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Allatotropin-like peptide released by Malpighian tubules induces hindgut activity associated with diuresis in the Chagas disease vector *Triatoma infestans* (Klug)

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Summary

Haematophagous insects incorporate a large amount of blood with each meal, producing a big quantity of urine in a few hours to eliminate the excess water and Na^+. Malpighian tubules (MTs) have traditionally been seen as a system that responds to neuroendocrine stimulus. In a related paper, we demonstrated that MTs of *Triatoma infestans* produce an autonomous endocrine secretion of an allatotropin-like (AT-like) peptide. In the present study, we report a myostimulatory activity of AT at the level of the hindgut (HG), associated with endocrine mechanisms regulating post-prandial diuresis. Allatotropin induced an increase in frequency and intensity of peristaltic contractions at the level of the HG. The release of the HG content in MTs–HG *in vitro* preparations undergoing an osmotic shock occurred at different times, depending on the number of MTs present, and there was no release in treatments without MTs. The application of an AT-antiserum to MTs–HG preparations undergoing osmotic shock produced a delay or a long-term blockade of diuresis, depending on the antiserum dilution applied. Similar results were obtained when AT-antiserum was applied *in vivo* prior to blood intake, decreasing the volume of urine eliminated during the first 2 h. Our results allow us to assign a specific endocrine function to the AT-like peptide released by MTs that is linked to the elimination of urine after blood meals.

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Key words: Malpighian tubules, allatotropin, *Triatoma infestans*, neuropeptide, hindgut, diuresis.

Introduction

Allatotropin (AT) is a neuropeptide isolated as a result of its ability to induce juvenile hormone synthesis in the tobacco hornworm *Manduca sexta* (Kataoka et al., 1989). Like other neuropeptides, it is multifunctional, acting as a myostimulator at the level of the foregut in two lepidopteran species (Duve et al., 1999; Duve et al., 2000) and at the level of the hindgut (HG) in the cockroach *Leucophaea maderae* (Rudwall et al., 2000). AT is also a cardioacceleratory peptide, both in lepidopterans (Koladich et al., 2002; Veenstra et al., 1994) and in cockroaches (Rudwall et al., 2000). In addition, a member of the AT family was isolated from male accessory glands of the locust *Locusta migratoria*, showing myostimulatory activity at the level of the HG and the oviducts in the species of origin, as well as in *L. maderae* (Paemen et al., 1991).

Haematophagous insects incorporate a large amount of blood with each meal, inducing them to produce a large quantity of urine in a few hours to eliminate excess water and mineral ions (Maddrell, 1964; Maddrell, 1978; Maddrell et al., 1993; O'Donnell et al., 2003; Ramsay, 1952). This process involves the coordinated activity of the crop, Malpighian tubules (MTs) and HG, regulated by neurosecretory mechanisms, including diuretic signals such as serotonin (Maddrell et al., 1991, Orchard, 2006). The presence of diuretic and anti-diuretic peptides acting together with serotonin has been also documented in the bug *Rhodnius prolixus* (Orchard, 2006; Paluzzi and Orchard, 2006; Quinlan et al., 1997; Te Brugge et al., 1999; Te Brugge et al., 2001; Te Brugge and Orchard, 2002; Te Brugge et al., 2005).

Malpighian tubules, the main excretory organ in insects, have traditionally been regarded as a system involved in water and mineral balance in response to neuroendocrine stimulus. New roles for this organ have started to emerge, such as a circadian rhythm regulator (Giebultowicz and Hege, 1997) and as an autonomous immune system producing anti-microbial peptides (Dow and Davies, 2006; McGettigan et al., 2005). In a related article, we communicate the ability of the MTs of the kissing bug *Triatoma infestans* (Klug) to secrete an AT-like peptide (M.S.S. and J.R.R., manuscript submitted). This activity was found to respond to changes in water and mineral composition and it is associated with the critical process of post-prandial diuresis (M.S.S. and J.R.R., manuscript submitted).

In the present study, we communicate the activity of AT as a myostimulator at the level of the hindgut and its association with the elimination of urine during post-prandial diuresis in *T. infestans*, confirming the proposed endocrine activity of MTs and assigning a first function to this new role.

Materials and methods

Insects

Fourth-instar *Triatoma infestans* (Klug) were obtained from an artificial colony maintained at 28±2°C and 45% relative humidity under a 12 h:12 h light:dark photoperiod. Insects reaching the 4th instar were isolated and starved for 21 days. After that, a meal was offered when necessary. All the insects were fed on chicken. Groups originally comprising 3–12 insects were used for different experimental designs.

Hindgut peristaltic contractions assay

As a first approach to assess the myostimulatory activity of AT on the HG, 4th-instar insects were dissected under *R. prolixus* saline (Maddrell et al., 1993). The HG was dissected together with MTs and the last portion of the midgut. Basal contractions were induced by bathing the dissected material in phosphate-buffered saline (PBS). A large variation in the response of the HG was observed between insects, apparently associated with nourishment state and the amount of material accumulated in the HG. After 3 min, samples in which signs of continued contractile activity were not evident were discarded. Only those preparations with intact HG (without loss of content during dissection) were used. Different doses of pure *Aedes aegypti* allatotropin (kindly provided by Dr Fernando G. Noriega) were diluted in PBS. Then, 20 μl of each dilution was applied to each preparation. The same preparation was used for assays with different doses. Between tests, preparations were washed with PBS (three washes, 2 min each) to restore basal conditions. Preparations were finally analysed under a dissection microscope, where the total number of contractions in a 2-min period was recorded for each dose applied (Duve et al., 2000). Results are expressed as number of contractions per minute (frequency of contractions).

Number of MTs and elapsed time to eliminate HG content after osmotic shock

We have recently shown that isolated MTs release an AT-like peptide into the incubation media in a constitutive way. The quantity of the peptide released increases when MTs undergo an osmotic shock (M.S.S. and J.R.R., manuscript submitted). In this assay, we analysed the time required by MTs–HG preparations to start content evacuation after an osmotic shock. Three groups of insects were used, one group retaining all four MTs (control), one group with two MTs and the remaining group with only one MT. All groups were exposed to 25 μl of diluted *R. prolixus* saline (20% saline, 80% distilled water), undergoing an osmotic shock and inducing AT-like peptide secretion (M.S.S. and J.R.R., manuscript submitted). The time at which the HG began eliminating its content was recorded. Results are expressed in seconds.

In vitro blockade of diuresis

To assess the relationship between AT-like peptide released by MTs and the ability of the HG to eliminate urine, we assayed the *in vitro* blockade of this process with an AT-antiseum (Hernadez-Martinez et al., 2005). We used two alternative experimental designs. In the first design, MTs–HG preparations preserving the complete set of renal tubules were incubated in 25 μl of diluted *R. prolixus* saline, including *A. aegypti* AT-antiserum. Different antiserum dilutions were applied; 1:100 000; 1:10 000; 1:1000 and 1:100. As a control, we used two different antiserum solutions preadsorbed with pure *A. aegypti* allatotropin (4°C overnight) (1:1000/20 nmol AT; 1:100/200 nmol AT). The amount of pure peptide used to preadsorb the antiserum was calculated based on previous experiments blocking immunostaining in cytological analysis of MTs (M.S.S. and J.R.R., manuscript submitted). The time required to fully evacuate the HG was recorded.

A second design was applied to corroborate the action of the AT-like peptide. MTs–HG preparations were incubated in a similar solution but, in this case, renal tubules were detached and maintained next to the HG, preventing their secretion into the HG. The presence of MTs in the same *in vitro* preparation allowed the AT-like peptide to diffuse into the medium.

In vivo blockade of diuresis

After *in vitro* experiments, we performed an *in vivo* assay to test the fate of the post-feeding diuresis alteration induced by AT blockade. The experimental design involved three groups of 4th-instar *T. infestans* larvae. In all treatments (12 insects each), 1 μl of solution was applied by intra-abdominal (ventral) injections three hours before offering a meal. We used an estimated haemolymph volume of 10 μl in non-fed 4th-instar insects to calculate the volume of injected solutions. The first group received an injection of *R. prolixus* saline, representing normal insects (general control). A second group was injected with AT-antiserum (1:100). The last group was injected with the same antiserum dilution, preadsorbed overnight (4°C) with pure AT (200 nmol). Of all the insects injected, only those that fed *ad libitum* after 30 min and without evident signs of internal damage caused by injection were used. After this sorting, none of the groups retained less than four insects. Fed insects were individually kept in microtubes (1.5 μl). The volume of urine produced by individual insects was recorded at five different times (45, 60, 90, 120 min and 24 h after blood intake) by the use of micropipettes. Data are expressed as μl of urine released per insect.

Statistical analysis

Differences between treatments were analysed by one-way or two-way analysis of variance (ANOVA), depending on the experimental design applied. In those experiments in which the interest was focused only in comparisons between treated groups and control, single *post-hoc* comparisons were tested by

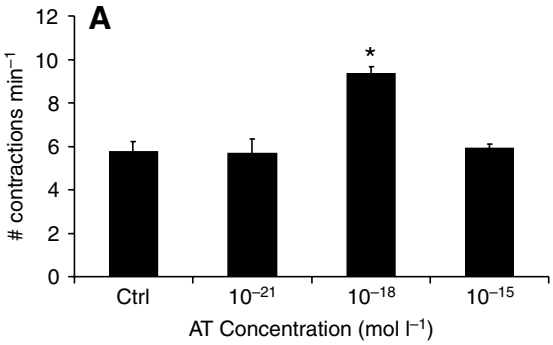
least significant difference (LSD). In experiments involving multiple comparisons, differences were analysed by the Tukey honest significant difference (HSD) test. Primary data were transformed to logarithms to improve normality and homoscedasticity when necessary. Only differences equal or less than 0.05 were considered significant. None of the samples analysed had less than three replications. Finally, data are expressed as means ± standard error. Each experimental design presented in this study was performed twice or more times showing similar results.

Results

AT induces peristaltic contraction in the HG

We first analysed the function of the AT-like peptide released by *T. infestans* MTs by the ability of the pure peptide to modify the frequency of contractions on the HG.

Our results showed a significant increase in the frequency of HG contractions with AT concentrations as low as 10^{-18} mol l^{-1} (Fig. 1A), producing a clearly defined peristaltic wave (see Movie in supplementary material). With a concentration of 10^{-15} mol l^{-1}, the peptide produced contractions with a lower frequency and longer duration of peristaltic waves. Higher concentrations of AT further increased the intensity of contractions, reaching a full and maintained contraction with AT concentrations of $\geqslant 10^{-12}$ mol l^{-1} (Fig. 1B,C). Again, and as previously described in the tomato moth *Lacanobia oleracea* (Duve et al., 2000), a large variation in the response to AT was observed between individual insects, apparently associated with nourishment state and the amount of material accumulated in the HG.



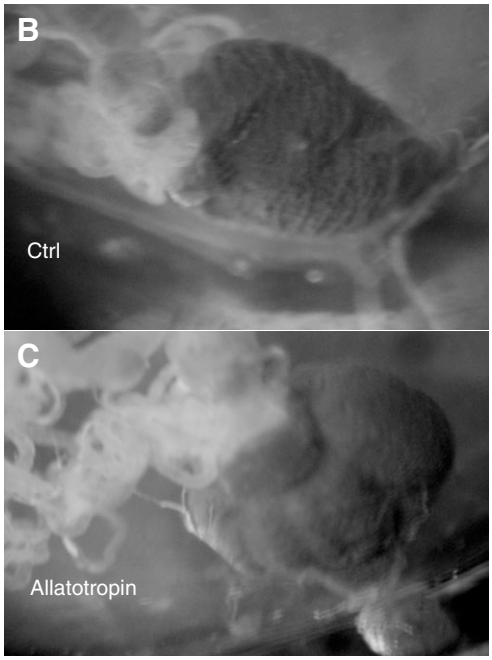


Fig. 1. Myostimulatory activity of *Aedes aegypti* allatotropin (AT) on the hindgut (HG) of *Triatoma infestans* 4th-instar larvae. (A) Changes in the frequency of contraction induced by *A. aegypti* allatotropin. *Significant difference between pure AT applied at 10^{-18} mol l^{-1} and the other groups analysed ($P \leqslant 0.05$). Each bar represents the mean ± s.e.m. of the number of contractions m^{-1} in one of three experiments performed showing similar results (N=3–5 samples per treatment). (B) Image obtained from a hindgut preparation maintained in saline (Control). (C) The same preparation after treatment with pharmacological concentrations of pure *A. aegytpi* AT, showing a full and long-term contraction of the HG.

Effect of MTs on HG voiding

In vitro MTs–HG preparations showed a differential response to diluted *R. prolixus* saline as a function of the number of tubules retained, while preparations undergoing the osmotic shock without MTs did not release the content, showing that renal tubules are involved in the process. A significant negative correlation was found between the number of tubules retained in each preparation and the time elapsed between the osmotic shock and HG evacuation (Fig. 2A).

In vitro AT blockade

The relationship between time to evacuation and MT number could be due simply to a larger influx of urine due to the higher number of tubules or, alternatively, to the need for a minimum concentration of the peptide to induce muscle contraction. To discriminate between these hypotheses, we blocked the AT by adding a high concentration of AT-antiserum (1:100) to the diluted incubation medium in preparations conserving the total number of tubules. A group of similar *in vitro* preparations was incubated with the same antiserum dilution (1:100) preadsorbed with 200 nmol of AT. A third group of samples was treated as general controls, receiving diluted saline.

Time to evacuation was similar in controls and preparations treated with preadsorbed antiserum. By contrast, MTs–HG preparations treated with AT-antiserum (1:100) showed a long-term blockade of the diuresis (>3 h), indicating the need for the AT-like peptide released by MTs to induce voiding. After this long-term blockade, the HG was swollen with liquid drained by MTs, showing that the excretory function continued, unaffected by the experimental blockage (Fig. 2B).

When similar MTs–HG preparations were treated with higher dilutions of the antiserum (1:1000; 1:10 000 and 1:100 000), the delay decreased proportionally, showing a dose–response behaviour. One of the dilution treatments used (1:1000) was compared with the analogous treatment with AT-

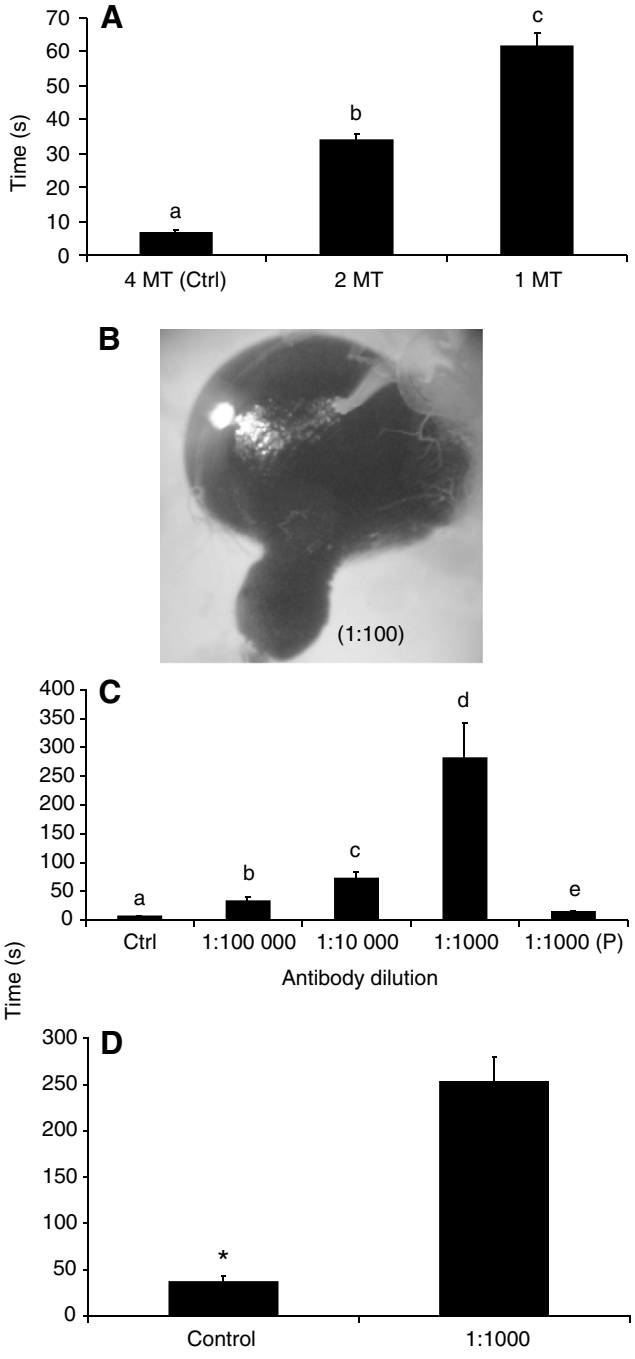


Fig. 2. (A) Elapsed time (seconds) necessary to release the hindgut (HG) content in *in vitro* preparations retaining different number of Malpighian tubules (MTs) undergoing an osmotic shock. Each bar represents mean ± s.e.m. of the elapsed time. Different letters (a–c) represent significant differences between samples in one of two experiments performed with similar results (N=3–6 samples per treatment; $P \leqslant 0.05$). (B) Aspect of the HG incubated with allatotropin (AT)-antiserum (1:100) undergoing an osmotic shock, after 3 h of the treatment (compare with the aspect of the HG under normal conditions in Fig. 1B). The same effect was obtained with three different preparations. (C) Elapsed time (seconds) until the beginning of the voiding in MTs–HG preparations retaining all MTs undergoing an osmotic shock, treated with different dilutions of the AT-antiserum [1:1000, 1:10 000, 1:100 000 and 1:1000 preadsorbed with 20 nmol of pure AT (P)]. Ctrl: samples without antiserum. Each bar represents mean ± s.e.m. of the elapsed time in one of two experiments performed with similar results (N=6 samples per treatment). Different letters (a–e) represent statistically significant differences between samples ($P \leqslant 0.05$). Note that preadsorbed samples are statistically different when compared with preparations treated with the same antiserum dilution (lower time), as well as with controls (higher). (D) Elapsed time (seconds) until the beginning of the voiding in MTs–HG preparations undergoing an osmotic shock. MTs were unattached and placed near the HG in the same diluted solution. Control, samples without antiserum; 1:1000, AT-antiserum dilution applied to the samples. Bars represent means ± s.e.m. *Significant differences between treatments ($P \leqslant 0.05$).

preadsorbed antiserum. A highly significant difference in the time elapsed was found, suggesting the specificity of the blockade (Fig. 2C).

To validate the action of AT on the HG, a new experimental design involving MTs–HG *in vitro* preparations was performed. *In vitro* preparations with unattached MTs placed near the HG in a diluted solution produced results very similar to those previous described, with a similar delayed response blocked by the AT-antiserum (Fig. 2D). The AT-like peptide diffusing through the incubation medium caused a direct response without MTs draining their content directly into the HG.

In vivo AT-like peptide blockade

The volume of urine produced at given times during the first 120 min after blood intake was lower in insects receiving AT-antiserum than in controls (Fig. 3A).

Cumulative urine released both at 90 and 120 min by the AT-antiserum-injected group was also lower than in controls. Although the total volume accumulated after 24 h is not statistically different, it was consistently smaller in insects receiving antiserum (Fig. 3B).

While the cumulative volume of urine eliminated by insects injected with AT-antiserum during the first two hours after a blood meal was lower than that of controls, differences disappear during the next 22 h. The lack of an inhibitory effect after the first 2 h may indicate that the AT-antiserum was either eliminated from the haemocoel or completely saturated by the AT-like peptide released by MTs (Fig. 3C).

Discussion

Our results show that AT has a myostimulatory activity on the HG of the kissing bug *T. infestans* (Hemiptera: Reduviidae). As previously reported in other insect species, very low doses are required to produce myostimulation (Duve et al., 1999; Duve et al., 2000). AT was first isolated in *M. sexta* (Kataoka et al., 1989) and then found in other insect and invertebrate species, showing a highly conserved sequence among all species. The results presented in the present study show that *A. aegypti* AT can modulate muscle contractions in the HG of *T. infestans* 4th-instar larvae, suggesting the

presence of specific functional receptors and that the AT-like peptide found in *T. infestans* MTs is closely related to that in other insects. In fact, an alternative splicing of AT was found in the MTs of *M. sexta* (Lee et al., 2002).

The response of the HG to AT in all experiments performed shows that after a maximum increment of contractions with a given dose, the frequency of contractions returns to that observed in controls. A similar behaviour was described in cockroaches (Rudwall et al., 2000). In our experience, this decrease is associated with a longer duration of contractions.

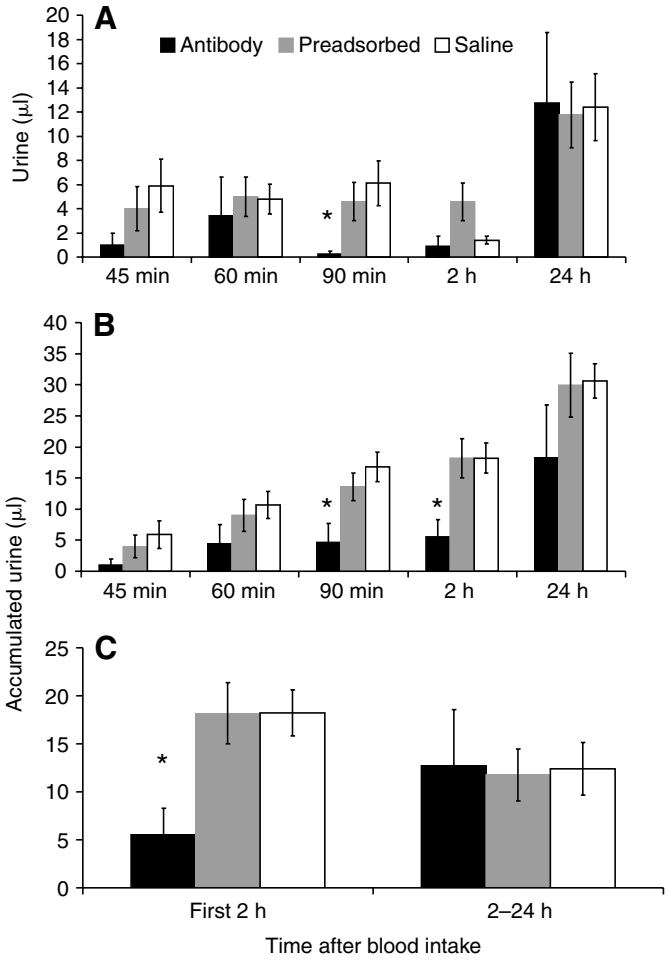


Fig. 3. *In vivo* blockade with allatotropin (AT)-antiserum of the AT-like peptide released by Malpighian tubules (MTs). Graphs represent the differences in the quantity of urine eliminated by insects injected with AT-antiserum (1:100), preadsorbed antiserum (1:100 plus 200 nmol of pure AT) or saline. (A) Urine eliminated at several time points after blood meal. (B) Accumulated volume of urine eliminated by groups with different treatments at several intervals after meal. (C) Comparison between treatments during the first 2 h after blood meal and during the next 22 h (2–24 h after blood intake). Each bar represents the mean ± s.e.m. of the volume of urine (N=4–6 samples per treatment). *Significant differences between insects injected with AT-antiserum and controls (saline and preadsorbed AT-antiserum). Data correspond to one of three experiments performed with similar results.

In fact, the highest doses led to a long-term contraction, possibly due to the saturation of the receptors. With regard to the direction of the peristaltic waves, as in the lepidopteran *Helicoverpa armigera* (Duve et al., 1999), we found considerable variations among different individuals. In some insects, the peristaltic waves seemed to be mainly in a posterior-to-anterior direction, while in other insects a combined pattern of anterior-to-posterior and posterior-to-anterior directed waves was found. The variability observed seems to be associated with the amount of material in the HG. A similar pattern of contractions observed in *H. armigera* crop has been proposed as a mechanism to generate a powerful mixing of the contents (Duve et al., 1999). Likewise, this pattern of peristaltic contractions in HG of *T. infestans* could serve to create a better mix of contents, facilitating the elimination of urine and faeces.

Our experiments show a correlation between the number of MTs and the time elapsed between an osmotic shock and voiding of HG contents. Our results also provide conclusive evidence that AT-like peptides released into the medium by MTs act directly on the HG, inducing its evacuation. In this way, MTs participate in an endocrine fashion, facilitating the voiding of the HG during post-prandial diuresis. *In vitro* treatments with different dilutions of AT-antiserum confirm this hypothesis. The use of different antiserum dilutions showed a dose–response pattern, decreasing the elapsed time when antibody dilutions were incremented. The delay must be related to the quantity of neuropeptide blocked by the antiserum applied, being greater when more antiserum is present in the medium. Furthermore, with the highest antiserum concentration tested, a long-term blockade was reached despite the fact that the HG was full with urine, showing that MT excretory function was active and not blocked during the experiment.

Haematophagous insects incorporate a large amount of blood with each meal, undergoing a critical post-feeding period during which they produce a large quantity of urine to eliminate the excess water and Na^+ incorporated (Maddrell, 1964; Maddrell et al., 1993). In our experiments, *T. infestans* 4th-instar larvae eliminated around 50% of the total urine produced during the first 24 h of post-prandial diuresis in the first 2 h.

In vivo blockade of peptide activity by the use of an antiserum has been previously used to analyse related physiological functions (Gebhardt, 2004; Patel et al., 1995; Tublitz and Evans, 1986). In our *in vivo* experiments, insects receiving AT-antiserum showed a significant decrease in urine eliminated during post-prandial diuresis. The effect was evident during the first 2 h after blood intake, when the injected antiserum decreased the accumulated volume of urine. A large intra-group variation was observed in the volume of urine produced over 24 h in the group treated with antiserum. This could be due to the fact that blockade depends on the action of an intra-haemocoelic injection of the antiserum, which could be biologically degraded at different rates in different insects.

We have shown that *T. infestans* MTs secrete an AT-like peptide in a constitutive way, increasing reversibly the quantity

of the peptide released when the surrounding medium is diluted. Furthermore, the content of AT-like peptide present in MTs decreases during the first hours after a blood meal, when diuresis is occurring at high rates. Taken together, our experiments clearly indicate the physiological nature of the processes involving the effect of AT-like peptides produced by MTs at the HG level and allow us to assign a first function to this new role. The importance of this peptide in the elimination of urine after a blood meal is clearly established.

T. infestans, like other triatominae insects, is implicated in the transmission of Chagas disease in several regions of Latin America, affecting a large number of people in several countries. The infection is naturally transmitted when the insect feeds, releasing, together with urine, faeces containing the infective form of the protozoan *Trypanosoma cruzi*. The possibility of delaying or even blocking urine elimination after a blood meal provides new ways in which to consider the potential control of this disease.

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