ANTI-DIURESIS IN THE BLOOD-FEEDING INSECT RHODNIUS PROLIXUS STÅL: THE PEPTIDE CAP$_{2b}$ AND CYCLIC GMP INHIBIT MALPIGHIAN TUBULE FLUID SECRETION

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Summary

*Rhodnius prolixus* eliminates NaCl-rich urine at high rates following its infrequent but massive blood meals. This diuresis involves stimulation of Malpighian tubule fluid secretion by diuretic hormones released in response to distention of the abdomen during feeding. The precipitous decline in urine flow that occurs several hours after feeding has been thought until now to result from a decline in diuretic hormone release. We suggest here that insect cardioacceleratory peptide $2b$ (CAP$_{2b}$) and cyclic GMP are part of a novel mechanism of anti-diuresis. Secretion rates of 5-hydroxytryptamine-stimulated Malpighian tubules are reduced by low doses of CAP$_{2b}$ or cyclic GMP. Maximal secretion rates are restored by exposing tubules to 1 mmol l$^{-1}$ cyclic AMP. Levels of cyclic GMP in isolated tubules increase in response to CAP$_{2b}$, consistent with a role for cyclic GMP as an intracellular second messenger. Levels of cyclic GMP in tubules also increase as urine output rates decline *in vivo*, suggesting a physiological role for this nucleotide in the termination of diuresis.

Key words: *Rhodnius prolixus*, CAP$_{2b}$, cardioactive peptide, cyclic GMP, diuresis, anti-diuresis.

Introduction

The hematophagous insect *Rhodnius prolixus* (Reduviidae) is faced with a potentially serious problem every time it takes one of its infrequent but massive blood meals. Fluid gains of 10–12 times the unfed mass are typical, and replete *R. prolixus* are notably immobile and more vulnerable to predation by comparison with unfed insects. To minimize the duration of this post-prandial sluggishness, *R. prolixus* has evolved a quickly activated, high-output excretory system that unloads 50% of the fluid mass of the meal and much of the excess NaCl within just a few hours. Indeed, the Malpighian tubules of this insect can, within a matter of minutes, increase their secretion rates by over 1000 times to levels as high as 50 nl cm$^{-2}$ s$^{-1}$ (Maddrell, 1991). Tubule secretion is stimulated *in vivo* by 5-hydroxytryptamine (serotonin, 5-HT) acting synergistically with one or more neuropeptide diuretic hormones (DH) released in response to abdominal distention (Maddrell et al. 1993). Both DH and 5-HT are quickly inactivated or excreted *in vivo*, so maximum rates of urine formation depend on the continuous release of these molecules by the nervous system (Maddrell, 1964). Given the high rates of fluid and ion excretion, the cessation of urine production must also be tightly controlled to avoid possible dehydration and excessive loss of NaCl. No separate anti-diuretic signal has previously been identified in *R. prolixus*, and the rapid decline in urine flow that occurs 3–4 h after feeding has been assumed until now to result from a decline in DH and 5-HT release (Maddrell, 1964).

Stimulation of secretion from the tubules is mediated by cyclic AMP in *R. prolixus* and many other insects (Maddrell et al. 1971; Aston, 1975). The role of other second messenger systems (cyclic GMP, inositol trisphosphate/Ca$^{2+}$) in controlling secretion from Malpighian tubules has been largely unexamined. However, recent work by Dow and his colleagues shows that both cyclic GMP and cyclic AMP stimulate secretion in *Drosophila melanogaster* tubules (Dow et al. 1994; Davies et al. 1995). It has been suggested that both modulate a vacuolar-type H$^+$-ATPase (O’Donnell et al. 1996). Exogenous cyclic GMP is effective at doses as low as 3.5 $\mu$mol l$^{-1}$ in *D. melanogaster*, and tubule levels of this nucleotide are elevated by both nitric oxide (NO) and a cardioactive peptide (CAP$_{2b}$) isolated originally from the hawkmoth *Manduca sexta* (Davies et al. 1995). CAP$_{2b}$ belongs to a group of at least five peptides that collectively influence cardiac function and various stage-specific patterns of behaviour in *M. sexta* (Tublitz et al. 1991).

We have tested cyclic GMP and several known or potential stimulants of cyclic GMP synthesis [nitric oxide (NO), atrial natriuretic peptide (ANP), CAP$_{2a}$, CAP$_{2b}$ and CAP$_{2c}$] on the secretory (upper) portion of Malpighian tubules from *R. prolixus*. We report here the unexpected finding that cyclic GMP...
and CAP2b are powerful inhibitors of fluid secretion from these organs. CAP2b depresses secretion in a dose-dependent manner and increases cyclic GMP levels in tubules. We also show with in vivo experiments that urine production is inversely related to tubule cyclic GMP levels. We conclude that CAP2b and cyclic GMP form a novel anti-diuretic mechanism in R. prolixus.

Materials and methods

Secretion assays

Fluid secretion rates of isolated Malpighian tubules were determined using Ramsay tubule preparations similar to those described by Maddrell et al. (1988). Briefly, upper (distal) tubules from third-instar R. prolixus nymphs were mounted under paraffin oil in 100 μl saline droplets such that the open ends extended into the oil to allow collection of the secreted fluid. Drugs were then added to the bathing droplets, and the volume of secreted fluid was determined at fixed intervals with an ocular micrometer. Tubules were only partially stimulated with 5-HT (typically 50% of the maximum rate; Maddrell et al. 1991) so that both stimulatory and inhibitory responses could be detected. Secretion rates were normalized using maximum rates obtained at the end of the experiments by exposing tubules to saturating levels of cyclic AMP.

Cyclic nucleotide determinations

Levels of cyclic GMP in tubules were determined using radioimmunoassay (RIA) kits from Amersham (RPA-525). Assays were conducted according to kit instructions except that reagent volumes were reduced by 50%. Tubules to be analyzed were first placed in vials containing ice-cold 80% methanol (Coast et al. 1991). Samples were then sonicated for 30 s, centrifuged for 10 min at 12,000 g, and the supernatant dried down in a vacuum centrifuge. Dried samples were rehydrated using RIA assay buffer.

Table 1. Summary of experiments testing cyclic GMP and several agonists of cyclic GMP synthesis on Malpighian tubules from third-instar Rhodnius prolixus nymphs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Controls</th>
<th>Treated</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic GMP</td>
<td>1 μmol l⁻¹</td>
<td>0.45±0.039 (11)</td>
<td>0.33±0.031 (11)</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Cyclic GMP</td>
<td>10 μmol l⁻¹</td>
<td>0.45±0.039 (11)</td>
<td>0.16±0.020 (11)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>SNP</td>
<td>10 μmol l⁻¹</td>
<td>0.70±0.058 (8)</td>
<td>0.66±0.055 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>ANP</td>
<td>0.1 μmol l⁻¹</td>
<td>0.64±0.040 (8)</td>
<td>0.67±0.053 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>CAP2a</td>
<td>0.1 μmol l⁻¹</td>
<td>0.41±0.049 (7)</td>
<td>0.46±0.055 (7)</td>
<td>NS</td>
</tr>
<tr>
<td>CAP2b</td>
<td>0.1 μmol l⁻¹</td>
<td>0.64±0.070 (8)</td>
<td>0.13±0.025 (8)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>CAP2c</td>
<td>0.5 NCE/Vn</td>
<td>0.45±0.065 (7)</td>
<td>0.55±0.066 (7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Reagent preparation and statistics

All drugs and peptides except the CAPs were obtained from Sigma, Inc. Synthetic CAP2a and CAP2b were obtained from Research Genetics (Huntsville, AL, USA). CAP2c was obtained from HPLC-purified fractions of homogenates of the central nervous system (CNS) from the tobacco hawkmoth Manduca sexta using the protocol of Huesmann et al. (1995). In brief, abdominal portions of the ventral nerve cord were removed from pharate adult M. sexta and frozen at −80 °C for further processing. After a brief heat treatment (80 °C for 5 min), nerve cords were homogenized in 0.5 mol l⁻¹ acetic acid and microfuged for 5 min. The supernatant was applied to a C-18 Sep-pak cartridge and eluted with step-wise increases in acetonitrile concentrations. CAP2c elutes in the 60% acetonitrile fraction and was subjected to further purification using a six-step HPLC protocol described in Huesmann et al. (1995). Secondary ion mass spectroscopic analysis of the CAP2c fraction revealed a single resolvable peak.

Unless otherwise stated, all chemicals were dissolved in distilled water or saline, sampled and frozen until time for use. Peptide samples were lyophilized before storage at −80 °C. The NO donor, sodium nitroprusside, was prepared immediately before each experiment and stored in darkness.

All values are reported as means ± standard errors of the mean. Statistical comparisons were performed using Student’s t-test; probabilities less than 0.05 were considered significant. When comparing normalized data, values were arcsine-transformed before applying t-tests.

Results

Both cyclic GMP and CAP2b significantly inhibited secretion from R. prolixus tubules (Table 1). CAP2b is clearly the more effective of the two (Table 1), and the threshold concentration for this substance is in the low nanomolar range (Fig. 1). The
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mechanism by which exogenous cyclic GMP inhibits secretion is considered in detail elsewhere (M. C. Quinlan and M. J. O'Donnell, in preparation). Note also in Fig. 1 that cyclic AMP completely reverses the inhibition (maximum rates for all groups were statistically indistinguishable). Neither sodium nitroprusside (10 mM) nor human atrial natriuretic peptide (ANP; 0.1 mM) affected secretion despite the comparatively high doses used in our experiments (Table 1). D. melanogaster tubules, for example, respond vigorously to 2 mM sodium nitroprusside (Dow et al. 1994). With respect to ANP, 0.1 mM is considered saturating for most vertebrate systems (Hamet et al. 1986).

These experiments suggested that cyclic GMP may act as an intracellular second messenger for CAP2b. This hypothesis was supported by the finding that exposure to CAP2b in vitro significantly increased intracellular levels of cyclic GMP in isolated tubules (Fig. 2). Intracellular levels of cyclic GMP in isolated tubules also increased in response to an extract of the metathoracic ganglion (Fig. 3).

The foregoing experiments demonstrated that an increase in intracellular cyclic GMP concentration was associated with an inhibition of secretion from the upper region of the tubules in vitro. It was therefore of interest to determine whether cyclic GMP levels in vivo correlate with rates of urine formation, so we measured the cyclic GMP content of tubules at various points in the diuresis cycle (Fig. 4). Urine formation followed a time course similar to that described by Maddrell (1964). Rates peaked quickly and remained high for approximately 2 h, after which they fell steadily for 3–4 h. At peak output, these insects divested themselves of 16.4% of the mass of their blood meal per hour (8.6 μl h⁻¹, or 1.35 times the unfed mass of the animals). The decline in urine output was correlated with a 56% increase in tubule cyclic GMP content (Fig. 4).

Discussion

Our results demonstrate that low doses of cyclic GMP and CAP2b suppress fluid secretion by Malpighian tubules stimulated with physiological doses of 5-HT. The inhibition can be reversed by application of sufficient concentrations of exogenous cyclic AMP. Tubule cyclic GMP content increases in response to CAP2b treatment, indicating that cyclic GMP
means ± S.E.M.) and cyclic GMP levels (bars) of tubules taken from four upper tubules from each animal were incubated together in 100 µl of saline. All four upper tubules from each animal were incubated together in 100 µl of saline with appropriate treatments for 10 min. The ganglionic extract dosage was 0.5 ganglia per 100 µl of saline. Reactions were terminated by adding ice-cold methanol. Values are means ± S.E.M. for 12 or more tubules. Bars marked with an asterisk differ significantly from the stimulated controls.

Fig. 3. Effects of ganglionic extract on intracellular cyclic GMP levels in tubules. Treatment groups were unstimulated controls, stimulated controls (5-HT) and stimulated tubules + ganglionic extract. Stimulated tubules received 70 nmol l⁻¹ 5-HT. Ganglionic extract was prepared by sonication of isolated metathoracic ganglia in saline. All four upper tubules from each animal were incubated together in 100 µl of saline with appropriate treatments for 10 min. The ganglionic extract dosage was 0.5 ganglia per 100 µl of saline. Reactions were terminated by adding ice-cold methanol. Values are means ± S.E.M. for 12 or more tubules. Bars marked with an asterisk differ significantly from the stimulated controls.

Fig. 4. Composite figure showing rates of urine formation (circles, means ± S.E.M.) and cyclic GMP levels (bars) of tubules taken from third-instar animals at various points in the diuresis cycle. Rates of urine formation were determined by weighing animals at 30 min intervals for 6 h immediately following feeding (N=8). Time 0 represents the termination of the 15–20 min feeding period. Urination rates are expressed as a percentage of the blood meal mass lost per hour. Tubules used for cyclic GMP determinations were removed quickly (<2 min) from donors under saline and immediately analyzed using radioimmunoassay (N=8). Histogram bars are positioned at the approximate time of tubule removal. The bar marked with an asterisk differs significantly from the stimulated controls (left-hand bar), i.e. from tubules removed from animals at the peak of diuresis.

functions as an intracellular second messenger for CAP₂b. Moreover, tubule cyclic GMP content increases as urine output from fed *R. prolixus* declines, consistent with a physiological role for cyclic GMP in the termination of diuresis.

A factor present in an extract of the metathoracic ganglionic mass also elevates tubule cyclic GMP content. The net effect of adding an extract will be a maintenance of or an increase in fluid secretion rate (cf. Maddrell, 1963), presumably because the large quantities of diuretic hormones present will mask the effects of factors leading to an increase in tubule cyclic GMP content. Nonetheless, our data indicate that a factor present within the CNS can elevate tubule cyclic GMP content, and that appropriate release of this factor into the haemolymph could counteract the effects of physiological levels of diuretic hormones and terminate diuresis.

The failure of NO to affect secretion by *R. prolixus* tubules is in contrast to the clear effect of NO on secretion rates of *D. melanogaster* tubules (Dow et al. 1994). Cyclic GMP synthesis is controlled by two classes of guanylate cyclase: a group of soluble enzymes stimulated by the highly diffusible NO (Schmidt et al. 1993), and a family of membrane-bound enzymes which are stimulated by peptide agonists such as ANP (Yuan and Garbers, 1992) and crustacean hyperglycaemic hormone (Goy, 1990). However, NO synthesis itself is frequently controlled, at least in vertebrates, by peptides (Schmidt et al. 1993). Hence CAP₂b might elevate cyclic GMP levels in *D. melanogaster* tubules by way of NO and in *R. prolixus* directly via a membrane-associated enzyme.

Our inclusion of ANP in the present study was motivated in part by its well-documented action via cyclic GMP in vertebrate systems and also because of early reports that plasma taken from dogs undergoing atrial distention caused inhibition of secretion from *R. prolixus* tubules (Kappagoda et al. 1979). In subsequent papers, Knapp et al. (1981a,b) were able to eliminate angiotensin II, bradykinin and vasopressin as potential mediators of this activity. At the time, ANP, a hormone responsible for hypervolaemic diuresis and natriuresis in mammals (Rosenzweig and Seidman, 1991) had not yet been identified and so escaped their attention. Pither et al. (1985) ultimately determined that the active factor had a low molecular mass (<1800 Da versus 3000 Da for ANP) and was lipophilic. This factor is unlikely to be ANP given these characteristics, and we have confirmed that ANP is without effect on secretion from upper Malpighian tubules. The inhibitory factor present in canine plasma remains unidentified.

The evidence presented here suggests that CAP₂b, or a related peptide, and cyclic GMP are part of an anti-diuretic mechanism in *R. prolixus*. Hormones with anti-diuretic effects on Malpighian tubules are rare in insects, however (Spring, 1990). Inhibition of Malpighian tubule fluid secretion by an anti-diuretic hormone has been demonstrated in crickets (Spring et al. 1988), but the identities of the hormone and its second messenger are unknown. Why does *R. prolixus* need a separate control system for reducing tubule secretion rates? In other words, why can termination of diuresis not be controlled simply through a reversal of the process that initiates diuresis? One reason is that haemolymph volume declines as diuresis progresses, and this will tend to elevate the concentration of
circuit of diuretic hormones, thereby increasing to stimulate the tubules further. In addition, the release of diuretic hormones is triggered by gross distention of the abdomen during feeding. However, plasticization of the cuticle during feeding will change the mechanical properties and dimensions of the cuticle. It is difficult to understand, therefore, how abdominal stretch receptors could be pre-set to permit subsequent detection of the appropriate reduced level of distention at which to signal the termination of diuretic hormone release. Even small errors in such an system could have deleterious consequences, since a 3% change in linear dimension corresponds to a 9% change in volume.

Our results raise several questions for further research on anti-diuresis. First, is the anti-diuretic response in R. prolixus mediated in vivo by CAP2b or a similar compound? Antibodies against CAP2b are already available (Tublitz et al. 1991), so it should be straightforward to test for the presence of this peptide within the nervous system of R. prolixus. Second, what could trigger the release of anti-diuretic hormone in vivo? One possibility is the haemolymph [Na⁺]:[K⁺] ratio, which declines in the future studies.

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References

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