



Allatotropin-like peptide in Malpighian tubules: Insect renal tubules as an autonomous endocrine organ

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ABSTRACT

Malpighian tubules (MTs) are recognised as the main excretory organ in insects, ensuring water and mineral balance. Haematophagous insects incorporate with each meal a large quantity of blood, producing a particularly large volume of urine in a few hours. In the present study, we report the presence of an allatotropin-like (AT-like) peptide in MTs of *Triatoma infestans* (Klug). The AT-like content in MTs decreased during the first hours after blood-intake, correlating with the post-prandial diuresis. *In vivo* artificial dilution of haemolymph showed a similar effect. Isolated MTs challenged with a diluted saline solution resulted in an autonomous and reversible response of the organ regulating the quantity of peptide released to the medium, and suggesting that MTs synthesise the AT-like peptide. While MTs are recognised as the target for several hormones, our results corroborate that they also have the ability to produce and secrete a hormone in an autonomous way.

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1. Introduction

Malpighian tubules (MTs) are the main excretory organ in insects and have been traditionally seen as a system involved in water and mineral balance. However, new roles for this organ have recently emerged (Dow and Davies, 2006; Giebltowicz and Hege, 1997; McGettigan et al., 2005; Santini and Ronderos, 2007).

During feeding, haematophagous insects incorporate a large quantity of blood. In the following hours high volumes of urine are produced to eliminate the excess of water and mineral ions incorporated (Maddrell, 1964, 1978; Maddrell et al., 1993; O'Donnell et al., 2003; Ramsay, 1952). In the kissing-bug *Rhodnius prolixus* (Hemiptera: Reduviidae) a decrease of the osmotic concentration of the haemolymph occurs during feeding (Maddrell, 1964). Indeed, *Triatoma infestans* (Hemiptera: Reduviidae) 4th instar larvae eliminate during the first 45 min after meal around 6 µl of urine, which represents the total volume of the insect haemolymph previous to food intake (Santini and Ronderos, 2007). MTs respond to this physiological stress by increasing their rate of secretion to produce an hypoosmotic urine and reestablish water and mineral balance (Maddrell, 1964; Maddrell and Phillips, 1975). All these processes, involving integrated activity of the crop, MTs and hindgut (HG), are regulated by neurohormonal mechanisms. They include diuretic signals like serotonin (Maddrell et al., 1991; Orchard, 2006), which

also induces K⁺ re-absorption at the lower MTs (Maddrell et al., 1993). The presence of diuretic and anti-diuretic peptides acting together with serotonin has been also proposed for *R. prolixus* (Orchard, 2006; Te Brugge et al., 1999, 2002, 2005).

In spite of MTs are considered as a target organ for both diuretic and anti-diuretic hormones in several insect groups, including triatominae (Clark and Bradley, 1997; Coast, 2001; Coast et al., 2005; Eigenheer et al., 2002; Furuya et al., 2000; Maddrell et al., 1991; Paluzzi and Orchard, 2006; Patel et al., 1995; Quinlan et al., 1997; Te Brugge et al., 2001, 2005; Te Brugge and Orchard, 2002; Veenstra et al., 1997; Wiehart et al., 2002), the presence of Peptidic hormone signals has been reported in MTs of the hornworm *Manduca sexta* (Digan et al., 1992; Lee et al., 2002) and in ampullae of the MTs in *Locusta migratoria* (Montuenga et al., 1996). Also, myoendocrine cells have been detected in MTs of *Drosophila melanogaster* (Sözen et al., 1997). Furthermore, we have recently shown that *T. infestans* MTs release an AT-like peptide which modulates the voiding of the HG during post-prandial diuresis (Santini and Ronderos, 2007).

AT is a neuropeptide isolated because of its ability to induce juvenile hormone synthesis in *M. sexta* (Kataoka et al., 1989). It has also been extensively characterized in other insect species (Abdel-latif et al., 2003; Lee et al., 2002; Park et al., 2002; Truesdell et al., 2000; Veenstra and Costes, 1999) while members of the family are present in several invertebrate phyla (Elekovich and Horodyski, 2003). As many other neuropeptides, AT is multifunctional, acting as myostimulator at the level of the foregut in

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lepidopteran species (Duve et al., 1999, 2000) and also as a cardio-acceleratory peptide in *M. sexta* (Veenstra et al., 1994). Also, it has been shown that AT acts as a myostimulator at the level of the HG in the cockroach *Leucophaea maderae* (Rudwall et al., 2000) and in *T. infestans* (Santini and Ronderos, 2007). An effect of AT on the activity of soluble alkaline phosphatases, involved in ionic balance regulation, has also been detected in MTs of the Colorado potato beetle, *Leptinotarsa decemlineata* (Yi and Adams, 2001). It has also been associated with the control of ion transport in epithelial cells of the digestive system (Lee et al., 1998). We have recently shown that AT induces peristaltic contractions in the HG of *T. infestans*. Performing *in vitro* experiments we have also shown that an AT-like peptide released by the MTs is responsible of the induction of peristaltic contractions facilitating the mixing of urine and faeces and the voiding of the HG. Indeed, *in vivo* blockade of the AT-like peptide released by MTs after blood meal resulted in a significant decrease of the volume of the urine eliminated by the insect during the first 2 h of post-prandial diuresis (Santini and Ronderos, 2007).

In the present study, we further characterize the presence of the AT-like peptide in the MTs of the kissing-bug *T. infestans*, the main Chagas disease vector in Latin-America, and its secretion after feeding. Our findings corroborate the role of the MTs as an autonomous endocrine organ.

2. Materials and methods

2.1. Insects

Triatoma infestans 4th instar insects were obtained from two different artificial colonies maintained at 28 ± 2 °C and 45% relative humidity under a 12:12 h light–dark period. Insects reaching 4th instar were isolated and starved during 21 days. After that, meal was offered when necessary. All the insects were fed on chicken during the light period. Groups originally conformed by 3–6 insects were used for different experimental designs. All experiments were performed during the light period.

2.2. AT-like immunoreactivity in the Malpighian tubules

4th instar *T. infestans* (Klug) MTs were dissected under binocular microscope and fixed in formaldehyde–phosphate buffered saline (PBS) (4%) at room temperature for 12 h. Tissue was then washed in PBS–Tween (0.05%) (PBS-T); permeabilised with Triton (1%) and blocked with 3% bovine serum albumin for 60 min. Then the tissue was incubated over-night at 4 °C with a polyclonal antiserum against *Aedes aegypti* AT (1/1000 in PBS-T) which previously proved to be specific in the species of origin (Hernandez-Martinez et al., 2005). The antibody recognises the following sequence of amino acids of the *A. aegypti* AT: APFRNSEMMTARGF (FG Noriega, personal communication). It is remarkable that 10 of the 14 amino acids are fully conserved in all the species in which AT is already characterized. In *T. infestans* the antibody produced the blockade of the voiding of the HG in a dose-dependent fashion. In the same series of experiments, preadsorption of the antibody with 20 nmol (1/1000 dilution) or 200 nmol (1/100 dilution) of pure AT reverts the voiding to the control conditions showing the specificity of the antibody (Santini and Ronderos, 2007). Finally, MTs were incubated with Alexa 488-labelled goat anti-rabbit secondary antibody (1/1000 in blocking-buffer) for 3 h at room temperature (whole-mounted preparations). After every step, tissue was washed (3 times \times 10 min) with PBS-T (0.05%). All incubations and washes were done in a rotative shaker. Histological sections were processed using the same primary antibody concentration but incubated with a goat anti-rabbit antiserum conjugated with

horseradish peroxidase (1/1000 in blocking-buffer) for 3 h at room temperature. Sections were finally developed with diaminobenzidine (DAB) and counterstained with Haematoxylin. To test the specificity of the reaction, two different controls were performed. To assay primary antibody specificity, polyclonal antiserum was previously incubated over-night at 4 °C with pure AT at a concentration of 40 nmol/ml of diluted antiserum (1/1000). For secondary antibody control, primary antibody incubation was replaced with PBS. As another way to check the specificity of the immunoreaction in *T. infestans* MTs, similar preparations were performed in MTs obtained from non-fed *A. aegypti* adults. The resulting material was analysed with a Laser Scan Microscope Zeiss LSM 510 Meta.

2.3. Variation in MTs AT-like content after blood meal

To assay the variations in AT-like content in MTs after meal, the presence of immunoreactive material before and during the first 48 h after a blood meal was evaluated in groups of 4th instar insects selected as described above. Insects were sacrificed and the MTs dissected 1.5, 3, 6, 12, 24 and 48 h after blood-intake. Non-fed insects were considered as time 0. The complete set of MTs of each insect was individually homogenised and immediately frozen for posterior analysis by ELISA. Another group of insects was fed and dissected 1 and 6 h after blood meal and processed for histological analysis.

2.4. Malpighian tubules response to haemolymph dilution

To further assay changes in AT-like content in MTs three groups of non-fed 4th instar insects were treated as follows. The first group was intra-abdominally injected with 3 μ l of saline; a second group received 3 μ l of distilled water. Finally, the third group was sham injected and used as a general control. Assuming that the volume of haemolymph of a non-fed IV instar *T. infestans* larva is around 5 μ l, the volume injected represents an approximated dilution of 60%. One hour after injection, insects were sacrificed and the complete set of MTs of each insect were independently homogenised in ELISA coating-buffer and frozen for posterior AT-like titres determination.

2.5. Autonomous secretion assay in isolated MTs

In order to analyse the autonomous response of MTs, 4th instar non-fed *T. infestans* (Klug) insects were dissected and MTs isolated. After several washes in *R. prolixus* saline (Maddrell et al., 1993), the complete set of renal tubules of each insect dissected was individually placed in plastic microtubes containing saline (50 μ l) at 4 °C until the beginning of the experiment. Once all the samples were ready, all microtubes were placed in a thermostated water bath at 27 °C for 30 min to standardise conditions. The maximum time of incubation of the isolated MTs was 3 h. After the finalization of the experiment, the acridine orange test was applied to one group of MTs to assay the viability of the tissue. Once the conditions for all the samples were standardised, the response of the organ to environment dilution was tested as follow. A group of microtubes, each one containing MTs from one insect (control group) received fresh standard saline and were maintained until finalisation of the experiment. The incubation media was then recovered and immediately frozen for posterior analysis. In a second set of microtubes, standard saline was replaced with a solution containing 20% of saline plus 80% of distilled water for 15 min. Again, incubation media was recovered and frozen. Finally, a third group of MTs underwent a similar treatment, but, after a 15 min shock, diluted saline was replaced by a standard one (control saline) for 3 h to check the reversibility of the effect. Finally media were recovered and frozen for posterior analysis.

2.6. AT-like quantification by ELISA

Quantitative analysis of AT-like immunoreactivity was performed by the method of ELISA. Samples were homogenised in coating-buffer and seeded in 96 wells (50 μ l) microplates (Nunc Maxisorp) over-night at 4 °C. After that, coating-buffer was eliminated and non-specific binding sites were blocked with PBS-skimmed milk (3%) for 3 h and then dried at room temperature (3 h). *Aedes aegypti* polyclonal antiserum (Hernandez-Martinez et al., 2005; Santini and Ronderos, 2007) diluted in blocking-buffer (1/1000) was incubated over-night at 4 °C. The excess of antibody was then eliminated with PBS-Tween (0.05%) (three washes, 5 min each), and goat anti-rabbit antiserum conjugated with horseradish peroxidase (1/2000) was added to the wells (3 h). After final washes, samples were developed with ABTS-H₂O₂ and optical density (OD) was determined at 405 nm. Briefly, ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid)¹ develops a blue-green water-soluble product when reacted with horseradish peroxidase labelled conjugates and H₂O₂. Each set of experimental samples determination was complemented with a standard curve, defined on the basis of known quantities of the pure AT synthetic peptide used to raise the polyclonal antibody (Hernandez-Martinez et al., 2005). For each standard curve, the linear portion was selected and a regression equation was established ($r^2 = 0.99$). Final results are expressed as ng AT-like peptide/insect (*in vivo* experiments), and as ng AT-like peptide released to 50 μ l of medium/insect MTs (*in vitro* experiments).

2.7. Statistical analysis

Differences were analysed by one way or two way analysis of variance. Individual comparisons were tested by Tukey test. All single comparisons were made against control or non-fed groups. Only differences with *p*-values equal or less than 0.05 were considered significant. After performing ELISA, samples with OD higher or lower than limits predicted by linear regression established for each experiment were discarded. None of the samples analysed had less than three replicates. Every graph presented in this study represents two or more similar experiments. Finally, data are expressed as means \pm standard error of ng AT-like material.

3. Results

3.1. AT-like presence in MTs

Histological analysis of renal tubules showed the presence of AT-like material in MTs of 4th instar *T. infestans* (Fig. 1A and B). The estimated average size of the immunoreactive cytoplasmic granules, measured in a confocal image (500 \times), was 0.22 \pm 0.01 μ m ($n = 25$). Preadsorption of the primary antibody with the immunising peptide clearly diminished the staining (Fig. 1C), while the omission of the primary antibody completely abolished it (data not shown), confirming the specificity of the primary and secondary antibodies, respectively. The images show the accumulation of immunoreactivity in the basal domain of some cells (Fig. 1G) and also the presence of the AT-like peptide around some nuclei in MTs obtained from non-fed and fed insects (Fig. 1E and F). Furthermore, the intensity of immunoreactivity observed between images obtained from non-fed and fed insects (1 h after blood-intake) suggest that the content of the peptide in MTs decrease after meal (Fig. 1B and D). AT-like immunoreactivity was similar in upper and lower regions of the tubules.

¹ Abbreviations used: ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); AT, Allatotropin; AT-like, Allatotropin-like; ELISA, enzyme-linked immunosorbent assay; HG, hindgut; MTs, Malpighian tubules peptide; OD, optical density; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline-Tween.

We also performed the immunolabeling of MTs in *A. aegypti*. A confocal analysis of the preparations also shows the presence of immunoreactivity against AT in MTs cells (Fig. 1H). Preadsorption of the primary antibody with the immunising peptide also diminished the staining and the omission of the primary antibody completely abolished it (data not shown).

3.2. Decrease of AT-like content in Malpighian tubules during post-feeding diuresis

To analyse changes of the AT-like peptide content after meal, we quantitatively assessed AT-like levels in MTs of 4th instar insects at different times after feeding.

A group of non-fed insects were processed and considered as time 0. As confocal microscopy previously suggested, non-fed insects clearly showed AT-like presence. We found a decrease in AT immunoreactivity between 1.5 and 3 h after blood-intake, corresponding with the period of maximum post-prandial diuresis. These changes were statistically significant at 1.5 h (non-fed: 5.11 \pm 0.45, $n = 6$; 1.5 h: 2.59 \pm 0.32, $n = 6$, $p = 0.05$). After that, the content of the peptide in MTs started to increase, showing similar values to those expressed in non-fed insects 24 and 48 h after meal (Fig. 2A).

3.3. Haemolymph dilution reduced AT-like content in MTs

To assay the probable autonomous response of MTs under osmotic stress, we decided to inject non-fed insects with saline, or distilled water to provoke an artificial dilution of the haemolymph. Another group of insects were sham injected as a general control. All the insects were sacrificed 1 h after treatment and amount of AT-like peptide determined by ELISA.

AT-like content was lower in insects receiving distilled water when compared with saline and sham injected animals (distilled against sham injected: $p = 0.01$; both treatment $n = 3$; distilled against saline injected $p = 0.01$; $n = 3$ for each treatment) (Fig. 2B).

3.4. Autonomous response of isolated MTs to osmotic challenges

To further characterize the mechanisms of the secretory activity of MTs, we analysed the ability of isolated tubules to release the peptide, and the autonomous response to a water and mineral ion challenge.

Results showed the presence in the control group of AT-like immunoreactivity in the medium, suggesting the constitutive secretion of the peptide. MTs undergoing an hypoosmotic shock released to the medium a significantly greater quantity of AT-like material, showing an increment in response to the shock ($p = 0.001$; ctrl: $n = 20$, diluted saline: $n = 24$). When compared with controls, the group undergoing the hypoosmotic shock and restored to control conditions, showed a similar quantity of AT immunoreactivity ($n = 22$) (Fig. 3A). When the content of AT-like peptide in MTs was evaluated, we found a decrease of the immunoreactivity after the shock, returning to the levels of the control when MTs were restored to an isotonic solution (Fig. 3B). These results demonstrate the ability of the tissue to revert to normal conditions and the viability of the cells. The increase in AT-like secretion to the medium after exposing MTs to dilute saline mirrored the decrease in AT-like content in MTs after diluting the haemolymph with water, suggesting that low salt concentration triggers secretion of AT.

4. Discussion

MTs have been traditionally seen as organs mainly involved in water and mineral balance and excretion. Recently, it has been

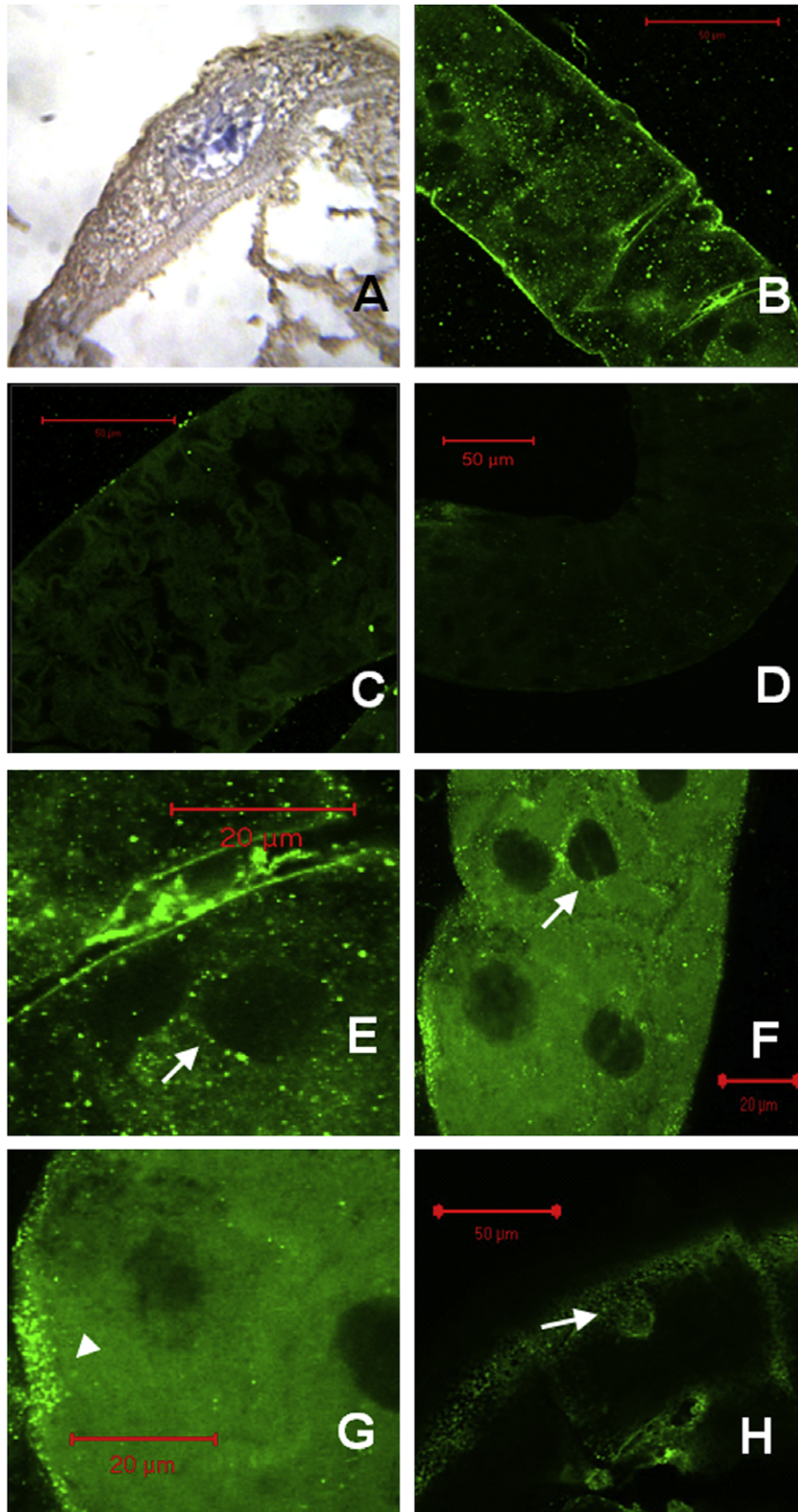


Fig. 1. Presence of the AT-like in MTs. Histological section and confocal images of MTs obtained from non-fed and fed 4th instar *T. infestans* (Klug) larvae and non-fed adult *A. aegypti*. (A) Histological section showing a MT cell presenting immunoreactive granules in the cytoplasm. Note the lack of immunoreactivity at the level of the nucleus and microvilli. (B) Confocal plane of a MT obtained from a non-fed insect showing AT-like presence in cells of the renal tubules. (C) Image obtained from similar MTs preparations performed with primary antibody preadsorbed with pure Allatotropin. Omission of the primary antibody completely abolished staining (data not shown). (D) *T. infestans* MT obtained 1 h after blood-intake showing a minor quantity of AT-like material. (E) Detail of a MT obtained from a non-fed larva showing immunoreactivity associated to one nucleus (arrow). (F) *T. infestans* MT obtained 6 h after blood-intake showing immunoreactivity around one nucleus (arrow). (G) Confocal slide obtained from a *T. infestans* MT 6 h after blood-intake showing immunoreactive granules associated to the basal membrane of the cell (arrow head). (H) Confocal slide of *A. aegypti* MTs cells immunolabeled with AT-antiserum, showing a granular pattern of immunoreactivity and the presence of immunoreactive material around the nucleus (arrow). All confocal images were taken to a depth around 5–20 μm .

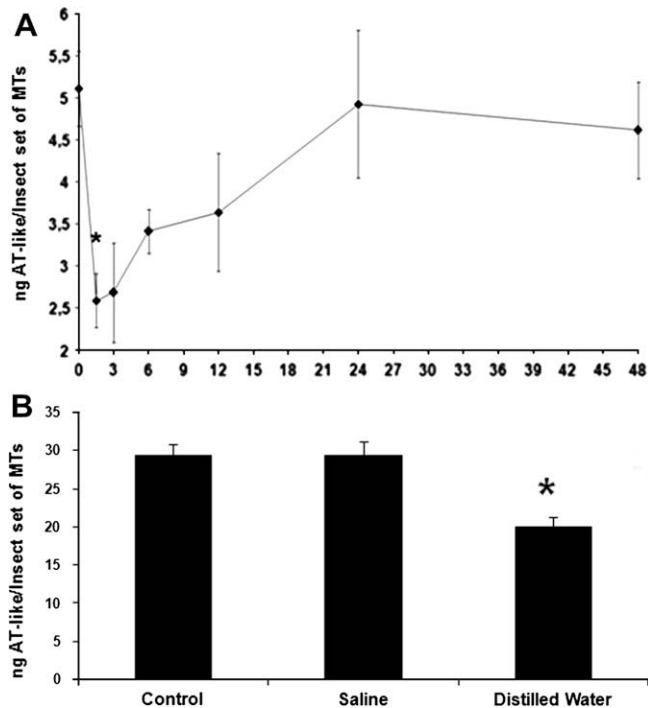


Fig. 2. AT-like content in MTs before and during the first 48 h after blood meal. (A) Variations in the AT-like content in MTs after blood meal. Each point represents the mean \pm SEM ($n = 6$ for each point represented) of ng of AT-like/insect in one of three similar experiments performed with similar results. *Significant difference compared to non-fed insects. (B) AT-like content in MTs of non-fed 4th instar insects 1 h after intra-abdominally injection with saline, distilled water, or sham injected (control). Each bar represents the mean \pm SEM ($n = 3$ for each point represented) of ng of AT-like/insect set of MTs in one of three similar experiments performed with similar results.

shown that they have other important physiological activities, acting as a cell-autonomous immune system (Dow and Davies, 2006; McGettigan et al., 2005), showing autonomous circadian activity (Giebultowicz and Hege, 1997), and also producing and secreting a myostimulatory neuropeptide acting during the process of post-prandial diuresis (Santini and Ronderos, 2007).

Previous work reported the presence of peptidic hormone signals (mRNA and protein) in MTs of lepidoptera (Digan et al., 1992; Lee et al., 2002), as well as cell populations with a myoendocrine origin in *D. melanogaster* (Sözen et al., 1997), but neither endocrine nor paracrine activity had never been previously reported in MTs. Unlike triatominae MTs which are composed by morphologically similar epithelial cells with functional specialisation (Maddrell, 1978) in *D. melanogaster*, MTs are conformed for at least two cell populations (principal and stellate cells) and these endocrine-like cells could be playing a similar role in both species.

Our results confirm the presence of AT-like material in *T. infestans* suggesting also the existence of AT in the MTs of the mosquito *A. aegypti*.

The evaluation of AT-like content in MTs during the first 48 h after blood meal demonstrates that there are significant variations throughout the first few hours, while diuresis is occurring at highest rates in *R. prolixus* (Maddrell, 1964), and *T. infestans* (Santini and Ronderos, 2007). In our experience, *T. infestans* releases during the first 2 h 60% of the total volume of urine excreted during the first day after blood-intake (Santini and Ronderos, 2007). Furthermore, the content of the peptide also decreased when haemolymph was artificially diluted by distilled water injection, mimicking post-prandial behaviour and suggesting an autonomous response of the renal tubules.

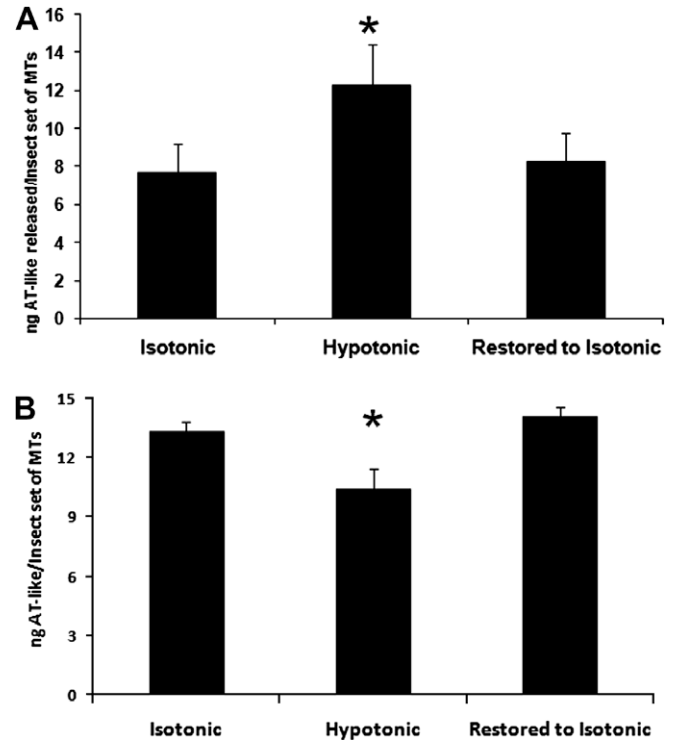


Fig. 3. *In vitro* autonomous response of MTs to osmotic challenge. (A) AT-like peptide released into the incubation medium by MTs from similar larvae under different conditions *in vitro*: control (isotonic), shock (hypotonic), returned to control solution (restored to isotonic) ($n = 20, 24$ and 22 , respectively). Differences were analysed by two way analysis of variance followed by Tukey test for individual comparisons. (B) Content of AT-like peptide in MTs exposed to isotonic (control) and hypotonic solutions. A third group of MTs was challenged to a hypotonic solution and then restored to isotonic solution. Each bar represents the mean \pm SEM ($n = 4$ for each point represented) of ng of the AT-like peptide by insect set of MTs in three similar experiments performed. *Significant difference compared to control group.

Isolated MTs responded to dilution of the saline solution increasing the amount of AT-like material released to the incubation medium, and reverting to control values when they were restored to control saline, showing the reversibility of the effect and suggesting the physiological nature of the process. This fact was correlated with changes in the amount of AT-like material in the tubules.

Despite that each experimental design presented in this study was performed twice or more times with a similar pattern of behaviour by *T. infestans* MTs, our results showed variations in the content of immunoreactive material in control groups between different experiments. This may be related to the use of insects coming from different colonies. The sensitivity of MTs to differences in the relative humidity of the environment could also be a factor affecting the basal AT-like peptide expression in the tissue. Preliminary results obtained in our laboratory suggest that after exposing MTs to a dry environment with high temperatures, AT-like immunoreactivity in MTs significantly decrease (Ma. S. Santini and J.R. Ronderos, unpublished observations).

If MTs have an endocrine function, it is logical to ponder about the activity of the AT released to haemolymph. As other peptidic messengers, AT proved to be multifunctional, having myostimulatory effects at the level of the digestive system in different insect species (Duve et al., 1999, 2000; Rudwall et al., 2000), and acting also as a cardioacceleratory peptide (Veenstra et al., 1994). Experiments performed in our laboratory showed that AT has myostimulatory activity on the HG of *T. infestans* (Klug) 4th instar larvae, modulating peristaltic contractions and facilitating both, the mix-

ing of urine and faeces and the voiding of the HG during post-prandial diuresis. The *in vitro* and *in vivo* blockade of the activity of the AT-like peptide secreted by MTs showed the relevance of both AT and the endocrine activity of the renal tubules during the physiological process of diuresis occurring after blood-intake (Santini and Ronderos, 2008). Furthermore, the analysis of the content of the AT-like peptide in the renal tubules obtained from non-fed insects along a 24 h period demonstrate the existence of a daily rhythm in the content of the AT-like peptide in the MTs, showing a peak next to the beginning of the dark phase, when the insect normally starts its feeding behaviour. As urine production begins while the insect is still feeding and the hindgut needs to be voided periodically during diuresis, the presence of high quantities of the AT-like peptide in the renal tubules should be relevant for the proper course of diuresis (Santini and Ronderos, 2008).

Together, this information shows that the MTs can detect changes in the ion composition of their environment responding with the secretion of the AT-like peptide. In this way, the after feeding decrease of the osmotic concentration in the haemolymph of triatominae insects (Maddrell, 1964) can be a factor triggering the release of the peptide, generating a response to co-operate with the reestablishment of the water and mineral balance of the insect.

Our results show for the first time, the *in situ* synthesis of an AT-like peptide in the MTs, and the autonomous regulation of its secretion to changes in the environment. Functional integration is a basic feature in any cell system, so mechanisms co-ordinating functions locally are likely to have appeared early in evolution. An autonomous endocrine system in MTs would provide a rapid and accurate mechanism to respond to sudden changes in insect internal equilibrium.

Triatoma infestans, as other triatominae species, is implicated in the transmission of the Chagas disease in several regions of Latin-America, affecting a large number of people in several countries. The infection is naturally produced when the insect feeds, releasing together with the urine, faeces containing the infective form of the protist *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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