAFGHSCFGGH-NH_2) was obtained from GenScript Corporation (Piscataway, NJ, USA) and dissolved in saline solution.

Statistics

Results are expressed as means±s.e.m. 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 midgut section of *R. prolixus* displayed a basal short-circuit current (I_{sc}) of 74.4±14.8 µA cm^{-2}, a transepithelial potential (V_t) of 4.9±1.2 mV basolateral side positive with respect to the lumen, and resistance (R) of 50.2±6.8 Ω cm^2 (N=10; Fig. 1). Addition of serotonin (0.1 µmol l^{-1}) to both sides (i.e. apical and basolateral) triggered a significant increase in I_{sc} to 279.6±42.6 µA cm^{-2}, V_t increased to 10.3±1.2 mV and R decreased to 43.4±6.4 Ω cm^2 (Fig. 1; $P<0.05$, ANOVA, Tukey's multiple comparison test), consistent with serotonin stimulation of transepithelial ion transport.

Serotonin-stimulated transport does not involve Na^+–K^+–2Cl^- or Na^+–Cl^- cotransporters

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 µmol l^{-1} added to both apical and basolateral sides) had no effect on I_{sc}, V_t or R displayed by the tissues 10 min after treatment (Fig. 1). Another

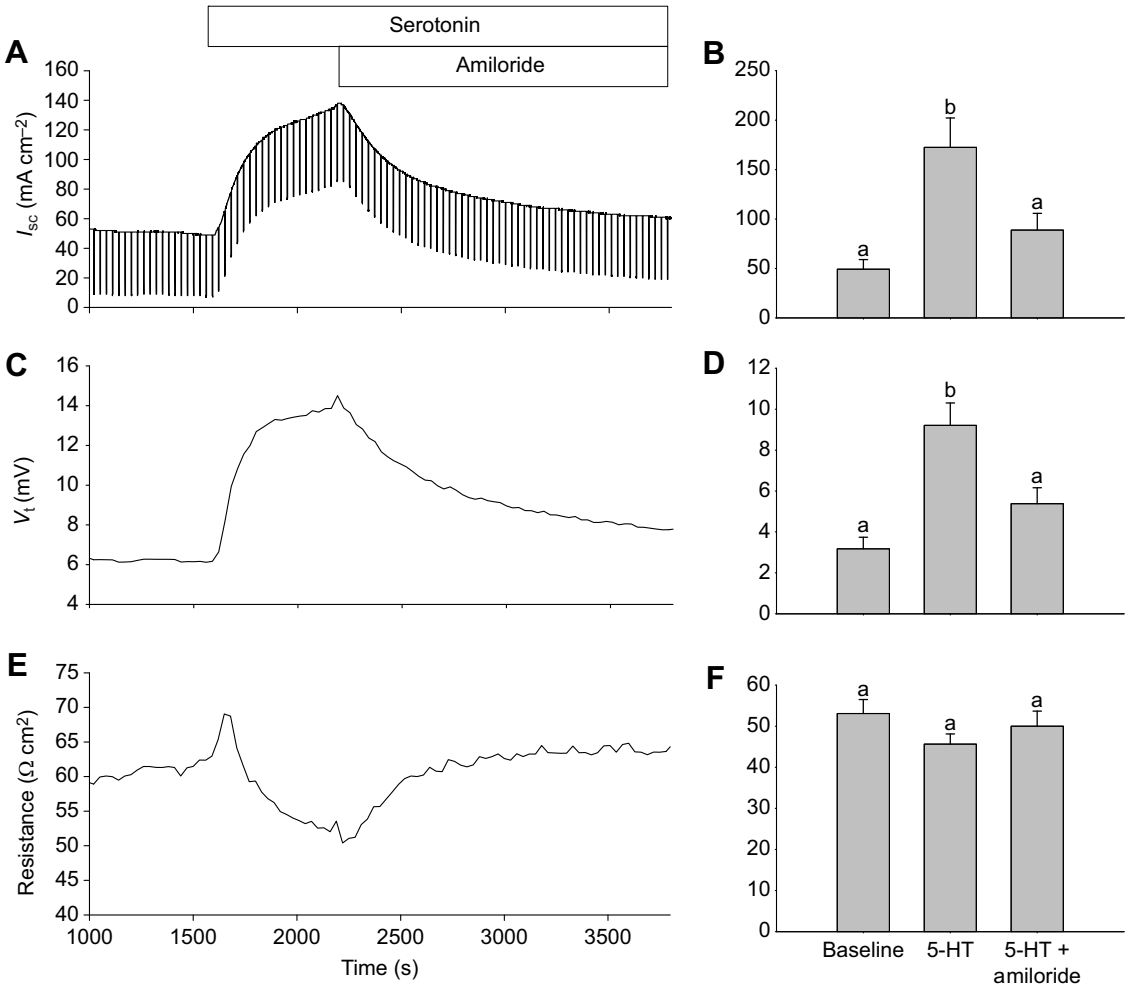

Fig. 4. Effect of amiloride on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in I_{sc} and V_t. Addition of amiloride (10 µmol l^{-1}) 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±s.e.m., repeated measures ANOVA, Tukey–Kramer multiple comparison test, $P<0.05$).

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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 (Hartmann et al., 2014), we decided to test a common NCC blocker used in vertebrates, hydrochlorothiazide. Adding hydrochlorothiazide (100 µmol l^{-1}) to both apical and basolateral sides had no effect on I_{sc}, V_t 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 conditions (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 µmol l^{-1}, 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, EIPA and benzamil in Ussing chamber assays (Giannakou and Dow, 2001; Petzel, 2000). Adding amiloride (10 µmol l^{-1}) on both the apical and basolateral sides of serotonin-stimulated preparations significantly reduced I_{sc} and V_t to values similar to the baseline condition (Fig. 4; N=18, P<0.05, Kruskal–Wallis test, Dunn's multiple comparison test). In contrast, the effect of amiloride on R was not statistically significant

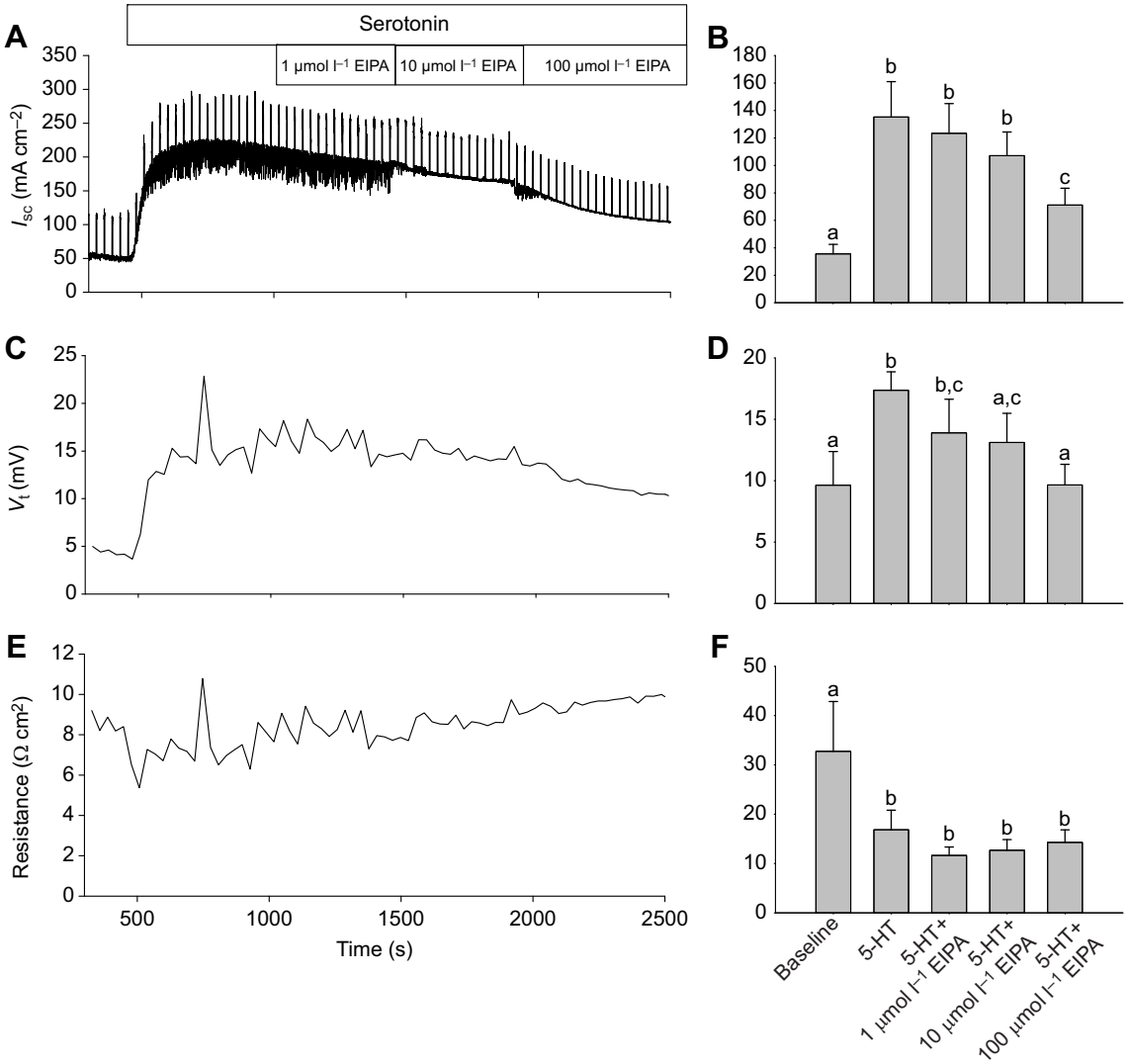

Fig. 5. Effect of EIPA on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in I_{sc} and V_t. Addition of 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA) (1, 10 and 100 µmol l^{-1}) 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±s.e.m., repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

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(Fig. 4F). Treatment with EIPA (Fig. 5) and benzamil (Fig. S1) on both the apical and basolateral sides resulted in a reduction in I_{sc} but at higher concentrations (100 µmol l^{-1}) than amiloride (N=6, P<0.05, ANOVA, Tukey's multiple comparisons test). Interestingly, V_t was more sensitive to both EIPA and benzamil, which caused a significant reduction in V_t at a concentration of 10 µmol l^{-1}. 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 Na^+/K^+-ATPase by treating the preparations with the blocker, ouabain (100 µmol l^{-1}). Serotonin-stimulated preparations responded to the addition of ouabain to the tissue by decreasing I_{sc} and V_t (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). As 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 (10 µmol l^{-1}). Blocking H^+-ATPase had no effect on I_{sc}, V_t 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 I_{sc} and V_t that resembled that of tissues bathed in control solution, and was blocked by amiloride (Fig. 7; N=13, 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 (Fig. 8; N=11, 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^- transport seem to be passive, e.g. through paracellular pathways.

Finally, we tested the effect of RhoprCCHamide2 on serotonin-stimulated preparations. The addition of RhoprCCHamide2 (1 µmol l^{-1}) significantly decreased I_{sc} and V_t but had no effect on R (Fig. 9; N=10, 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 RhoprCCHamide2-treated preparations. Treatment with RhoprCCHamide2 reduced I_{sc} by 50% (Fig. S3). Adding amiloride further blocked I_{sc} by 50%, thus fully blocking the effect of 5-HT. Ouabain had a larger effect, completely blocking the I_{sc} to 0 µA cm^{-2}. These results indicate

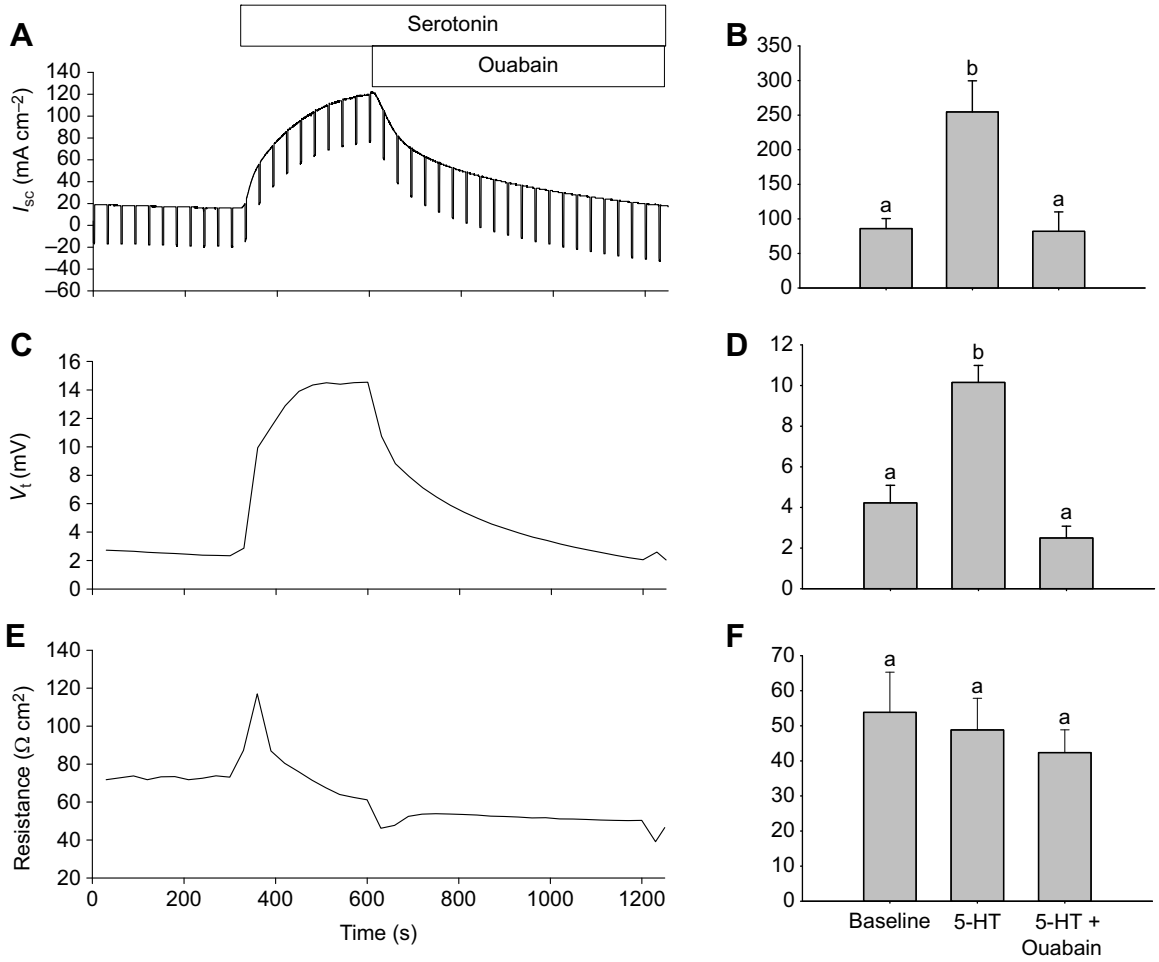

Fig. 6. Effect of ouabain on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in I_{sc} and V_t. Addition of ouabain (100 µmol l^{-1}) 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 lower case letters are significantly different (means±s.e.m., repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

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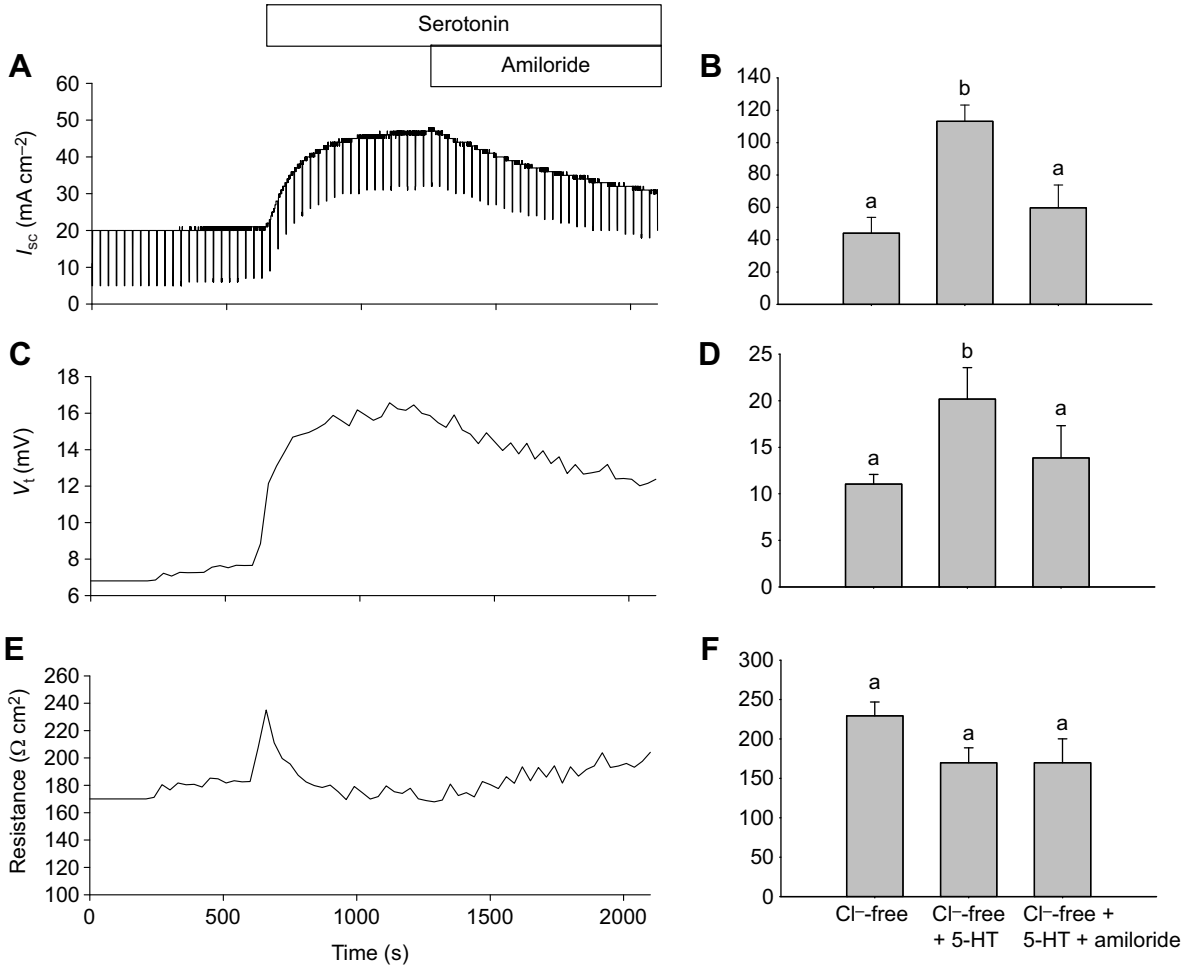

Fig. 7. Effect of Cl$^-$-free bath on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus* (N=13). Serotonin (5-HT) and amiloride were added during the times indicated by the horizontal bars in A. Columns marked with different letters are significantly different (means±s.e.m., repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

RhoprCCHamide2 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 RhoprCCHamide2.

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 an 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 conditions 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 I_{sc} 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 I_{sc} 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 µmol l^{-1}) that is an order of magnitude larger than that for amiloride (IC_{50} 80 µmol l^{-1}) or benzamil (IC_{50} 70 µmol l^{-1}; Giannakou and Dow, 2001; Petzel, 2000). Thus, based on the pattern of sensitivity to EIPA, amiloride and benzamil, our data suggest that Na^+ flux across anterior midgut of *R. prolixus* is likely to be 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) and 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 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 the serotonin-stimulated hemolymph-side positive transepithelial potential observed in our

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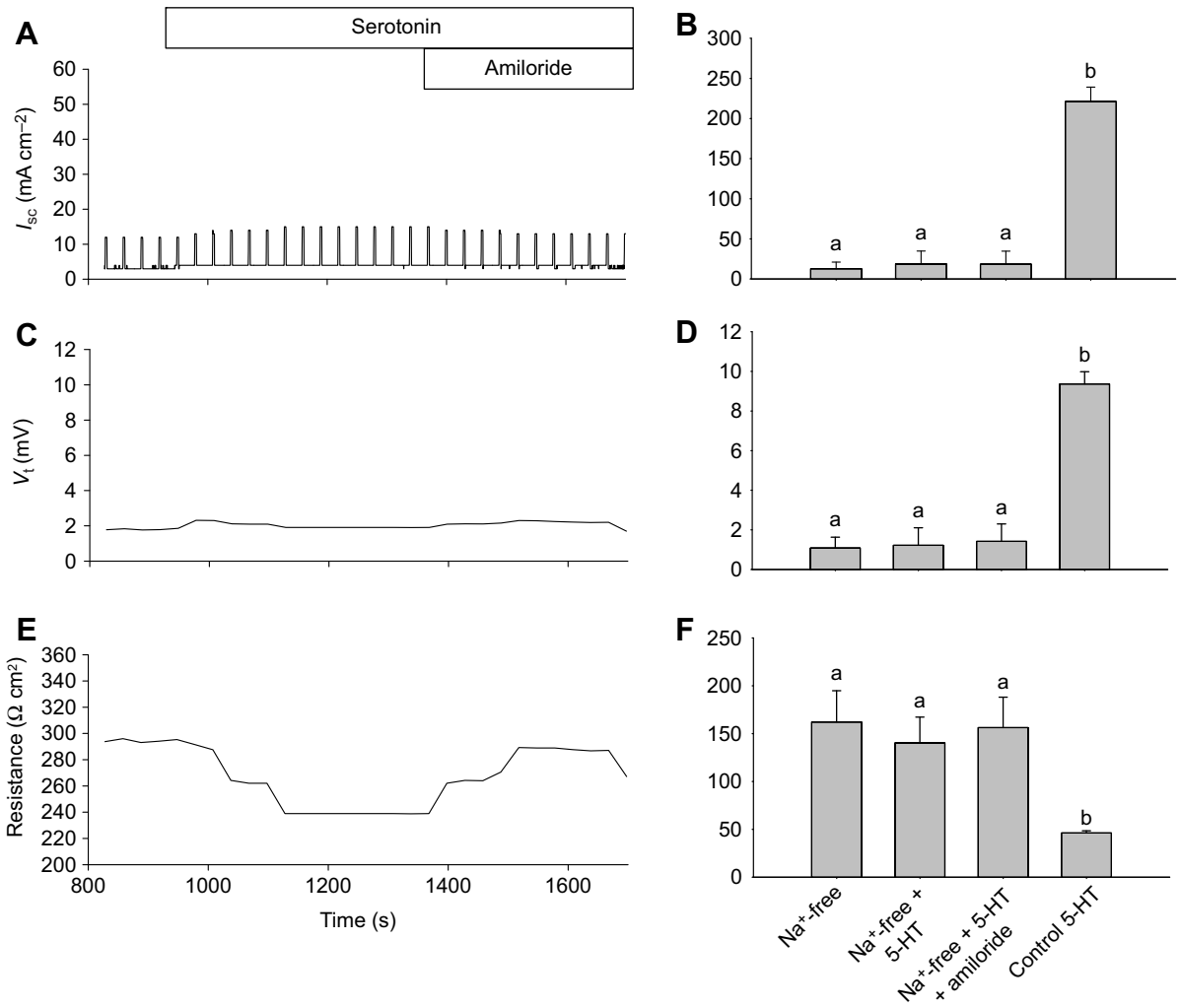

Fig. 8. Effect of Na$^+$-free bath on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth-instar *R. prolixus* (n=11). Serotonin (5-HT) and amiloride were added during the times indicated by the horizontal bars in panel A. Columns marked with different letters are significantly different (means±s.e.m., repeated-measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

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), as 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 *A. aegypti*. Ion transport across the gut of adult *A. aegypti* involves Na^+/K^+-ATPase-energized Na^+ and K^+ transepithelial transport (Pacey and O'Donnell, 2014). However, there is also evidence of an apical H^+-ATPase that drives H^+-linked amino acid transport, basolateral Na^+–H^+ exchange (Pacey and O'Donnell, 2014), and also HCO_3^-–Cl^- exchange involved in pH regulation in larval stages (Filippov et al., 2003; Onken et al., 2004a,b). Similarly, the resorptive transport across the gut of locust, one of the best-studied tissues in insects, 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 (Audsley et al., 1992, 1994, 2013; Phillips and Audsle, 1995). 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 RhoprCCHamide2 downregulates I_{sc} in Ussing chamber assays. RhoprCCHamide2 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. RhoprCCHamide2 would also reduce the driving force for passive Cl^- transport as it would reduce transepithelial potential. Interestingly, ion transport across anterior midgut is reduced, but not completely blocked by RhoprCCHamide2, consistent with our previous results (Capriotti et al., 2019). The effect of RhoprCCHamide2 on ion flux observed here seems to be moderate compared with the activity of the anti-diuretic hormone RhoprCAPA2, which completely blocks the effect of serotonin on the anterior midgut (Ianowski et al., 2010). Among the neuropeptides controlling diuresis in *R. prolixus* identified to date, RhoprCCHamide2 plays a unique dual role: it is an anti-diuretic factor on the anterior midgut and a diuretic factor on the Malpighian tubules where it stimulates urine formation. These

Journal of Experimental Biology (2021) 224, jeb242272. doi:10.1242/jeb.242272

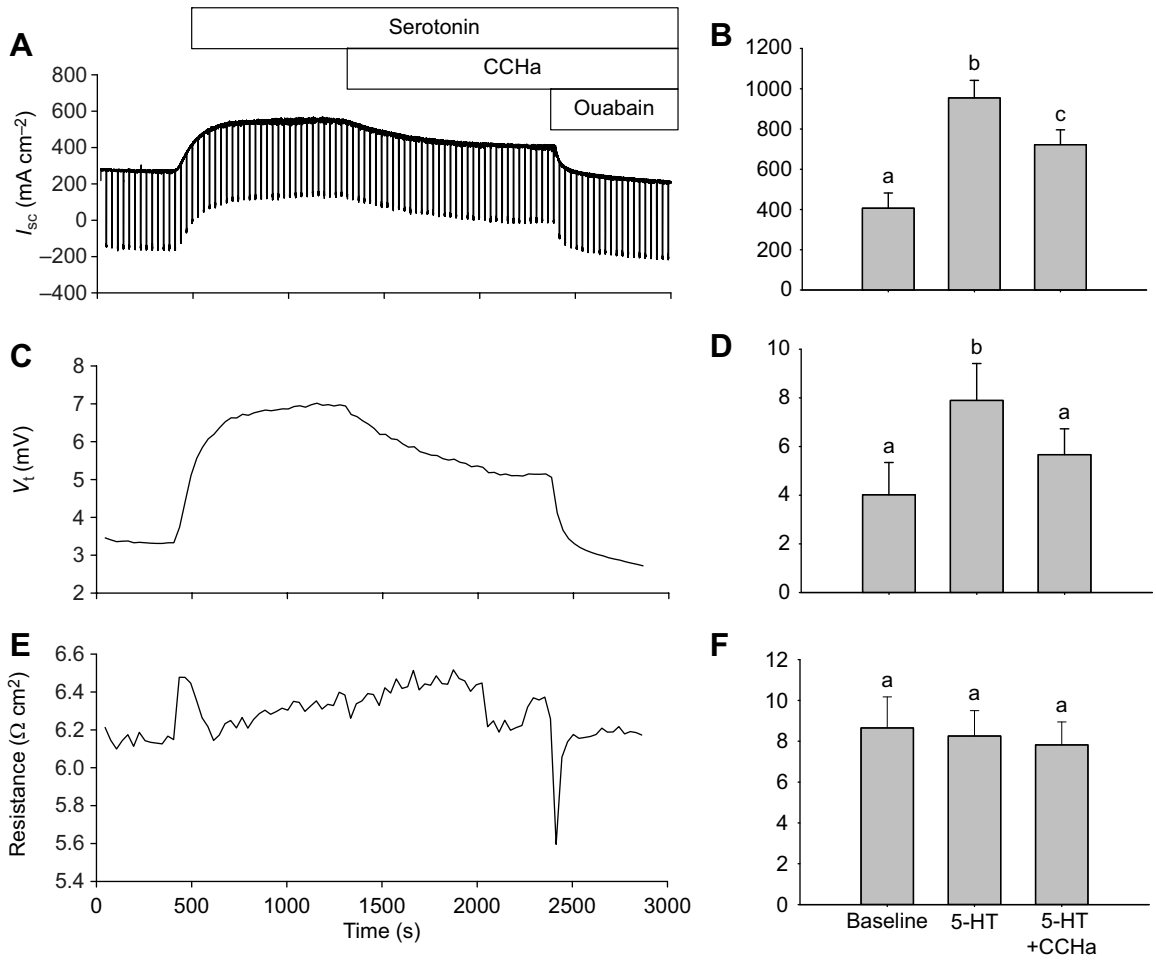

Fig. 9. Effect of RhoprCCHamide2 on serotonin-stimulated ion transport. (A,B) Short-circuit current (I_{sc}); (C,D) transepithelial voltage (V_t); (E,F) resistance (R) across the anterior midgut from fifth instar *R. prolixus*. Application of serotonin (5-HT) on both the basolateral and apical sides induced a positive deflection in I_{sc} and V_t. Addition of RhoprCCHamide2 (CCHa; 1 µmol l^{-1}) reduced the I_{sc} (N=10), which was further inhibited by ouabain. Serotonin, RhoprCCHamide2 and ouabain 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 B, D and F. Columns marked with different letters are significantly different (means±s.e.m., repeated measures ANOVA, Tukey–Kramer multiple comparison test, P<0.05).

subtle and dual modulatory effects could contribute to a fine tuning of changes in volume and ion composition of hemolyph during post-prandial diuresis.

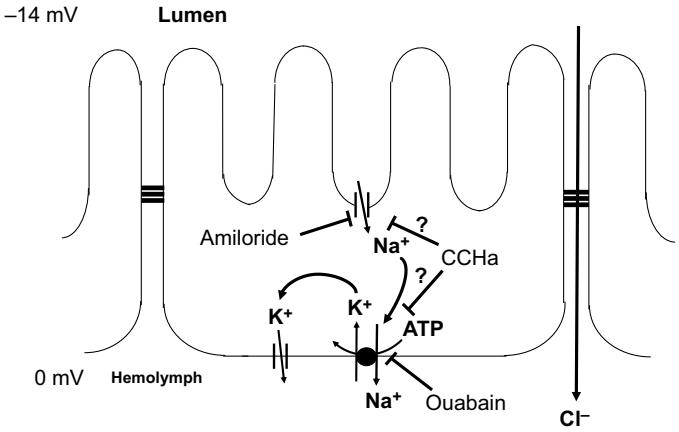

Fig. 10. Proposed working model of serotonin-stimulated ion transport across the anterior midgut of *R. prolixus*. The model incorporates our results and the published work by Farmer et al. (1981). The model includes the inhibitory effects of ouabain and amiloride, and the potential effect of RhoprCCHamide2 (CCHa).

Acknowledgements
We thank Dr Santosh Jagadeeshan and Mr Brendan Murray for their generous assistance in the rearing of insects.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.C., J.P.I.; Methodology: N.C., P.G., S.O., J.P.I.; Formal analysis: P.G., S.O., J.P.I.; Investigation: N.C., P.G., S.O., J.P.I.; Resources: J.P.I.; Writing - original draft: J.P.I.; Writing - review & editing: N.C., S.O., J.P.I.; Supervision: S.O., J.P.I.; Project administration: J.P.I.; Funding acquisition: J.P.I.

Funding
This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (RGPIN-2021-03575 to J.P.I.); the Agencia Nacional de Promoción Científica y Tecnológica (Argentina; PICT2018-00862 to S.O. and J.P.I.); and a Travelling Fellowship from The Company of Biologists to N.C. S.O. and N.C. are researchers of Consejo Nacional de Investigaciones Científicas y Técnicas.

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