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

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Submitted 13 April 2009; accepted in final form 2 December 2009

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Ianowski JP, Paluzzi JP, Te Brugge VA, Orchard I. The antidiuretic neurohormone RhoprCAPA-2 downregulates fluid transport across the anterior midgut in the blood-feeding insect *Rhodnius prolixus*. Am J Physiol Regul Integr Comp Physiol 298: R548–R557, 2010. First published December 9, 2009; doi:10.1152/ajpregu.00208.2009.—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 (the neuropetide RhoprCAPA-2) acting on the Malpighian tubules of *Rhodnius prolixus*. Here we describe a third, novel mechanism central to the antidiuretic strategy of *R. prolixus*, the inhibition of ion and fluid transport across the anterior midgut by RhoprCAPA-2. Our results show that RhoprCAPA-2 (1 μmol/l) reduces serotonin-stimulated fluid transport from 83 ± 11 to 12 ± 12 nl/min and equivalent short-circuit current from 20 ± 4 to 5 ± 0.7 μA/cm² 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 volume and ion composition of haemolymph.

Neuropeptide; serotonin; ion transport; Ussing chamber; cGMP; cAMP

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 (12, 13, 25, 39, 44).

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 (7, 21–23), calcitonin-like peptides (17), and the cardioacceleratory peptide 2b/CAPA family of peptides that have diuretic effects in dipterans (9, 52, for review see Ref. 8). Most diuretic factors increase urine production by stimulating the Malpighian tubules, but in *Rhodnius prolixus* they also stimulate the midgut (15, 57), 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 *R. 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 (8). 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 (1, 2) and Cl⁻ transport stimulating hormone (51), elevate intracellular cAMP in the ileum and rectum, respectively, and stimulate reabsorption of fluid from the primary urine produced by the Malpighian tubules (8). Similarly, neuroparins 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 neuroparins (27).

More recently, a second antidiuretic strategy was described by Quinlan and colleagues (53, 54). They demonstrated that the cardioacceleratory peptide 2b, isolated from the tobacco hornworm *Manduca sexta* (ManseCAP2b, also known as MascAPA-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* (53, 54). 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 *R. 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): 129 NaCl, 8.6 KCl, 8.5 MgCl₂, 2 CaCl₂, 20 glucose, 10.2 NaHCO₃, 4.3 Na₂HPO₄, and 8.6 HEPES with a pH 6.2. The incubation tubes were immediately placed in a boiling water bath for 5 min and then stored at 4°C for 10 min at 8,800 g. The anterior midguts were incubated for 10 min, and the experiment was terminated by adding 500 l of boiling 50 mmol/l sodium acetate (pH 6.2). The incubation tubes were immediately placed in a boiling water bath for 5 min and then stored at −20°C. To prepare the samples for analysis, tubes were thawed, sonicated briefly on ice, and centrifuged at 4°C for 10 min at 8,800 g. The supernatant was then collected and assayed using a cGMP or cAMP radioimmunoassay kit (PerkinElmer/NEN, Boston, MA). 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 wk 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) while being viewed under a dissecting microscope. The chamber was maintained at room temperature, and apical and basolateral compartments were air bubbled.

**Fig. 1.** Effect of RhoprCAPA-2 on fluid transport rate (means ± SE) by anterior midguts from adults (A) and fifth instar (B) *Rhodnius prolixus*. The anterior midguts were incubated with saline (n = 7), 0.1 μmol/l serotonin (5-HT; n = 10), or 0.1 μmol/l 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).
Statistics. Results are expressed as means ± SE. Significance of differences between means was determined using unpaired or paired parametric or nonparametric 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-HT, 0.1 \( \mu \)mol/l) by increasing the rate of fluid transport to 83 ± 11 (\( n = 10 \)) and 74 ± 7 nl/min (\( n = 24 \)), respectively. The serotonin-stimulated fluid transport was inhibited by RhoprCAPA-2 in a dose-dependent manner (Fig. 1). Similarly, treatment with serotonin increased \( e_{sc} \) and \( V_t \) and reduced resistance of the epithelium (Fig. 2). Addition of 5-HT increased \( e_{sc} \) 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. 2, A and B; \( n = 13 \); repeated-measures ANOVA, Tukey-Kramer multiple comparison test, \( P < 0.05 \)). \( V_t \) 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. 2, C and D; \( n = 13 \); repeated-measures ANOVA, Tukey-Kramer multiple comparison test, \( P < 0.05 \)). The resistance decreased from 286 ± 25 \( \Omega \)-cm\(^2\) to the lowest value of 158 ± 14 \( \Omega \)-cm\(^2\), finally reaching a plateau of 183 ± 21 \( \Omega \)-cm\(^2\) (Fig. 2, E and F; \( n = 13 \); repeated-measures ANOVA, Tukey-Kramer multiple comparison test, \( P < 0.05 \)), consistent with previous reports (15, 57). Addition of RhoprCAPA-2 (1 \( \mu \)mol/l) blocked the effects of serotonin (Fig. 2). RhoprCAPA-2 reduced both serotonin-stimulated \( e_{sc} \) and \( V_t \) to 29 ± 3 \( \mu \)A/cm\(^2\) and 4 ± 0.4 mV, respectively, while increasing resistance to 258 ± 24 \( \Omega \)-cm\(^2\). 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 *M. sexta*, ManseCAP2b, triggers antidiuresis through an intracellular second messenger pathway that may involve cGMP (53, 54). 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 (53, 54). cGMP is involved in the effect of *Tenebrio molitor* antidiuretic neurohormone Tenmo ADFa on Malpighian tubules of *T. molitor* and mosquito (14, 41). We decided, then, to study the effect of RhoprCAPA-2 (1 \( \mu \)mol/l) on intracellular cAMP and cGMP levels in anterior midgut from adult *R. prolixus*. Radioimmunoassay results showed that stimulation with serotonin (0.1 \( \mu \)mol/l) increases intracellular cAMP (Fig. 3A, \( n = 10 \); ANOVA, Tukey-Kramer multiple comparison test, \( P < 0.05 \)). 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-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; 1 mmol/l) stimulated fluid transport to 192 ± 60 nl/min (Fig. 3B, n = 6 to 15, ANOVA, Tukey-Kramer multiple comparison test, 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, Fig. 3B).

The effect of 8-Br-cAMP was also detected in Ussing chamber preparations (Fig. 4, n = 11, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). The eIsc increased from 46 ± 8 to a stable value of 142 ± 22 μA/cm² after the addition of 8-Br-cAMP (1 mmol/l, Fig. 4, A and B). The addition of RhoprCAPA-2 (1 μmol/l) reduced eIsc to 75 ± 14 μA/cm² (Fig. 4, A and B). Similarly, Vt was reduced by the addition of RhoprCAPA-2 (Fig. 4, C and D). In contrast, the resistance suffered a small increase but did not fully recover after RhoprCAPA-2 treatment (Fig. 4, E and F).

Radioimmunoassays also showed that the intracellular cGMP level is significantly lower in anterior midguts treated with serotonin (0.1 μmol/l) than those treated with serotonin (0.1 μmol/l) plus RhoprCAPA-2 (1 μmol/l, 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) in the absence of serotonin produced a small but significant decrease in eIsc from 56 ± 11 to 29 ± 3 μA/cm² (Fig. 5, B and C; n = 6; repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05).

To further analyze the role of cGMP we treated anterior midgut preparations with the membrane-permeable analog of cGMP, 8-Br-cGMP (1 mmol/l). 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 multiple comparison test, P < 0.05). 8-Br-cGMP also had a significant effect on the eIsc, 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 eIsc and Vt and the decrease in resistance (Fig. 6, A and B; n = 8; repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). Treatment with RhoprCAPA-2 (1 μmol/l) reduced eIsc and Vt of anterior midguts stimulated with serotonin (50 nmol/l) and 8-Br-cGMP (1 mmol/l) from 132 ± 23 to 57 ± 11 μA/cm² and 26 ± 5 to 17 ± 3 mV, respectively (Fig. 6, A 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. 6, C and D). Both eIsc and Vt increased in response to 8-Br-cGMP (1 mmol/l) treatment from 41 ± 5 to 181 ± 47 μA/cm² and 17 ± 1 to 20 ± 2 mV, respectively (Fig. 6, C and D; n = 7; repeated-measures, ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). The effect of 8-Br-cGMP was blocked by treatment with RhoprCAPA-2 (1 μmol/l) (Fig. 6, C and D; n = 7). Even a lower dose of 8-Br-cGMP (50 μmol/l) increased transepithelial transport as indicated by the increase in eIsc and Vt and the reduction in resistance (Fig. 6, E and F; n = 6; repeated-measures ANOVA, Tukey-Kramer multiple comparison test, 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 (CAP2h) 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 EGTA or the membrane-permeable BAPTA-AM. The results show that serotonin stimulation of ion transport was not affected by Ca²⁺-free saline (Fig. 7A). BAPTA-AM was dissolved in a 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 Ca²⁺-free saline (Fig. 7A, n = 9, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). Addition of...
of RhoprCAPA-2 (1 μmol/l) reduced eIsc of anterior midguts stimulated with serotonin (50 nmol/l) from 61 ± 10 to 27 ± 5 μA/cm² (Fig. 7A, n = 9). To minimize the possibility of Ca²⁺ contamination of our Ca²⁺-free solution, we added the Ca²⁺ chelator EGTA. The preparation responded to serotonin by increasing the eIsc (Fig. 7B, n = 9, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). Treatment with RhoprCAPA-2 reduced the serotonin-stimulated eIsc from 52 ± 5 to 25 ± 3 μA/cm² (Fig. 7B, n = 9). These results suggest that RhoprCAPA-2 effect on ion transport across the anterior midgut is independent of extracellular Ca²⁺.

To test the possible role of intracellular Ca²⁺ 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 Ca²⁺-free saline, 1 mmol/l EGTA, and 50 μmol/l BAPTA-AM in 0.15% DMSO. Stimulation with serotonin (50 nmol/l) increased eIsc from 19 ± 3 to a peak of 120 ± 25 μA/cm² that plateaus at 98 ± 5 μA/cm² (Fig. 7C, n = 4, repeated-measures ANOVA, Tukey-Kramer multiple comparison test, P < 0.05). Treatment with RhoprCAPA-2 (1 μmol/l) in the presence of BAPTA-AM reduced the serotonin-stimulated eIsc from 98 ± 5 to 25 ± 5 μA/cm² (Fig. 7C, n = 4). Similarly, the inhibition of intracellular Ca²⁺ availability with 1 mmol/l 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8-HCl) that blocks intracellular Ca²⁺ mobilization (6, 16, 56), 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 EGTA responded to 5-HT (0.1 μmol/l) by increasing the rate of fluid transport to 86 ± 18 nl/min (n = 16) and was inhibited by 1 μmol/l RhoprCAPA-2 (Fig. 7D). Most importantly, treatment of the preparation with 1 mmol/l TMB-8-HCl 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.
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 (26, 49), which in turn activates the phospholipase C (PLC) pathway, leading to increased levels of inositol trisphosphate and Ca\(^{2+}\) release from intracellular stores (5). The resultant rise in intracellular Ca\(^{2+}\) activates a Ca\(^{2+}\)–calmodulin-sensitive nitric oxide (NO) synthase increasing production of NO (12, 30). NO in turn activates a soluble guanylate cyclase to increase production of cGMP 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 independently of NO (53, 54). Similar effects of cGMP have been described in T. 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 those in anterior midguts stimulated with serotonin only. These results are consistent with those in R. prolixus tubules (47, 48, 53, 54). 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 cGMP. This suggests 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 analog, 8-Br-cGMP. The concentration of 8-Br-cGMP used in these experiments (1 mmol/l) is likely to saturate the cGMP pathway, and thus it could not be increased any further by RhoprCAPA-2. Since RhoprCAPA-2 is very effective in downregulating cGMP-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) stimulates e\(_{st}\), 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 the 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). Similarly, Ca\(^{2+}\) does not seem to be the intracellular messenger triggered by RhoprCAPA-2 in R. prolixus anterior midgut. Our results show that RhoprCAPA-2 blocks serotonin-stimulated e\(_{st}\) 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-2-2 has on serotonin-stimulated fluid secretion rate was not affected by treatment with TMB-8-HCL, which inhibits availability of intracellular Ca\(^{2+}\). Thus, we conclude that in R. prolixus anterior midgut, the effect of the CAPA peptides is independent of Ca\(^{2+}\). These results contrast with those reported in Drosophila 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 (48, 53, 54). 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; 54). In contrast, treatment of the anterior midgut of *R. prolixus* with RhoprCAPA-2 does not result in a reduction of intracellular cAMP. Moreover, RhoprCAPA-2 blocked fluid transport and $e_{Isc}$ even when the anterior midgut was stimulated with high levels of 8-Br-cAMP (1 mmol/l) 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 downregulate 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 the lepidopteran posterior midgut, two families of peptides, allatotropins and extended FLRFamides, downregulate active ion transport by goblet cells to modulate nutrient absorption and K$^{+}$ regulation (11, 32, 35, 42, 60, 61). Similarly, the ion transport machinery involved in establishing pH gradients in the midgut of the mosquito *Aedes aegypti* larvae is downregulated by several peptides, including allatostatin, proctolin, and neuromodulin.
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

R. 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 min (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, which downregulates 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).

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada. We are grateful to Dr. Ron Nachman for synthesizing and purifying RhoprCAPA-2.

GRANTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada.
DISCLOSURES
No conflicts of interest are declared by the author(s).

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