Peptides and serotonin control feeding-related events in *Rhodnius prolixus*

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**1. ABSTRACT**

*Rhodnius prolixus* periodically gorges on a blood meal that could compromise salt and water balance. This, however, is prevented by rapid production of urine within minutes of feeding. The Malpighian tubules increase their rate of secretion 1,000 fold leading to the production of a hypo-osmotic urine that is high in NaCl content. Feeding and post-prandial diuresis in *R. prolixus* are tightly coordinated events, involving a variety of neurons within the central nervous system. The present review considers how neurohormones provide flexibility in signaling for the maintenance of hemolymph homeostasis in response to challenges associated with blood-gorging. As will be demonstrated, the overall control of events associated with gorging is complex, and utilizes a range of neuropeptide families and serotonin acting upon a variety of tissues to bias them towards a new functional state.

**2. INTRODUCTION**

There are a large number of neuroactive chemicals within nervous systems, represented, for example, by acetylcholine, amino acids, biogenic amines, and the very diverse neuropeptides. One may question the reason for this large number and diversity and how these translate into a versatile messaging system (see 1-4). Ultimately, of course, the nervous system needs to be flexible and plastic and the diversity of neuroactive chemicals, their location, and site of release can lead to flexibility (see 1,2) in the specificity of the message (private or non-private), speed of delivery of the message (fast or slow), and duration of message (short or long). Thus, neurotransmitters released at synapses tend to carry highly specific, short messages which are private (e.g. accessible by receptors only in the very immediate vicinity). Neurohormones released into the circulatory
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System are delivered relatively slowly, are longer lasting, and the messages are non-private (e.g. accessible by most tissues, limited only by the possession of receptors). In between these extremes is a continuum of activities served by the chemical messengers loosely termed neuromodulators. In addition, the same neuroactive chemical can be expressed in a variety of cell types, including interneurons, neurosecretory neurons, sensory neurons, etc. and so it is possible that a single neuroactive chemical might represent a functional unit that interacts to modulate behavior (e.g. the neuroactive chemical may be the common feature shared by the entire neuronal pathway involved in a behavior). These functional units and their neuroactive chemicals might bias the nervous and endocrine system, and subsequent physiological and behavioral events, towards a new functional state of the animal. In addition, neuropeptides and biogenic amines are also components of the brain-gut axis and therefore serve as a link between the endocrine system of the digestive tract, the neuroendocrine system and the central nervous system (CNS).

As an example within insects, the functional state might be one associated with events that compromise salt and water balance, such as feeding, high metabolic activity (flight), habitat, etc. Thus, activities such as feeding do not occur in isolation, but are part of a behavioral sequence with distinct phases. Neuroactive chemicals involved in feeding behavior might therefore coordinate quite disparate physiological and endocrinological events associated with a common behavior.

We are particularly interested in the principle that neurohormones can be used to coordinate a variety of events associated with a common behavior (see 3,5). As an example, this review will focus on feeding-related physiological events in *Rhodnius prolixus*. *Rhodnius prolixus* is an obligatory hematophagous insect found in Central and South America. This hemipteran is one of 12 Triatominae species acting as a vector for the parasite *Trypanosoma cruzi* (6) that causes Chagas’ disease in humans. As such, *R. prolixus* has considerable medical importance, but separately, and historically, was the model insect used by Sir Vincent Wigglesworth, a pioneer of insect physiology and endocrinology (see 7,8). Interest in *R. prolixus* as a model insect has recently been reinvigorated by the announcement of the sequencing of the *R. prolixus* genome (http://www.genome.gov/13014443) which will greatly enhance our understanding of this medically-important insect.

Unfed *R. prolixus* remain in a state of arrested development, and gorging upon a blood meal triggers endocrinological events associated with growth and development to the next instar. Short-term physiological / endocrinological changes are also initiated in response to the consumption of a blood meal that can be up to 10-12 times the insect’s initial body weight (see 9). In particular, to lower its mass and also concentrate the nutrients of the blood meal across the anterior midgut epithelium into the hemolymph. Following this, the upper (secretory) portions of the Malpighian tubules rapidly secrete a fluid that is iso-osmotic with the hemolymph, but containing high NaCl and KCl content. Finally, the lower (reabsorbing) portions of the Malpighian tubules modify the fluid by reabsorbing KCl with very little H2O, resulting in primary urine entering the hindgut which is now similar in ionic and osmotic composition to the plasma of the blood meal (9).

The fluid and ionic movement across the anterior midgut and Malpighian tubules occur rapidly (within minutes of the start of feeding) and continue at such high speed that within 3 h of a fifth instar gorging, a volume of urine equivalent to 10 times the hemolymph volume is excreted. Indeed, the cells in the epithelium of *R. prolixus* Malpighian tubules have been described as “the fastest fluid-secreting cells known” (10). Each cell of the upper Malpighian tubules can secrete a volume of fluid equal to its own volume every 15 sec (10). Therefore, the epithelium of the Malpighian tubules is a useful model for studying ion transporters in insects, and much is known about the various transporters and co-transporters (for review see 11). In addition, Malpighian tubules are not innervated and the epithelial cells are under the control of neurohormones released from neurosecretory cells (NSCs) found within the CNS; they are thus very useful model systems for studying neurohormones that regulate epithelial transport and that might coordinate other feeding-related processes (3, 12-14). As one can imagine there must be a precise control over the activities of the varied transporters and urinary excreting mechanisms that need to respond to blood feeding. The present review will consider how neurohormones provide the flexibility for maintaining hemolymph homeostasis (volume, ionic and osmotic balance) in response to these challenges.

3. DISTRIBUTION OF DIURETIC HORMONES

In an intricate and delicate series of experiments, Maddrell (12-14) showed that diuresis in response to gorging in *R. prolixus* is under neurohormonal control. Thus, hemolymph from freshly fed insects stimulates secretion by Malpighian tubules in vitro, whereas hemolymph from an unfed insect does not. Ligature experiments and nerve transection experiments reveal the source of the hormone to be in the mesothoracic ganglionic mass or MTGM (the fusion of the mesothoracic, metathoracic and abdominal neuromeres) and more specifically, abdominal nerve neurohemal sites. Finally, microdissection of identifiable posterior lateral NSCs of the MTGM and bioassay reveal them to be the source of a diuretic hormone (DH), with release occurring from neurohemal sites on the abdominal nerves (15, 16). The DH, at that time, was assumed to be peptidergic in nature; however, as we will see later, serotonin is a true DH in *R. prolixus*, working in concert with peptidergic DHs (see 17, 18). The following description concentrates on the distribution of putative DHs in the MTGM of *R. prolixus*, although it must be stated that these factors are also present elsewhere in the CNS, with the potential for contributing to diuresis during feeding, or possibly at other times (e.g. flight). The putative DHs are also in nerve processes that
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Figure 1. Location of neurosecretory cells in the mesothoracic ganglionic mass believed to be involved in diuresis in *Rhodnius prolixus*. A) Dorsal unpaired median cells that are serotonin-like and RhoprDH31-like immunoreactive. These cells produce extensive neurohemal areas on all 5 abdominal nerves (lower panel). B) Posterior-lateral neurosecretory cells that are Locmi-DH-like and kinin-like immunoreactive. These cells produce extensive neurohemal sites on abdominal nerves 1 and 2 (lower panel). C) Ventral posterior-medial neurosecretory cells that are CAP2b-like immunoreactive. These paired cells produce extensive neurohemal sites on abdominal nerves 2, 3, and 4 (lower panel). Redrawn by Paul Hong from 21, 26, 29, 53.

Project directly to tissues that are involved in feeding-related activities, such as salivary glands, digestive system, and epidermis (but not Malpighian tubules).

3.1. Immunohistochemistry

There is an extensive peripheral serotonergic system in *R. prolixus* originating from neurons within the MTGM (19-21). Of particular relevance, are five serotonin-like immunoreactive dorsal unpaired median (DUM) neurons in the abdominal neuromeres of the MTGM (Figure 1) that each project axons through their respective left and right abdominal nerves, on which they produce neurohemal terminals (21). The processes also continue on, projecting over the entire surface of the epidermis on the dorsal cuticle (20). Serotonin-like immunoreactive DUM neurons are interesting, in light of the fact that most DUM cells in insects are considered to be octopaminergic (see 22). The immunoreactive staining of neurohemal sites on the abdominal nerves suggests that serotonin might be a neurohormone in *R. prolixus*. Evidence for this is now quite conclusive, including: the presence and quantification of serotonin in the abdominal nerve neurohemal areas, and its calcium-dependent release from such areas (17, 19, 23, 24); the increasing titer of serotonin within the hemolymph following feeding (17 and see later); and finally, the responsiveness to serotonin of tissues that are not innervated (e.g. Malpighian tubules, Table 1, and see later). In addition to serotonin acting as a neurohormone, serotonin-like immunoreactive processes also project directly to feeding-related tissues (see 3). Thus, the digestive tract, salivary glands, and epidermis of the dorsal cuticle (which plasticizes at feeding) each possess a complex array of serotonin-like immunoreactive processes over their surfaces (Table 1 and 2), suggesting that serotonin might directly control activities of these tissues, in addition to its possible neurohormonal influence (see 3).

Peptides with diuretic activity have been identified in other insects (see 4, 25) and typically belong to one of at least 4 families of peptides; the calcitonin (CT)-related DHs (e.g. DippuDH31 from *Diploptera punctata*), the corticotropin-releasing factor (CRF) – related DHs (e.g. Locmi-DH from *Locusta migratoria*), the kinin-like DHs (e.g. leucokinin 1 from *Leucophaea maderae*), and the CAPA-like DHs (e.g. ManseCAP2b from *Manduca sexta*). Double-labeling, using antisera generated against serotonin and against various neuropeptide families, illustrates that serotonin is co-localized with Dippu-DH31-like (CT-related) immunoreactivity in the 5 DUM neurons of the MTGM (26) (Figure 1). The *R. prolixus* CT-related DH31 (RhoprDH31) has recently been sequenced, and found to be identical to DippuDH31 (27). Locmi-DH-like immunoreactivity (CRF-related) is co-localized with kinin-like immunoreactivity in posterior lateral NSCs of the MTGM (Figure 1), which also release their product into the hemolymph via neurohemal sites on the abdominal nerves (28, 29). These posterior lateral NSCs are bilaterally paired and consist of 5-6 NSCs in each lateral cell group. They are likely the cells microdissected and bioassayed by Berlind and Maddrell (16). The axons of these cells bifurcate anteriorly to the cell body, with one set of branches passing into the neuropile and the other branches projecting through abdominal nerves 1 or 2 where they
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Table 1. Biological activity and presence of diuretic hormones/factors on tissues associated with diuresis in *Rhodnius prolixus*.

<table>
<thead>
<tr>
<th>Diuretic Factor</th>
<th>Anterior Midgut</th>
<th>Upper Malpighian Tubule</th>
<th>Lower Malpighian Tubule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>Increases cAMP, stimulates absorption, myotropic. IR present.</td>
<td>Increases cAMP, stimulates secretion, myotropic. IR present.</td>
<td>Increases cAMP, stimulates reabsorption.</td>
</tr>
<tr>
<td>RhodDH₁⁺</td>
<td>No increase in cAMP, no effect on secretion, myotropic. IR present.</td>
<td>No increase in cAMP, Simulates minor increase in secretion.</td>
<td>No effect on reabsorption.</td>
</tr>
<tr>
<td>CRF₂-related</td>
<td>Increases cAMP, stimulates absorption, myotropic.</td>
<td>Increases cAMP, stimulates secretion.</td>
<td>No effect on reabsorption.</td>
</tr>
<tr>
<td>(e.g. Zoone-DH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinin-related</td>
<td>No effect on cAMP or absorption, myotropic.</td>
<td>No effect on cAMP or secretion.</td>
<td>No effect on reabsorption.</td>
</tr>
<tr>
<td>(e.g. leucokinin 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ‘’immunoreactive processes; ‘’corticotropin-releasing factor

Table 2. Biological activity and presence of diuretic hormones / factors on other feeding-related tissues in *Rhodnius prolixus*.

<table>
<thead>
<tr>
<th>Diuretic Factor</th>
<th>Salivary Glands</th>
<th>Hindgut</th>
<th>Epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>Increases cAMP, stimulates secretion, myotropic. IR present.</td>
<td>Increases cAMP, myotropic. IR present.</td>
<td>Increases cAMP, induces plasticization. IR present.</td>
</tr>
<tr>
<td>RhodDH₁⁺</td>
<td>Myotropic. IR present on principal gland.</td>
<td>Increases cAMP, myotropic. IR present.</td>
<td>Activity not determined.</td>
</tr>
<tr>
<td>CRF₂-related</td>
<td>Increases cAMP, stimulates secretion, myotropic.</td>
<td>Increases cAMP, myotropic. IR present.</td>
<td>No effect on cAMP.</td>
</tr>
<tr>
<td>(e.g. Zoone-DH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinin-related</td>
<td>No effect on cAMP, myotropic.</td>
<td>No effect on cAMP, myotropic. IR present.</td>
<td>Activity not determined.</td>
</tr>
<tr>
<td>(e.g. leucokinin 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ‘’immunoreactive processes; ‘’corticotropin-releasing factor

result in neurohemal sites lying on the nerves (28, 29). Immunoreactive processes can also be seen in the other abdominal nerves, but do not generate the extensive neurohemal sites (29). In addition to their likely actions as neurohormones, processes immunoreactive to these neuropeptides are also found directly on other feeding-related tissues (see Table 1 and 2, and later).

3.2. Circulating Neurohormones

There is a reduction in staining intensity of serotonin-like immunoreactive neurohemal areas on the abdominal nerves and over the body wall during feeding in fifth instars (19, 20). This change is coincident with a rapid rise in the titer of serotonin in the hemolymph (Figure 2), to a peak of 115 nM reached 5 min after the onset of gorging, from an unfed level of about 7 nM (17). The titer drops to 40nM by the time gorging is completed, 10 - 15 min later. Similar titers are found when fifth-instars are fed on an artificial diet, eliminating the possibility that the serotonin appearing in the hemolymph is derived from the blood meal. Rather, as referred to above, the serotonin appears to be released from abdominal neurohemal areas and processes on the body wall during feeding (19, 20). In addition, serotonin is releasable from these neurohemal areas in vitro (19) in response to a high K⁺ depolarizing stimulus. Thus, serotonin is indeed a neurohormone in *R. prolixus* released by the natural stimulus of blood-feeding (17, 18). The peak titer of serotonin in the hemolymph is sufficient (see later) to induce cAMP elevation and fluid transport in Malpighian tubules and anterior midgut in vitro (30-32); leading to the conclusion that serotonin is a true diuretic neurohormone in *R. prolixus*, released by the natural stimulus of feeding, and controlling diuresis.

There is also evidence for the release of peptidergic DHs in response to feeding, though not as direct as the evidence for serotonin. Te Brugge and Orchard (33) examined quantitative and qualitative changes in immunoreactivity of the CRF-related and kinin-related peptides in the CNS and neurohemal sites before and after feeding. The study also assayed the hemolymph for evidence of these neuropeptides. Again, for the purposes of this review, attention is given to the MTGM and the abdominal nerves, since these were shown to be the source of DHs associated with feeding (12-14); however, again, it should be noted that these peptide families exist in NSCs elsewhere in the CNS. The intensity of staining for CRF-like immunoreactivity and the co-localized kinin-like immunoreactivity in the posterior lateral NSCs of the MTGM is reduced in intensity 1.5 and 2.5 h after the start of feeding, relative to unfed controls (kinin-like immunoreactivity shows some decrease at 30 min). This reduction in staining (33) remains for up to 24 h (although there is some variability at this time). Concomitantly, at 1.5 and 2.5 h, the neurohemal sites are reduced in number of blebs and intensity of staining (although again for kinin-like immunoreactivity this is evident at 20 min). In addition, the fine immunoreactive axonal processes in the abdominal nerves are more apparent at these times. By 24 h after the start of feeding the neurohemal sites have recovered to their unfed appearance. Using an RIA for kinin-like peptides it was found that there is significantly less kinins in the MTGM 1.5 and 2.5 h after feeding. The data suggests co-release of the two peptides from neurohemal sites and restocking of these sites from their cell bodies in the MTGM (33). This data is consistent with the findings of Berlind and Maddrell (16) and Berlind (34) who determined by microdissection and bioassay that the posterior lateral NSCs lost 50 percent of their diuretic activity 1 h after feeding. Furthermore, Berlind (34) showed that axonal transport is necessary for the restocking of the neurohemal sites at the end of diuresis. Bioassays further indicate that restocking of the cell bodies is slow, needing about 17 d. The immunoreactive staining indicates some restocking by 24 h although there is considerable variability in this (33). The immunoreactivity of course may not be a true reflection of the content of the biologically active material. During the post-feeding period, there might also be some continued release of DHs from neurohemal sites and restocking from cell bodies, to accommodate the clearance of waste products and water recycling. It is worth noting that the CRF-related Locmi
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**Figure 2.** Blood-feeding in *Rhodnius prolixus* 5th instars results in an increase in the hemolymph titer of serotonin. Redrawn from 17.

DH has been shown to be a true DH in *L. migratoria*, decreasing in content in neurohemal areas and increasing in titer in the hemolymph during feeding (35, 36). In a similar fashion, RhoprDH31-like immunoreactivity of the DUM neurons of the MTGM (co-localized with serotonin) is reduced in intensity 1 h after feeding, suggestive of release of RhoprDH31 (27).

The hemolymph of recently fed insects contains diuretic activity (12-14). Clearly this will in part reflect serotonin which is present in the hemolymph as a DH within minutes of the start of feeding (17). However, the evidence also indicates peptidergic DHs in *R. prolixus* (14, 37, 38). The effects of serotonin on Malpighian tubules can be blocked by the serotonergic antagonist ketanserin (29). This antagonist has been used to gain further insight into the timing of release of DHs and their cocktail in the hemolymph after the start of feeding. Hemolymph collected at 5 min and 1.5 h after the start of feeding can be tested on Malpighian tubule secretion in the absence or presence of ketanserin, thus providing the relative contributions to diuresis made by serotonin and peptidergic DHs. Seventy percent of the diuretic activity found in 5 min hemolymph is eliminated by ketanserin, whereas only 30 percent of the diuretic activity of 1.5 h hemolymph is eliminated by ketanserin. Furthermore, partial purification of hemolymph collected 30 min to 1.5 h after feeding using Sep-Pak and/or RP-HPLC reveals a number of fractions with diuretic activity which cannot be accounted for by serotonin (29). The overall evidence suggests serotonin and peptidergic DHs are released at differing times after the start of feeding, and contribute to diuresis in differing relative proportions.

4. **NEUROHORMONAL CONTROL OF DIURESIS**

4.1. **Control of fluid absorption by anterior midgut**

The blood meal enters the expanded anterior midgut of *R. prolixus* where the nutrient components are concentrated by fluid absorption into the hemolymph. Rather little is known about this fluid transport and indeed, there are only two published reports about its control (31, 39). Farmer and colleagues (31) used *in vitro* preparations of the anterior midgut to demonstrate transport of a NaCl-based fluid which is iso-osmotic to the blood meal. The fluid transport is attributed to active transport of Na\(^+\) from the lumen, possibly involving a Na\(^+\)/K\(^+\) exchange pump on the basolateral membrane. Water and Cl\(^-\) are thought to move passively into the hemolymph. Absorption into the hemolymph occurs at about 20 nl/min in unstimulated preparations, but can be increased at least 6 fold by serotonin (200nM) or cAMP (2mM). Hemolymph titer of serotonin peaks at 115nM (Figure 2), which is capable of increasing absorption by about 4 fold (calculated from 31). Barrett (39) confirmed the likelihood of a Na-dependent pump in the anterior midgut *in vivo*, by using low Na diets and monitoring absorption. As mentioned earlier, serotonin-like immunoreactive processes project directly over the anterior portion of the anterior midgut, and serotonin is released into the hemolymph from DUM cells of the MTGM soon after the start of feeding. Serotonin is therefore ideally suited to activate fluid absorption from the anterior midgut. This issue has been more recently revisited (Table 1), and serotonin shown to elevate cAMP in anterior midgut and to stimulate fluid absorption (Te Brugge and Orchard, unpublished). The resting rates of absorption are lower, using this *in vitro* assay, than observed by Farmer and colleagues (31), but stimulated rates are comparable. In a similar fashion, CRF-related DHs which are present in the lateral NSCs of the MTGM (but do not project over the anterior midgut) also act on the anterior midgut *in vitro* to stimulate an increase in cAMP and an increase in fluid absorption (tested with the CRF-related Zoom-DH, Te Brugge and Orchard, unpublished). Thus, absorption across the anterior midgut appears to be under the control of serotonin (as a neurohormone and / or neuromodulator) and a CRF-related DH (as a neurohormone), each acting via cAMP (Table 1). If serotonin were acting as a neuromodulator released directly onto the anterior midgut, rather than a neurotransmitter, then this might explain the inability of hemolymph from freshly fed insects (time not noted) to stimulate absorption from anterior midgut *in vitro* (9). Perhaps hemolymph collected later in the diuretic time-course, and containing the peptidergic DHs, might be active. Interestingly, and in contrast, the CT-related RhoprDH31, which is co-localized with serotonin in the DUM cells, and kinins, which are co-localized with the CRF-related DHs in the posterior lateral NSCs, do not stimulate absorption across the anterior midgut (Te Brugge and Orchard, unpublished). RhoprDH31 does, however, produce a small but significant increase in cAMP (Table 1). It is possible that the absorption assay is not sensitive enough to detect the effects of RhoprDH31, or there are other activities controlled by RhoprDH31 and cAMP that are not being monitored by this absorption assay (see later).

4.2. **Control of fluid secretion by upper Malpighian tubules**

Fluid crosses the anterior midgut into the hemolymph rapidly (*in vivo* at about 400nl/min, see 31) but
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**Figure 3.** Secretion by upper Malpighian tubules of *Rhodnius prolixus* in vitro. A) Serotonin produces a dose-dependent increase in secretion rate. B) Zoone-DH also produces a dose-dependent increase in secretion rate (plotted as a percentage of that produced by a maximal dose of serotonin). Redrawn from 38.

There are only small changes in the composition of the hemolymph. Its volume, ionic, and osmotic balance are maintained. This is because the upper Malpighian tubules secrete fluid at a very high rate from the hemolymph into the lumen (10, 18). The upper Malpighian tubules secrete a fluid containing approximately equimolar NaCl and KCl, with ion transport stimulated 1000 fold after feeding. The cells of the Malpighian tubules are stimulated within 2 – 3 min of the onset of feeding by DHs released from the abdominal nerves of the MTGM (12-14). This stimulation and diuresis persist for about 3 h. The upper Malpighian tubules are not innervated and this diuresis is controlled by the release of the neuropeptides serotonin and at least one neuropeptide which is likely to be a CRF-related DH. Thus, serotonin and Zoone-DH (CRF-related) each dose-dependently increase the rate of secretion from upper Malpighian tubules (Figure 3 and Table 1) in the presence or absence of IBMX (38), and exogenously applied cAMP can stimulate maximum rates of secretion.

The electrophysiological actions of serotonin have been studied in some detail (see 11, 40, 41). In brief, serotonin produces a characteristic triphasic change in transepithelial potential (TEP). In the unstimulated state the TEP is -25mV (lumen negative). Serotonin results in a phase 1 shift in the TEP to -33 mV, then a phase 2 shift to +30mV and finally a phase 3 shift to -32 mV. Each phase has been attributed to a particular ion transporter (41, 42). Thus, phase 1 is a result of an apical Cl− conductance, phase 2 an apical V-type H+ -ATPase, and phase 3 a basal Na⁺−K⁺−Cl⁻ cotransporter. The resulting movement of ions from the hemolymph across the Malpighian tubule cells and into the lumen is thought to drive the flow of osmotically obligated water through aquaporin-like channels (43, 44). The expression of the aquaporin mRNA is upregulated in Malpighian tubules after the blood meal and in Malpighian tubules treated with serotonin or cAMP (44), although the osmotic permeability is very high in the upper Malpighian tubules and is not increased by serotonin. The effects of peptidergic DHs has only recently been investigated (45). Upper Malpighian tubules respond to the CRF-related peptide, Zoone-DH, with the characteristic triphasic change in TEP, mimicking that of serotonin. Similarly, the fluid secreted by the upper Malpighian tubules in response to Zoone-DH is rich in Na⁺ and K⁺. RhoprDH31, which produces relatively small increases in rate of secretion induces a positive deflection of the TEP of about 11 mV, suggestive of an influence on the V-type H⁺ -ATPase, while leucokinin 1 has no effect. RhoprDH31 also slightly modifies the triphasic change in TEP induced by serotonin (see 45).

It would appear that serotonin and a native CRF-related neuropeptide similar to Zoone-DH activate the same second messenger systems and ion transporters in the upper Malpighian tubules, resulting in very high rates of fluid secretion; the fluid being rich in Na⁺ and K⁺. As we shall see, the lower segments of the Malpighian tubules (representing 30 percent of the tubule length) are activated to reabsorb KCl, such that hemolymph K⁺ is not depleted.

**4.3. Control of KCl reabsorption by lower Malpighian tubules**

Reabsorption of KCl occurs very rapidly (see 11) as the fluid passes through the lower Malpighian tubules. The concentration of KCl in the lumen falls at a rate of 20nmol/l/sec (9, 11, 46). The membrane transporters in the lower Malpighian tubules are different from those in the upper Malpighian tubules. A model for this KCl reabsorption suggests that K⁺ is pumped from lumen into the cell by an H⁺/K⁺ -ATPase (47, 48). Cellular K⁺ then leaks into the hemolymph via Ba²⁺ -sensitive K⁺ channels.
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Figure 4. cAMP as a second messenger in *Rhodnius prolixus* Malpighian tubules. A) Serotonin stimulates a dose-dependent increase in cyclase activity. Redrawn from 18. B) Zoone-DH elevates cAMP content of Malpighian tubules in a dose-dependent manner. Redrawn from 38.

The Cl⁻ moves from lumen to cell through a stilbene-insensitive Cl⁻/HCO₃⁻ exchanger and then exits the cell into the hemolymph through basolateral Cl⁻ channels. The lower Malpighian tubule has a low permeability to H₂O (49).

Hemolymph from freshly fed insects stimulates KCl reabsorption from isolated lower Malpighian tubules, whereas hemolymph from unfed insects does not (9, 46). This observation suggests a neurohormonal control over KCl reabsorption of the lower Malpighian tubules. The location and release sites of the neurohormones again appear to be the MTGM and associated abdominal neurohemal sites (see 9). Interestingly, however, extracts of the posterior lateral NSCs of the MTGM, which stimulate secretion from upper Malpighian tubules, fail to stimulate KCl reabsorption from the lower Malpighian tubules (Table 1, 45). This suggests that the two regions of the Malpighian tubules are not necessarily controlled by the same DHs (9). Threshold doses of serotonin coupled with either hemolymph from a recently fed insect or MTGM extracts, result in maximum stimulation of Malpighian tubules when applied together (50). This is again somewhat surprising since cAMP appears to be the second messenger for serotonin and the peptidergic DH in *R. prolixus*. Serotonin and the extracts also cooperate synergistically to activate adenylate cyclase in broken membrane preparations of Malpighian tubules. Similarly, synergism is evident when forskolin is tested in conjunction with serotonin (38). The mechanisms for such synergism include the possibility of isozymes of adenylyl cyclase differing in their mode of regulation (51). However, the details of this synergism are not understood in *R. prolixus*. In addition the actual peptidergic DHs that might be present in the hemolymph or MTGM extract have not been identified in *R. prolixus*. Neither Locmi-DH, Zoone-DH, leucokinin 1, nor RhoprDH31 produce any synergism when combined with serotonin on *R. prolixus* Malpighian tubules (25, 27, 38). The native *R. prolixus* CRF-related peptide or kinin have not been sequenced and therefore have not been tested. It is possible that the native peptides may show synergism, or there may be some other peptidergic DH in the MTGM and hemolymph, or other material that enables serotonin to act at lower concentrations. Synergism has not been tested in detail on anterior midgut or lower Malpighian tubules.

4.4. Synergism between diuretic hormones

Synergism steepens the dose-response curve and shifts it to the left (lower concentrations) providing a functionality for multiple diuretic hormones. The result is that the upper Malpighian tubules can be stimulated more quickly and at lower concentrations of the DHs. Synergism has been shown for DHs in *L. migratoria* (Locmi-DH and Locmi-K), which act via different second messenger pathways, and in *Musca domestica* with Musco-DP and Musco-K (see 4). Interestingly, although acting via cAMP, DippuDH₄₀ (CRF-related) and DippuDH₃₁ (CT-related) act synergistically in *D. punctata* (see 4). In *R. prolixus*, threshold doses of serotonin coupled with either hemolymph from a recently fed insect or MTGM extracts, result in maximum stimulation of Malpighian tubules when applied together (50).
4.5. Termination of diuresis

Diuresis rapidly ceases in *R. prolixus* when a sufficient amount of hypo-osmotic fluid has been excreted (12). It is not known how this is monitored or indeed what is being monitored, but diuresis needs to be terminated in order to avoid excessive loss of water and salts, thereby maintaining volume, ionic and osmotic stability of the hemolymph. Following diuresis, hemolymph no longer possesses diuretic activity when tested *in vitro* and so one assumes the DHs are no longer being released and are of low titer in the hemolymph. Maddrell (9) postulates that so long as the anterior midgut does not cease activity before the upper or lower Malpighian tubules, then the absence of release of DHs coupled with an increasing hemolymph volume would dilute the titer of DHs in the hemolymph and effectively slow the transport processes occurring in the Malpighian tubules. The circulating DHs would then be broken down / excreted / removed from circulation (mechanisms that have been little studies in *R. prolixus*). Intriguingly, the anterior midgut receives innervation from serotonin-like immunoreactive neurons, and so could be neurally-controlled at this time to maintain absorption. The topic of cessation of diuresis has been revisited (52), with the thought of an anti-diuretic hormone. These authors found that cGMP content of Malpighian tubules elevates *in vivo* at a time when diuresis is ceasing, and that ManducaCAP$_{2b}$, a cardioacceleratory peptide that is a potent diuretic factor in *Drosophila melanogaster* (see 4), inhibits serotonin-induced secretion *in vitro* and also elevates cGMP in serotonin-stimulated Malpighian tubules. It is suggested that a peptide similar to Manduca CAP$_{2b}$ might be an anti-diuretic hormone in *R. prolixus*. Recently, these observations were confirmed (53) and CAP$_{2b}$-like immunoreactive NSCs were described in the posterior midline of the MTGM (Figure 1) and in neurohemal terminals on the abdominal nerves. The *R. prolixus* CAPA gene has now been cloned, revealing the sequence of the CAP$_{2b}$-like peptide named RhoprCAPA-2. The gene is expressed in the posterior midline NSCs of the MTGM, matching the results from immunohistochemistry (53, 54). RhoprCAPA-2 dose-dependently inhibits Malpighian tubule secretion stimulated by either serotonin (Figure 5) or Zoone-DH, and also stimulates an elevation in cGMP content in serotonin-stimulated Malpighian tubules. Furthermore, RhoprCAPA-2 inhibits serotonin and Zoone-DH-stimulated absorption across the anterior midgut (Paluzzi and Orchard, unpublished). It seems clear that RhoprCAPA-2 might be an anti-diuretic hormone in *R. prolixus*, acting on anterior midgut and Malpighian tubules to rapidly inhibit the post-prandial diuresis.

5. DIURETIC HORMONES AND OTHER FEEDING-RELATED EVENTS

Much is known in *R. prolixus* about probing of the host, the gorging stimulus (ATP) and the feeding apparatus (55). Having started to probe and ingest a blood meal, a series of events must occur, including continued salivary gland secretion, plasticization of the abdominal cuticle to accommodate the large blood meal, diuresis (including anterior midgut, and upper and lower Malpighian tubules), and then regular expulsion of the urine from the hindgut. The DHs that control diuresis also appear to be involved in a number of these other physiological events (Table 2).

5.1. Salivary glands

The salivary glands in *R. prolixus* are paired, cherry-red structures that lie on either side of the gut in the thorax. Injection of saliva into the host is an essential component of successful and efficient gorging (e.g. 56, 57), since the saliva counteracts the hemostasis of the host. The salivary glands are composed of a single epithelial layer of binucleate cells and a double layer of visceral muscle cells surrounding a large secretory cavity. Muscles associated with insect salivary glands have not been reported in other insects. In *R. prolixus* these muscles are under polyneuronal control from the salivary nerve (58). Serotonin-like immureactivity is seen in the nerve supply to the salivary glands and over the principal gland and salivary duct. RhoprDH1$_{31}$-like immunoreactivity is seen in processes over the principal gland (26), but no immunoreactivity is seen for CRF-related or kinin-like peptides. Serotonin, Zoone-DH, RhoprDH1$_{31}$, and leucokinin 1, each increase the frequency of contractions of muscles associated with salivary gland (see 58, Te Brugge and Orchard, unpublished). These contractions could contribute to the mixing of salivary gland contents, as well as potentially propelling the saliva out of the principal gland. Indeed, recent results corroborate this latter suggestion and the secretion of saliva from a semi-intact preparation has now been shown to be stimulated by serotonin and Zoone-DH (Table 2). These two DHs also increase cAMP content of salivary glands (Te Brugge and Orchard, unpublished).
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5.2. Epidermis

The ingestion of a blood meal that can be up to 10 times the unfed body mass of an unfed fifth instar, results in considerable extension of the abdomen, with the surface area increasing about 4-fold coupled with a thinning of the cuticle. This plasticization is dependent upon an intact nerve supply (59), since transecting the abdominal nerves on one side of the insect prevents plasticization of the denervated half, but not the other (59). Injection of serotonin results in plasticization that is comparable to that induced by ingestion of a blood meal (60), with EC₅₀ at 330nM and threshold at 50nM. The axons that project to the epidermis are indeed serotoninergic and the staining of the serotonin-like immunoreactive processes over the epidermis disappears following a blood-meal (20). Serotonin likely influences the epidermal cells via cAMP (61). It is interesting that the peak titer of serotonin in the hemolymph at the start of feeding (115nM) is apparently insufficient to induce plasticization (concluded from the results of the denervation experiments), and so one assumes the serotonin released directly from the nerves onto the epidermal cells must reach a higher concentration. Adult integument, which does not appear to plasticize, also responds to serotonin with an increase in cAMP content (61). Serotonin may have effects on epidermal cells in addition to those that lead to plasticization, and cAMP may therefore mediate serotonin-induced changes in epidermal cells that are unrelated to plasticization (this may of course also be true in fifth instars). The peptidergic DHs have not been tested for their ability to plasticize the cuticle, but the nerve processes projecting to the epidermis are not immunoreactive to Zoone-DH, RhoprDH₂, or leucokinin 1 (Table 2, Te Brugge and Orchard, unpublished), and an intact nerve supply is necessary for plasticization.

5.3. Anterior midgut

As discussed earlier, diuresis in *R. prolixus* requires rapid movement of water and ions from the lumen of the anterior midgut into the haemolymph. The previously described peak in haemolymph serotonin concentration 5 min after the onset of gorging in fifth-instar *R. prolixus* might well elicit such an effect *in vivo*, although serotonin-like immunoreactive processes also project over the anterior midgut from the stomatogastric nervous system leading to a possible direct control over the midgut. There is also a serotonin-induced elevation in cAMP content of the anterior midgut (32), and cAMP stimulates fluid absorption (Table 1). There are no processes immunoreactive for CRF-related DHs or kinin-like DHs on the anterior midgut, but processes immunoreactive for CT-related DH are present (26). As referenced earlier, a CRF-related peptide, probably acting as a neurohormone, stimulates an increase in cAMP and fluid absorption. RhoprDH₂ also stimulates an increase in cAMP (though small), but does not appear to stimulate fluid absorption (Te Brugge and Orchard, unpublished).

The rate of contraction of anterior midgut increases within 30 sec of feeding (13), and serotonin, Zoone-DH, leucokinin 1, and RhoprDH₂ are all capable of increasing the frequency of contractions of the anterior midgut *in vitro* (Table 2). This effect is mimicked by application of 8-bromo-cAMP or forskolin, suggesting a cAMP-dependent mechanism (TeBrugge and Orchard, unpublished). It should be noted, however, that leucokinin 1 increases the frequency of contractions in the absence of an increase in cAMP. The increase in the rate of contraction is believed to contribute to the mixing of the ingested blood meal in the anterior midgut, thus minimizing unstirred layers, and hence facilitating ion transport. The contractions may also help mix the surrounding haemolymph and also aid in the circulation of DHs towards the Malpighian tubules (13). This is of some physiological relevance, since Malpighian tubules do not possess muscle fibers and are therefore incapable of independent movement, and the expanded anterior midgut might also hinder the flow of hemolymph posteriorly in the insect. *In vivo*, unstirred layers surrounding the Malpighian tubules are considerably reduced relative to the *in vitro* situation (62).

5.4. Hindgut

The visceral muscles of the hindgut receive innervation from the MTGM, but could also be under neurohormonal control. Secretion by the Malpighian tubules begins within seconds of feeding (13) but waste from the hindgut is only eliminated once feeding has stopped. Voiding of this waste occurs through a protrusion from the anus, with synchronized contraction of the three vertical tergal-sternele muscles in segments 2-7 coupled with contractions of the hindgut (13). As with the other tissues, there is a fine, serotonin-like immunoreactive nerve plexus over the hindgut. In addition immunoreactivity to CRF-related, CT-related peptides and leucokinin-like peptides is also seen (26, 28, 29). Serotonin, Zoone-DH, RhoprDH₂, and leucokinin 1, increase the frequency of hindgut contractions (Table 2). Serotonin and Zoone-DH also stimulate an increase in cAMP content (32, Te Brugge and Orchard, unpublished). The action of serotonin and the peptidergic DHs might well aid in expulsion of waste, but also aid in mixing of gut contents as well as mixing and flow of the hemolymph. These latter effects may be quite important because of the location of two of the Malpighian tubules, which lie on either side of the hindgut in a tangled mass.

6. PERSPECTIVES

It is reasonable to conclude that much is known about the neurohormonal control of osmotic and ionic balance in *R. prolixus* associated with blood-gorging. It is also reasonable to conclude that much is to be discovered. For example, the CRF-related DH, likely to work in concert with serotonin to control the anterior midgut, Malpighian tubules, and other feeding-related tissues is yet to be isolated and sequenced. Similarly, the sequence of the kinin which is co-localized with the CRF-related DH is yet to be published. Little detail is known about the receptors for serotonin and the peptidergic DHs or about the ionic transporters involved. The sequencing of the *R. prolixus* genome is imminent, and one can anticipate that work in these areas will be accelerated. Indeed, probing a cDNA of *R. prolixus* has identified the CAPA gene (54) and also the
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RhoprDH31 and kinin genes (Paluzzi, TeBrugge and Orchard, unpublished), and the CRF-related DH sequence cannot be far off.

Much of our understanding of diuresis in R. prolixus relies upon in vitro work, and a comprehensive understanding of osmotic and ionic balance will require knowledge of the factors normally controlling these processes in vivo and how their release is controlled, coordinated, and monitored. Indeed, it is still unclear what factors associated with gorging act as the primary stimulus for release of DHs, and what factors are monitored to determine when diuresis should be terminated. We should also not lose sight of the fact that the DHs are released from NSCs, which are neurons, and therefore can be expected to participate more generally in the processing of events that result in the animal entering a new physiological state. Thus, the NSCs release their neurohormones into the hemolymph, but also possess central projections that can provide links to other networks of neurons within the CNS. Little is known about the neurobiology of these NSCs, no concept of the uniquely identifiable cell, and no information about integration of these cells within the neuronal and endocrine systems. Other gaps in our knowledge include the involvement of DHs at times other than feeding. For example, it is known that adult R. prolixus produce urine and lose 50 percent of their hemolymph volume during flight (63). Are DHs associated with this diuresis, and if so, which ones? Some other interesting questions to which answers can be postulated include the matter of why there are multiple diuretic factors and why these diuretic factors are co-localized? It is possible that there are multiple diuretic factors to provide synergy (50, 51, 64) and/or because they each have different physiological effects and/or are released at different times. Although serotonin and CRF-related DHs (e.g. Zoone-DH, Locmi-DH) have similar actions (probably identical) on the upper Malpighian tubules (and released from different neurons), it is important to note that Zoone-DH cannot activate KCl reabsorption by the lower tubules, and neither can RhoprDH31, or leucokinin (which are co-localized with serotonin and the CRF-related peptide respectively). During post-feeding diuresis there is a rapid release of serotonin from DUM cells within the first 5 minutes of gorging which might serve to activate absorption from the anterior midgut, KCl reabsorption by the lower tubule and rapid fluid secretion by the upper tubule. The serotonin titers subsequently fall and may not be sufficient to maintain the high rates of fluid secretion by the upper tubules (the lower Malpighian tubules may be more sensitive to serotonin and maintain activity, and anterior midgut may be neurally-controlled). It is possible that a native CRF-related peptide is released from the lateral NSCs at this time, which can maintain high rates of absorption and secretion but which need not stimulate KCl reabsorption. This scenario also implies that the native CRF-related peptide might be used at other times when diuresis is needed, but KCl reabsorption is not. For example, there is a period of digestion of the red blood cells of the blood meal which could lead to a K+ load that the insect needs to eliminate. Thus, this latter diuresis may be controlled by the native CRF-related peptide in the absence of serotonin. Similarly, as mentioned earlier, diuresis occurs during flight, but no studies have examined its control.

The DUM cells of the MTGM co-localize the diuretic hormone serotonin and the very weakly diuretic peptide RhoprDH31. It is possible that RhoprDH31 might influence the actions of serotonin and thereby contribute to diuresis, although synergism or cooperativity has not been demonstrated. In addition both of these factors have biological effects on other tissues that are associated with feeding, such as contractions of salivary glands, anterior midgut, and hindgut. Thus, the frequency of contractions of the anterior midgut is increased following feeding and this increase in frequency might be mediated through serotonin and co-localized RhoprDH31 (3, 27, 38, unpublished observations). Contractions of the hindgut void the urine, and the frequency of contractions of the hindgut is increased by both serotonin and RhoprDH31. These factors, therefore, might be co-localized in order to produce complementary actions on feeding-related tissues. It is also possible that there are other tissues, yet to be identified, that are responsive to serotonin but not to RhoprDH31, and vice-versa. In a similar manner the lateral NSCs co-localize a CRF-related DH (potent diuretic), and a kinin-like peptide that has no diuretic activity. Again, both of these factors are capable of increasing the frequency of contractions of salivary glands, anterior midgut and hindgut and so might participate with serotonin and RhoprDH31 in the overall control of feeding-related tissues.

It should also be emphasized that this review has concentrated on the DUM cells and posterior lateral NSCs of the MTGM of R. prolixus, since these regions of the insect appear to be primarily involved with diuresis associated with blood-feeding. Serotonin, and the peptidergic DHs, are present in other neurons throughout the CNS, including other NSCs (26, 28, 29, 33). In addition, in a number of these cells the serotonin and/or peptidergic DHs are not co-localized. Thus, all manner of combinations are possible with respect to independent or co-release of these neuroactive chemicals. The possession of multiple diuretic hormones, co-localized factors, and their presence in a variety of neuronal types, allows for a flexibility of messaging (2, 64) in both timing and physiological responsiveness that might allow for a fine tuning of diuresis under differing osmotic challenges, such as presented following gorging on a massive blood meal.

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