Neurohormones implicated in the control of Malpighian tubule secretion in plant sucking heteropterans: The stink bugs *Acrosternum hilare* and *Nezara viridula*

Geoffrey M. Coast, Victoria A. TeBrugge, Ronald J. Nachman, Juan Lopez, Jeffrey R. Aldrich, Angela Lange, Ian Orchard

*School of Biological and Chemical Sciences, Birkbeck (University of London), Malet Street, London WC1E 7HX, UK*
*Department of Biology, University of Toronto Mississauga, 3359 Mississauga Rd, Mississauga, ON, Canada L5L 1C6*
*Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center, USDA, 2881 F/B Road, College Station, TX 77845, USA*
*Invasive Insects Biocontrol & Behavior Laboratory, Beltsville Agricultural Research Center-West, 10300 Baltimore Avenue, Beltsville, MD 20705, USA*

**ARTICLE INFO**

**ABSTRACT**

Plant sucking heteropteran bugs feed regularly on small amounts of K⁺-rich plant material, in contrast to their hematophagous relatives which imbibe large volumes of Na⁺-rich blood. It was anticipated that this would be reflected in the endocrine control of Malpighian tubule (MT) secretion. To explore this, neuroendocrine factors known to influence MT secretion were tested on MT of the pentatomid plant sucking stink bugs, *Acrosternum hilare* and *Nezara viridula*, and the results compared with previously published data from *Rhodnius prolixus*. Serotonin had no effect on *N. viridula* MT, although it stimulates secretion by *R. prolixus* MT >1000-fold, and initiates a rapid diuresis to remove excess salt and water from the blood meal. Kinins had no effect on stink bug MT, but secretion was increased by Zoone-DH, a CRF-like peptide, although the response was a modest 2–3-fold acceleration compared with 1000-fold in *R. prolixus*. Native CAPA peptides, which have diuretic activity in dipteran flies, had antidiuretic activity in MT of the stink bug (*Acrh/Tvz*-CAPA-1 and -2), as previously shown with Rhopr-CAPA-2 in *R. prolixus*. The antiuretic activity of Rhopr-CAPA-2 has been linked with terminating the rapid diuresis, but results with stink bugs suggest it is a general feature of heteropteran MT.

© 2009 Elsevier Inc. All rights reserved.
serotonin increases to >100 nM within 5 min of feeding [13], which is sufficient to maximally stimulate fluid uptake from the anterior midgut and MT secretion [2,9,17]. Serotonin levels fall after 5 min, and the rapid diuresis is sustained by release of a corticotropin releasing-factor (CRF)-like DH from the axons of posterior lateral neurosecretory cells in the MTGM that also project into the abdominal nerves [39]. A CRF-like DH of *R. prolixus* has yet to be identified, but Zoone-DH, a CRF-like peptide from the termite *Zootermopsis nevadensis*, maximally stimulates fluid transport across both the anterior midgut and the upper MT segment [38,40]. The rapid diuresis ceases after about 3 h, which was originally thought to be due to the removal of the stimulus for DH release and the degradation/excretion of circulating hormone. More recently, however, evidence has emerged for the release of an antidiuretic peptide toward the end of the rapid diuresis, which decreases stimulated rates of fluid transport across both the upper MT and the anterior midgut [29,36]. This peptide belongs to the family of cardioacceleratory 2b (CAP2b) peptides, which are designated CAPA peptides, because they are encoded on the *capa* gene (capable of encoding CAP2b) of the fruit fly, *Drosophila melanogaster*. The *capa* gene of *R. prolixus* is expressed in three bilaterally paired cells on the ventral surface of the MTGM, and CAPA-like immunoreactive material is released from their axons, which project bilaterally to the abdominal nerves, toward the end of the rapid diuresis [31,32].

In contrast to *R. prolixus*, plant sucking bugs feed on material with low salt content and do not imbibe large volumes of fluid. The rapid diuresis and natriuresis that enables the speedy removal of much of the volume and salt load consumed by *R. prolixus* is therefore inappropriate for a plant sucking insect. The only study to date of MT function in a plant sucking heteropteran [20] focussed on the handling of the cardiac glycoside ouabain by the large milkweed bug *Oncopeltus fasciatus* (Heteroptera; Lygaeidae). Little is known of the control of MT secretion in plant sucking bugs, but serotonin appears to be a neurohormone in *O. fasciatus*, although the serotoninergic DUM cells that are found in *R. prolixus* are absent [21]. Moreover, serotonin does not stimulate cAMP production by *O. fasciatus* MT, although this is the diuretic second messenger in *R. prolixus*. The plant sucking bug may, therefore, not use serotonin as a DH, which is consistent with its not requiring the rapid diuresis of its blood feeding relative.

The present study examines the control of MT secretion in two related plant sucking bugs (*Heteroptera: Pentatomidae*). The green stink bug *Acrosternum hilare*, and the Southern green stink bug *Nezara viridula*, polyphagous pentatomids that have a detrimental effect on product quality and yield in cotton and other row, fruit and nut crops [33]. More recently, adult southern green stink bugs have been shown to vector plant pathogens in cotton [19]. These bugs feed by inserting their proboscis into the host plant and sucking up nutrients. They employ a macerate-and-flush strategy [10]: the mandibular and maxillary styles contained within the proboscis sheath cut into the plant and saliva, which contains digestive enzymes, is injected into the wound to liquefy the tissues [19]. The macerated and partially digested material is “flushed out” by the saliva and ingested by sucking. Relatively small volumes of liquefied material, much of which is the injected saliva, are imbided and there is little obvious abdominal distension during feeding. In this paper, attention has focussed on the effects of serotonin and of neuropeptides implicated in the control of MT secretion, namely kinin, CRF-related peptides and CAPA peptides. Kinin and CRF-related peptides have yet to be identified from stink bugs, but two CAPA peptides (Nezvi-CAPA-1 and -2) are known from *N. viridula* [34] and identical peptides are present in *A. hilare* [35]. The effect of CAPA peptides on stink bug MT was of particular interest since these peptides have diuretic activity in dipteran insects [6,22,27,26], but antidiuretic activity in *R. prolixus*. The immunocytochemical localization of CAPA cells in *N. viridula* is identical to that described in *R. prolixus* and the presence of neurohemal release sites on the abdominal nerves suggests CAPA peptides function as circulating hormones.

### 2. Materials and methods

#### 2.1. Insects

Adult green stink bugs (*A. hilare*) were captured in 40 W light traps (with live insect canisters) located adjacent to fields cultivated with corn, cotton, sorghum and soybeans in Burleson County, Texas. Adult Southern green stink bugs (*N. viridula*) were obtained from a colony maintained at the USDA Invasive Insects Biocontrol & Behavior Laboratory, Beltsville Agricultural Research Center in Beltsville, MD.

#### 2.2. Fluid secretion assay

Stink bugs have four MTs that are situated in the dorsal abdomen with their closed distal ends surrounding the heart. Female bugs were fixed ventral side uppermost in a wax dish. The dish was then flooded with *O. fasciatus* saline [20] (Table 1) and the ventral abdominal sterna cut away. The ovaries were removed and the gut carefully pulled to one side to expose the two urerets each with an attached pair of MT. Although it was possible to dissect out intact MT this was an extremely slow process and, in general, studies were made using shorter lengths (~1.5 cm) that did not include the most distal (from the gut) closed ends of the tubules. A length of MT was removed by gripping the region closest to the ureret with a pair of watchmaker’s forceps, cutting it away from the ureret, and then gently teasing it free of tracheal connections using a fine glass rod. Once a sufficient length of tubule was dissected free it was severed and transferred to a 10 μL drop of *O. fasciatus* saline resting on the Sylgard-lined based on a Petri dish containing water-saturated paraffin oil. Each end of the tubule segment was withdrawn into the surrounding paraffin oil and wrapped around separate minutin pins set close to the drop of bathing fluid. With practice, all four MTs could be removed from an insect in <10 min.

Stink bug MT secreted spontaneously in *O. fasciatus* saline, and fluid generally emerged only from the lower end of the tubule which would have emptied into the ureret. Secreted droplets were collected using a fine glass micropipette at 20–40 min intervals when the bathing fluid was replenished. The collected fluid was discharged under the paraffin oil and droplet diameter (*D*) measured with an eyepiece micrometer. The volume (*V*) of the secreted droplets could then be calculated (*V* = *D*²π/6) assuming they were perfect spheres when resting on the non-wettability Sylgard. Rates of secretion (in nL min⁻¹) were obtained after dividing the volume (in nL) by the time interval over which the droplet was collected. For *A. hilare* MT, basal rates of secretion were measured over 60 min before they were challenged with test substances dissolved in *O. fasciatus* saline. To correct for differences in the rate of secretion by MT of different lengths, the effects of test

#### Table 1

<table>
<thead>
<tr>
<th>Composition of <em>O. fasciatus</em> saline [17].</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mM)</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>CaCl₂</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>3.5</td>
</tr>
<tr>
<td>6.7</td>
</tr>
</tbody>
</table>
substances were expressed as a percentage of the basal rate of secretion measured over the first hour. Malpighian tubules removed from *N. viridula* did not survive well in *O. fasciatus* saline, which probably reflects the poor condition of the insects when they arrived in Toronto (Canada). Single measurements were therefore made of fluid secretion either under basal conditions or in the presence of test compounds.

2.3. Peptides and chemicals

The following compounds were tested separately or in combination for effect on MT secretion: serotonin (Sigma–Aldrich, St Louis, MO); a kinin from the house cricket *Acheta domesticus* (Achdo-K2); the CRF-related DH of the termite *Z. nevadensis* (Zoone-DH; a gift from David Schooley); two isoforms of CAP2b identified in *A. hilare* and *N. viridula* (Acrhi/Nezvi-CAP2b-1 and -2). The two stink bug CAP2b isoforms and Achdo-K2 were synthesized as previously described [3,24,34]. All other chemicals were obtained from Sigma.

2.4. Data handling

Graphs were prepared using GraphPad Prism 5.02 (GraphPad Software Inc., La Jolla, CA) and appropriate statistical tests were performed using GraphPad InStat 3.06 with *P* < 0.05 accepted as significant. Because only single measurements were made of fluid secretion by *N. viridula* MT, the data could only be analyzed using unpaired *t*-tests, which have lower resolving power than paired tests.

3. Results

3.1. Performance of *A. hilare* MT in *O. fasciatus* saline

The MT of *A. hilare* functioned well in *O. fasciatus* saline as illustrated in Fig. 1, which shows the mean rate of secretion by four tubules of similar length removed from one insect. The rate of secretion measured over the first 30 min was 1.83 ± 0.52 nL min⁻¹, which does not differ significantly from that measured after 3 h (1.31 ± 0.31 nL min⁻¹; *P* = 0.418, unpaired *t*-test).

3.2. Effect of serotonin

Serotonin acts via CAMP to stimulate maximal secretion by MT of the blood sucking bug *R. prolixus*, but does not increase CAMP production by MT from the plant sucking *O. fasciatus* [21] and may therefore not function as a DH. Consistent with this suggestion, 1 μM serotonin had no significant effect (*P* = 0.90; unpaired *t*-test) on secretion by MT of the plant sucking bug *N. viridula* (Fig. 2), although this concentration is sufficient to elicit maximal secretion by *R. prolixus* tubules.

3.3. Effect of Achdo-K2 and Zoone-DH

Kinin and CRF-related peptides have diuretic activity in many insect species from a number of different orders [5]. Representatives of these two families of DH, the kinin Achdo-K2 and the CRF-related Zoone-DH were tested alone and together on the MT of *A. hilare*. Zoone-DH stimulates maximal secretion by *R. prolixus* tubules, whereas none of the kinins that have been tested have diuretic activity [40]. Fig. 2A shows the effect of challenging *A. hilare* MT with 100 nM Achdo-K2 alone and then together with 100 nM Zoone-DH. The kinin had no effect on secretion, which remained at 100 ± 8% (*N* = 4) of the basal rate, but after the addition of Zoone-DH it increased to 271 ± 32% of the basal rate. The reverse of this experiment is shown in Fig. 2B with 100 nM Zoone-DH added in advance of 100 nM Achdo-K2. In this experiment, Zoone-DH increased fluid secretion to 312 ± 44% (*N* = 6), but this did not change significantly (*P* = 0.667; paired *t*-test) after the addition of Achdo-K2.

3.4. Effect of Acrhi-CAPA-1 and -2 on basal and peptide-stimulated secretion

CAPA peptides have diuretic activity on the MT of dipteran insects, the fruit fly *D. melanogaster* [6], the housefly *Musca domestica* [22,26] and the stable fly *Stomoxys calcitrans* [27], but antidiuretic activity on *R. prolixus* tubules [31,36]. The stink bugs *A. hilare* and *N. viridula* have identical CAPA isoforms (Acrhi/Nezvi-CAPA-1 and -2) [34,35] and these were tested for effects on basal and/or peptide-stimulated (Zoone-DH) fluid secretion. Fig. 3 shows the effect of 1 μM Acrhi-CAPA-2 on basal secretion. In the 20 min immediately following the addition of CAPA peptide, fluid secretion decreased significantly from 1.39 ± 0.37 nL min⁻¹ (*N* = 4) to 0.52 ± 0.13 nL min⁻¹. The peptide was washed off after 40 min using five changes of the bathing medium and over the next 40 min fluid secretion increased to 1.14 ± 0.16 nL min⁻¹.

The effect of 10 μM Acrhi/Nezvi-CAPA-2 on MT stimulated with 100 nM Zoone-DH is shown in Fig. 2A and B. In the experiment shown in Fig. 2A, secretion fell from 271 ± 32% to 130 ± 16% (*N* = 4) of basal following the addition of CAPA peptide. The effect of Acrhi/Nezvi-CAPA-2 was less pronounced in the experiment shown in Fig. 2B, with secretion falling from 322 ± 43% to 229 ± 19% (*N* = 6) of
basal after adding the peptide. In this instance, the effect of Acrhi/Nezvi-CAPA-2b-1 was not quite significant (P = 0.057; paired t-test), although secretion by each of the six tubules decreased after adding the peptide. Even at a lower concentration (1 μM), Acrhi/Nezvi-CAPA-2 significantly (P < 0.05; paired t-test) reduced secretion by six MT expressed as a percentage of the basal rate of secretion. Different letters indicate mean values that differ significantly.

176 ± 30% (N = 5) of basal (Fig. 4A). The second CAP2b isoform, Acrhi/Nezvi-CAPA-1, was also tested for its effect on MT stimulated with 100 nM Zoone-DH, and at 10 μM it significantly (P < 0.01; paired t-test) reduced secretion from 277 ± 39% to 219 ± 35% (N = 6) of basal (Fig. 4B).

Similar data were obtained with N. viridula MT in that the rate of fluid secretion measured in the presence of 1 μM Zoone-DH was greatly reduced when it was combined with 5 μM Acrhi/Nezvi-CAPA-2 (Fig. 5), although the difference was not significant in an unpaired t-test (P = 0.34) (Fig. 6).
4. Discussion

Although the stink bugs, A. hilare and N. viridula, and the triatomid bug, R. prolixus, are all heteropteran insects, their feeding habits are very different as are the requirements placed on their excretory systems for hemolymph homeostasis. R. prolixus imbibes a large volume and salt (NaCl) load, which must be voided for the insect to regain its maneuverability and this is achieved during the rapid postprandial diuresis and accompanying natriuresis. Stink bugs, on the other hand, feed regularly and imbibe much smaller fluid meals, which comprise partially digested plant tissues rich in K+ rather than Na+. The rapid diuresis and natriuresis that is a feature of R. prolixus would therefore be inappropriate for a plant sucking bug, and it is likely that the regulation of excretion will differ between the two groups. The present study has confirmed this, but has also revealed some surprising similarities.

The rapid diuresis of R. prolixus is initiated by a massive release of serotonin from neurohemal sites on the lateral abdominal nerves into which axons from the five serotonergic DUM cells of the MTGM project [13,30]. DUM cells are absent, however, from the MTGM of O. fasciatus [21], and serotonin has no effect on fluid secretion or cAMP production by MT of the stink bug, N. viridula, and milkweed bug, respectively. The use of serotonin as a DH in heteropteran insects, therefore, appears to be a specialization associated with rapid postprandial diuresis of blood sucking species. The lack of effect of serotonin on stink bug MT is surprising given that the biogenic amine has diuretic activity on MT from many other species of insects [5], although in these animals CAMP is not used as a second messenger.

The rapid diuresis of R. prolixus lasts about 3 h, but circulating levels of serotonin fall below those needed to stimulate secretion by the upper MT segment within 20 min of the completion of the blood meal [2,13]. For the continuation of diuresis, a CRF-like DH is released, which in common with serotonin acts via cAMP to stimulate maximal secretion by the upper MT segment. Although a CRF-like DH has yet to be identified in R. prolixus, Zoone-DH has been shown to act via cAMP to stimulate maximal tubule secretion [29,40]. Similarly, Zoone-DH is here shown to stimulate secretion by stink bug MT. However, secretion by stink bug MT is increased just 2–3-fold by Zoone-DH, which is very modest compared with the 1000-fold increase obtained in R. prolixus tubules, and is consistent with plant sucking bugs not needing to rapidly void large amounts of salt and water after a meal.

A notable feature of R. prolixus MT is their failure to respond to insect kinins, which have diuretic activity in a number of orthopteran, dictyopteran, dipteran and lepidopteran insects [5]. Kinins also have no effect on secretion by MT of the coleopteran, A. hilare [31], and neither a receptor nor a kinin precursor are encoded by the genome of the red flour beetle Tribolium castaneum [14]. Kinin-like immunoreactive material is present, however, in the MTGM of R. prolixus, but this material also has no effect on tubule secretion [40] even though kinin receptors are present, as evidenced by myotropic actions on the hindgut and the anterior midgut [38,39]. The present study has shown that kinins have no effect on secretion by the MT of A. hilare, which suggests this is a general feature of heteropterans. Kinins act by opening a transepithelial electrochemical gradient for Cl− diffusion into the bathing fluid, the reverse of that required for fluid secretion [12]. This might explain why kinins have no effect on secretion by the MT of R. prolixus, and possibly other heteropteran insects, although transepithelial voltages have not been measured in the MT of A. hilare. It is worth noting, however, that kinins open a transepithelial conductance through either stellate cells or principal cells in the MT of D. melanogaster [28] and the house cricket, A. domesticus [4], respectively. Hence the driving force for Cl− movement into the MT lumen of these insects is not the transepithelial electrochemical gradient.

A particularly interesting result from the present study is that CAPA peptides native to A. hilare and N. viridula have antidiuretic activity on stink bug MT, reducing basal and Zoone-DH-stimulated rates of fluid secretion. The first CAPA peptide (Manse-CAPa20; renamed Manse-CAPA-1) was identified from the tobacco hornworm, Manduca sexta on the basis of its cardioacceleratory activity [11]. Manse-CAPA-1 was later shown to have diuretic activity on MT from D. melanogaster [6], although it has no effect on secretion by the MT of M. sexta [37]. CAPA peptides native to dipteran insects have since been shown to have diuretic activity in D. melanogaster, M. domestica and S. calcitrans [22,27,26]. In R. prolixus, however, Manse-CAPA-1 has antidiuretic activity, reducing secretion by MT partially stimulated with serotonin [36]. Manse-CAPA-1 has also been shown to have antidiuretic activity on MT of the beetle, T. molitor [41], but it is considerably less potent than either of the native antidiuretic factors ADFA and ADFB [7,8], both of which are unrelated to CAPA peptides. The antidiuretic activity of Manse-CAPA-1 on R. prolixus MT has since been confirmed using the native Rhopr-CAPA-2 peptide [32], which also reduces fluid uptake from the anterior midgut [29].

The actions of Rhopr-CAPA-2 in R. prolixus are believed to be associated with the management of the rapid diuresis following a blood meal, when large amounts of water and NaCl are imported into the hemolymph from the expanded anterior midgut and exported into the lumen of the upper segment of the MT [29]. These two processes must at all times be precisely matched and both are stimulated by serotonin and a CRF-like DH [29,38,40]. The rapid diuresis ceases about 3 h after feeding, when the stimulus for DH release (abdominal distension [15]), is removed, and DH already present in the hemolymph is inactivated and/or removed. However, it is unlikely that fluid transport across the anterior midgut and upper segment of the MT would remain precisely matched as the circulating DH titer gradually declines. For this reason another level of control is required, namely the release of CAPA peptide toward the end of the rapid diuresis [31], which shuts down both DH-stimulated transport processes. The antidiuretic activity of Rhopr-CAPA-2 in R. prolixus MT is therefore seen as a special requirement for terminating the rapid diuresis. But, stink bugs do not imbibe large meals and have no need for a rapid diuresis; indeed MT secretion is only modestly increased by serotonin-containing DUM cells in other heteropteran insects (O. fasciatus and N. viridula), and serotonin has no effect on secretion by stink bug MT. On the other hand, the lack of responsiveness to insect kinins and the antidiuretic activity of CAPA peptides are shared by MTs from stink bugs and R. prolixus, and would therefore appear to be a general feature of heteropteran insects. The data generated here on the regulation of water and ion balance by neuropeptide hormones in these important plant pests
may guide future studies focused on development of mimetic neuropeptide analogs [23,25] with enhanced bioavailability and bioavailability for use in novel pest management strategies that target these critical processes.

Acknowledgements

We wish to acknowledge the able technical assistance of Allison Strey and Chris Parker, and the financial support of a Collaborative Research Grant (#LST.CLG.979226) from the North Atlantic Treaty Organization (NATO) (GMC, RJN) a Binational Agricultural Research and Development Grant (BARD # IS-4205-09C) (RJN), and a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (IO).

References


References

[20] Paluzzi J-P, Orchard I. Distribution, activity and evidence for the release of an antidiuretic peptide in the kissing bug, 

References


References

[26] Paluzzi J-P, Orchard I. Distribution, activity and evidence for the release of an antidiuretic peptide in the kissing bug, 

References

[28] Orchard I, Paluzzi J-P, Orchard I, Distribution, activity and evidence for the release of an antidiuretic peptide in the kissing bug, 

References