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ALLATOSTATIN-C ANTAGONIZES THE SYNERGISTIC MYOSTIMULATORY EFFECT OF ALLATOTROPIN AND SEROTONIN IN *RHODNIUS PROLIXUS* *(Stal)*.

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ABSTRACT:

Haematophagous insects can ingest large quantities of blood in a single meal and eliminate high volumes of urine in the next few hours. This rise in diuresis is possible because the excretory activity of the Malpighian tubules is facilitated by an increase in haemolymph circulation as a result of intensification of aorta contractions combined with an increase of anterior midgut peristaltic waves. We have recently shown that haemolymph circulation during post-prandial diuresis is modulated by the synergistic activity of allatotropin (AT) and serotonin, resulting in an increase in aorta and crop contraction rates. In the present study we describe the antagonistic effect of allatostatin-C (AST-C) on the increase of aorta frequency of contractions and crop peristaltic waves induced by serotonin/AT in *Rhodnius prolixus*. The administration of AST-C in unfed adult males counteracted the increase in the frequency induced by the treatment with serotonin/AT, but did not affect the increase of the frequency induced by the administration of serotonin alone, suggesting that AST-C is altering the synergism between serotonin and AT. Furthermore, the treatment with AST-C of individuals undergoing post-prandial diuresis induced a decrease of both the frequency of contractions of the aorta and of the crop peristaltic waves. The AST-C receptor is expressed in the HG, MG and DV, three critical organs involved in post-prandial diuresis. All together these findings provide evidence that AST-C plays a key role as a myoregulatory and cardioacceleratory peptide in *R. prolixus*.

1. INTRODUCTION

Juvenile individuals of the kissing bug *Rhodnius prolixus* (*Stal*) (Hemiptera: Reduviidae) can ingest in a single meal a volume of blood up to 12.5 times its unfed weight (Buxton, 1930). Consequently large quantities of mineral salts and water must be quickly eliminated in order to decrease weight and restore water and mineral homeostasis. Therefore large volumes of urine are produced during the first few hours after feeding (Ramsay, 1952; Maddrell, 1964; Maddrell, 1978; Maddrell, et al., 1993; O'Donnell et al., 2003). During this physiological stress, Malpighian tubules (MTs) respond by increasing their rate of secretion to produce hypo-osmotic urine to re-establish the osmotic balance (Maddrell, 1964, Maddrell and Phillips, 1975). This physiological process is controlled by diuretic and anti-diuretic hormones; serotonin being one of the most important regulator of MTs activity (Maddrell and Phillips, 1975, Maddrell et al., 1991). Water and ion homeostasis also depends on the ability of the dorsal vessel (DV) to pump haemolymph in a posterior-anterior direction (Chiang et al., 1990). Furthermore, *R. prolixus* diuresis also depends on the ability of the anterior midgut (crop) to move haemolymph in an antero-posterior direction (Maddrell, 1964). In fact, almost immediately after the beginning of ingestion of blood there are increases in the number of peristaltic waves of the crop and the frequency of heart contraction, facilitating haemolymph recirculation (Maddrel, 1964).

In *R. prolixus*, in addition to the role as a diuretic factor, serotonin also controls other processes during feeding, including salivation and plasticization of the cuticle (Orchard, 2006). Furthermore, serotonin is also involved in the regulation of visceral and cardiac muscle contractions in *Drosophila melanogaster* (Dasari and Cooper, 2006), and *R. prolixus* (Villalobos-Sambucaro et al., 2015). Allatotropin (AT), a neuropeptide isolated on the basis of its activity stimulating juvenile hormone synthesis in the lepidoteran *Manduca sexta* (Kataoka et al., 1989), has also proved to be multifunctional, acting in different insect species as myoregulator and cardioaccelerator (Duve et al., 1999 and 2000; Koladich et al., 2002; Rudwall et al., 2000; Veenstra et al., 1994). In *Triatoma infestans* (Hemiptera: Reduviidae) (another kissing-bug species acting as the most important vector of Chagas disease in several South American countries), AT increases the frequency of contractions of the DV, crop and hindgut (HG) (Santini and Ronderos, 2007; Sterkel et al., 2010). In unfed male adults of *T. infestans*, AT has no myoregulatory effect by itself, but synergizes the stimulatory effect of serotonin on the frequency of the dorsal vessel contractions (Sterkel et al., 2010). In *R. prolixus*, it was shown that AT has no effect modulating heart beat frequency, nor contractions of the digestive tract under basal conditions (Masood and Orchard, 2014). However, a recently published study described a synergistic activity of serotonin and AT in *R. prolixus* (Villalobos-Sambucaro et al., 2015). In the same study it was also shown that the AT receptor is expressed in whole midgut (MG), rectum and DV (organs modulated by AT in triatominae) (Santini and Ronderos, 2007, 2009 a,b; Sterkel et al., 2010).

Allatostatins (ASTs) are a group of three structurally unrelated families of peptides originally associated with the control of *corpora allata* activity (Bendena and Tobe, 2012; Nässel, 2000). Like AT, ASTs are pleiotropic peptides, having myoregulatory functions in several insect species (Duve et al., 1999, 2000; Matthews et al., 2007; Robertson et al., 2012).

In the present study, we report the expression of an AST-C receptor in several organs of *R. prolixus,* including MG and DV, and demonstrate that treatment of unfed adult males with AST-C during the period of highest serotonin/AT stimulatory activity results in a decrease of the beat frequency of the aorta. Furthermore, AST-C also induces a decrease of both, DV frequency of contractions and peristaltic wave frequencies in adult males undergoing post-prandial diuresis. All together these results suggest that AST-C is involved in the regulation of haemolymph recirculation during the diuresis occurring after a blood meal in *R. prolixus*.

2. MATERIAL AND METHODS

2.1 Insects: Adult males of *R. prolixus* were obtained from a colony maintained at 28 ± 2°C, 45% relative humidity and a 12:12 h light-dark period. For those experiments performed with non-fed insects, adult males were immediately isolated after molting and starved during 14 to 21 days. For the experiments performed with fed insects, again individuals were isolated just after the last molt (i.e. fifth instar to adult), and starved for the same period before a blood-meal was offered. The insects were fed on chicken. All the experiments were performed during the light period. Only those insects fed *ad libitum* were used.

2.2 Myoregulatory bioassays: The effect of AST-C on the contractions of the aorta and anterior midgut were analyzed *in vivo*. To perform these experiments, the wings of the insects were removed to expose the dorsal cuticle of the abdomen. Due to the transparent nature of the cuticle, the contractions of the aorta and the peristaltic waves of the anterior midgut were clearly seen and could be recorded (Sterkel et al., 2010, Villalobos-Sambucaro et al., 2015). We tested the effect of *Aedes aegypti* AT (10^{-9}M) and AST-C (10^{-14}, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6}M) (Biopeptide, San Diego, CA) (Hernández-Martínez et al., 2005). The sequences of both peptides tested are AT: APFRNSEMMTARGF and AST-C: QIRYRQCYFNPISCF. Peptides were diluted in 3 µl of *R. prolixus* saline (Maddrell et al., 1993). Controls received only saline. Peptides were administered with a 5 µl syringe through an incision at the conexive of the first abdominal segment. Due to the incision, and cut wings, the pressure of the injection in each treatment displaces a similar volume of haemolymph which is eliminated, causing that the final volume remains constant throughout the experiment (Sterkel et al., 2010, Villalobos-Sambucaro et al., 2015). To minimize the effect of the stress caused by handling, previously to the administration of the first treatment (saline injection), insects were rested for 30 minutes. The contractions of the aorta and peristaltic waves of the anterior midgut were observed through the dorsal cuticle (segments IV and V of the abdomen) under a dissection microscope. The number of contractions in a 3-min period was recorded at 5, 15 and 30 minutes after each dose was applied (Santini and Ronderos, 2007; Sterkel et al., 2010, Villalobos-Sambucaro et al., 2015). To evaluate the effect on the peristaltic waves of the crop, only those contractions that produce an anterior-posterior wave through the abdomen were recorded. Local contractions (usually observed at the level of the segments II and III of the abdomen) were not recorded. All data were collected by the same operator. As in previous studies, forty minutes after the treatments, the frequency of contractions observed resembled the frequency of the control, showing that the insects tend to return to basal conditions (Sterkel et al., 2010). The same individual was used to assay different doses. Results are expressed as number of contractions or peristaltic waves per minute (frequency of contractions). Experiments involving fed insects were started after feeding. Taking into account that it takes about 15 minutes to feed, followed by a 30 minutes resting period, the first treatment was applied around 45 minutes after the beginning of the blood intake; a time at which post-prandial diuresis is at maximum rates (Maddrell, 1964) and both, peristaltic contractions of the crop and the contraction frequency of the dorsal vessel are at highest rates.

2.3 Statistical analysis: Significant differences were evaluated by multifactorial or repeated measures Analysis of Variance (ANOVA). Single post-hoc comparisons were tested by the LSD test. Each experimental group was constituted by 6 or 7 individuals. Only differences equal or less than 0.05 were considered significant. Data are expressed as means ± standard error.

2.4 Identification of the *RpAST-C* gene: Based on the sequences of the *Tribolium castaneum* AST-C receptor (XP_971178.2), the sequence of the corresponding ortholog gene was searched by TBLASTN algorithm and the BLOSUM62 matrix in the *R. prolixus* genome (http://vectorbase.org). The structure of the genes (ORF, introns and exons) were predicted using the software *Augustus (http://augustus.gobics.de/).*

2.5 Analysis of the sequences: Sequences analyses were performed using holometabolous and hemimetabolous sequences available in GeneBank. The accession numbers of the AST-C receptor sequences are: XP_003486456.1 (*Bombus impatiens*), XP_003394391.1 (*Bombus terrestris*), XP_396335.1 (*Apis mellifera*), XP_003698610.1 (*Apis florea*), XP_003706519.1 (*Megachile rotundata*), EFN80627 (*Harpegnathos saltator*), EFN69671.1 (*Camponotus floridanus*), XP_001600654.1 (*Nasonia vitripennis*), XP_971178.2 (*T. castaneum*), AAZ66058.2 (*D. melanogaster*), AAL02125.1 (*D. melanogaster*), AAF49259.2 (*D. melanogaster*), XP_001662510.1 (*A. aegypti*), EDS34469.1 (*Culex quinquefasciatus*), EDS35110.1 (*C.

quinquefasciatus), XP_001663106.1(*A. aegypti*), XP_003246151.1 (*Acyrthosiphon pisum*) and AHE41430.1 (*R. prolixus*). These sequences were aligned using the Clustal Wallis algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and further analyzed by the JalView 2.7 (Waterhouse et al., 2009). The seven transmembrane domains of the putative G protein-coupled receptors (GPCRs) encoded were determined using the online software *Interproscan* (Jones et al., 2014).

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2.6 mRNA expression: To amplify fragments of the *RpAST-Cr* transcript, the following primers were designed: Primer Forward 5´ - AATCTAAGCGGCCAGACAGCG -3´; Primer Reverse 5´ - TAGATGTGAGCGCCGTTGTGG -3´, corresponding to a 577 bp fragment of *RpAST-Cr*; and Primer Forward 5´ - AAGCGTGCACTTGTGCTGCTGG - 3´; Primer Reverse 5´ - ATGTGAGCGCCGTTGTGGAATG - 3´ for further characterization. The expression of the receptor was analyzed on RNA obtained from different organs (MTs, rectum, ovaries, MG, and DV) of pooled adults *R. prolixus* collected at different times before and after a blood meal.

RNA was isolated using the RNeasy kit according to the manufacter specifications (Qiagen). RNA was treated with RNAse-free DNAse (Qiagen), cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA) and used as template in a PCR reaction with the primers indicated above. PCR products were sequenced at the Unidad de Genómica - Instituto de Biotecnología - CICVyA - CNIA – INTA (Argentina).

3. RESULTS:

3.1 Antagonistic effect of AST-C on the cardio acceleratory activity of AT: AST-C (10^{-6} M) was applied to insects after they have reached the maximum increase of dorsal vessel frequency due to consecutive treatments with serotonin (10^{-9}M) and AT (10^{-9}M). The frequency of contractions of the aorta decreased significantly after treatment with AST-C (Fig. 1A, supplementary File 1). In a new set of insects (control), the AST-C treatment was replaced by a saline injection. On these insects the frequency of contractions of the aorta was not altered (Fig. 1B). Notably, after treatment with AST-C, the frequency of the contractions of the aorta decreased to a frequency similar to that previously reached by the serotonin treatment (Fig. 1A); suggesting that AST-C is antagonizing the synergistic effect of AT on tissues previously exposed to serotonin. The analysis of the data by Repeated Measures ANOVA showed that the inhibitory effect of AST-C occurred mainly during the first 15 minutes after injection (Fig. 2A). When AST-C was applied just after the serotonin treatment, the frequency of contractions of the aorta was not modified (Fig. 2B).

3.2 Activity of AST-C after blood ingestion: We analyzed the activity of AST-C during the post-prandial diuresis period. When recently fed insects (i.e. 45 min after blood ingestion) were treated with AST-C 10^{-6} M, we observed a significant decrease in the number of contractions of the aorta, as well as in the rate of peristaltic waves of the crop (Fig 3A). Furthermore, both tissues responded to the AST-C treatment in a dose-dependent manner (Fig. 3B).

3.3 Genomic characterization and expression of AST-C receptors in *R. prolixus*: We identified and cloned the putative *R. prolixus* AST-C receptor (Fig. 4A). The intronless ORF has 1260 bp and encodes a 419 AA protein (Fig. 4A and 5A; supplementary file 2). The predicted protein includes the seven transmembrane domain characteristics of the receptor family (Fig. 5A). A detailed analysis of the sequence shows that Rp-*AST-Cr* presents the amino acid sequence DRY at the cytoplasmic face of the transmembrane 3 that is characteristic of the GPCRs (Fig. 4A and B). Furthermore, all the conserved features of a somatostatin-like receptor are present, including several N-linked glycosylation sites in the N-terminal domain and several probable palmitoylation sites (Fig. 4A). In addition, the highly conserved sequence YSNSAMNPILYA is also present (Fig. 4A and B). The alignment of *Rp-AST-Cr* indicated a high degree of homology with AST-C receptors from other insect species (Fig. 4B). Transcripts for AST-C were present in all the organs analyzed, including those two relevant for these studies, namely the MG and dorsal vessel (Fig. 5B).

4. DISCUSSION:

Previous studies described cardioaceleratory and myostimulatory activities of AT on the crop and HG in *R. prolixus* (Villalobos-Sambucaro et al., 2015) and *T. infestans* (Santini and Ronderos, 2007; Sterkel et al., 2010). The presence of allatotropic nerves innervating aorta, crop and HG in *R. prolixus* and *T. infestans* were also described (Masood and Orchard, 2014, Riccillo and Ronderos, 2010; Sterkel et al., 2010). In *T. infestans*, AT increased the contractions of the digestive tract (midgut and HG) and dorsal vessel (Santini and Ronderos, 2007, Sterkel et al., 2010). AT regulatory activity on the peristaltic waves of the HG was also confirmed by injecting juvenile individuals with anti-AT antiserum (Santini and Ronderos, 2007). In addition, feeding juvenile and adults individuals of *R. prolixus* anti-AT antiserum resulted in a decrease in the frequency of contractions of the DV, the peristaltic activity of the crop and the total quantity of urine eliminated by larvae (Villalobos-Sambucaro et al., 2015). AST-C also inhibits foregut contractions in the Lepidoptera *Lacanobia oleracea* (Duve et al., 2000; Matthews et al., 2007) and heart contractions in *D. melanogaster* (Price et al., 2002).

Genes encoding AST-C related peptides have been found in several insect groups including hemimetabola such as Orthoptera and Hemiptera, as well as in mites and crustacean species (Veenstra, 2009). Surprisingly, only the sequence defined as its paralogue (AST-CC) has been annotated in the *R. prolixus* genome (Veenstra, 2009). Comparison of the *A. aegypti* AST-C used in this study with the predicted sequence of *R. prolixus* AST-CC showed a 58.3% of identity and 83.3 % of similarity for 12 out of 16 amino acids at the C-terminal of the active peptide (Fig. 5C), suggesting that *A. aegypti* AST-C peptide could bind to the AST-C receptor in *R. prolixus* tissues.

AST-C decreased contraction frequencies in target tissues to values similar to those observed before the addition of AT (i.e. the frequency after treatment with serotonin). Furthermore, AST-C had no effect when applied just after serotonin treatment, suggesting that this peptide is acting specifically on the synergistic increment caused by AT.

AST-C had no effect on the crop basal peristaltic wave frequencies, as well as on crops treated with serotonin and AT in unfed adult (data not shown). On the contrary, during post-prandial diuresis, AST-C showed a dose-response reduction of aorta beat frequency, as well as peristaltic waves of the crop. These results suggest that AST-C is already regulating haemolymph recirculation during post-prandial diuresis. The lack of response of the crop in unfed insects suggests that besides serotonin, additional factor/s might be implicated in crop muscle activity regulation.

Our results showed that AST-C antagonized the synergistic myostimulatory effect of AT. The existence of a somatostatin-like receptor for AST-C in insects raises the possibility that this peptide shares an evolutionary relationship with vertebrate somatostatin (SST), a neuropeptide originally isolated from the hypothalamus based on its ability to inhibit growth hormone secretion. SST has also pleiotropic functions and inhibits the secretion of several hormones, acting through the activation of five different G-protein-coupled receptors (Patel, 1999). Finally, SST acts by inducing a hyperpolarisation of the cell membrane and diminishing intracellular Ca^{2+} (Barbieri et al., 2013; Patel, 1999). AST-C receptors in insects might act similarly and antagonize AT activity by inducing a membrane hyperpolarisation and a decrease of intracellular Ca^{2+} necessary for muscle contraction.

In summary, our results suggest that the process of post-prandial diuresis is facilitated by synergistic and antagonistic actions of AT, serotonin and AST-C, which might play an important role by regulating haemolymph circulation as a result of modulation of aorta contractions and the anterior midgut peristaltic waves during this critical physiological process.

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Author contributions: Conceived and designed the experiments: JRR. Performed the experiments: MJVS. Analyzed the data: JRR; MJVS; FGN; LAD. Contributed reagents/materials/analysis tools: JRR; FGN; LAD. Wrote the paper: JRR. Critically revised the manuscript: FGN; LAD

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Legends for the figures:

Figure 1: Antagonistic effect of AST-C on the cardio acceleratory activity of AT. **A**: Addition of AST-C (10^{-6} M) decreased the frequency of contractions of the aorta after stimulation with serotonin and AT. **B**: Addition of saline did not modify the synergistic activity of serotonin/AT. Data analyzed by Multifactorial ANOVA. Each bar represents Mean ± Standard error

Figure 2: Time-dependent effect of AST-C on the frequency of contractions of the aorta contractions. A: The inhibitory effect of AST-C on the frequency of contractions of the aorta after being stimulated with serotonin/AT was significant during the first 15 min of the treatment. **B:** AST-C had no effect on serotonin treated aortas. Data analyzed by Repeated Measure ANOVA. Each bar represents Mean ± Standard error

Figure 3: *In vivo* activity of AST-C on the frequency of contractions of the aorta and crop during post-prandial diuresis. A: inhibitory effect of AST-C (10^{-6} M) on the frequency of the aorta and on the peristaltic waves of the crop when applied immediately after a blood meal (empty columns: saline; filled columns: AST-C). **B**: Dose response of AST-C in recently fed insects, showing the decrease of the frequency of contractions of both aorta and anterior midgut. Data analyzed by multifactorial ANOVA. Each bar represents Mean ± Standard error

Figure 4: Analysis of the *R. prolixus* AST-C receptor structure and alignment with other insect species. A: Predicted sequence of the protein showing the characteristic features of a GPCR and somatostatin-like receptors. Note the presence in the sequence of several SST-like receptor features. **Red frames:** glycosylation sites; **Black frames:** cysteine residues representing probable palmitoylation sites; **Green frame:** Sequence characteristic of GPCRs**; Blue frame:** Highly conserved sequence in SST receptors; **B:** Sequence alignment of *R. prolixus* AST-Cr with several insect orthologues showing the high level of conservation.

Figure 5: Gene structure and mRNA expression of *R. prolixus* AST-C receptor. A: Structure of the AST-C receptor gene, showing the existence of only one exon codifying for the seven transmembrane domains. **B:** Expression of the AST-C receptor in several organs of the adult male. **C:** alignment of *A. aegypti* and *R. prolixus* peptides showing the degree of conservation of the C-terminal domain.