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Research Paper

Functional characterization of the dual allatostatin-A receptors in mosquitoes

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ABSTRACT

The neuropeptide allatostatin-A (AstA) and its cognate receptors (AstARs) are involved in the modulation of feeding behavior, which in hematophagous insects includes the regulation of the disease vector-related behaviors, host seeking and blood feeding. In mosquitoes and other dipterans, there are two copies of AstAR, contrasting with the single copy found in other insects. In this study, we identified and cloned the dual AstAR system of two important disease vectors *Aedes aegypti* and *Culex quinquefasciatus*, and compared them with those previously described, including those in *Anopheles coluzzii* and *Drosophila melanogaster*. Phylogenetic analysis of the AstARs revealed that the mosquito AstAR1s has retained a similar amino acid sequence as the AstARs from non-dipteran insect species. Intron analysis revealed that the number of introns accumulated in the *AstAR2s* is similar to that in other insects, and that introns are conserved within the receptor types, but that only the final two introns are conserved across *AstAR1s* and *2s*. We functionally characterized the dual AstARs in *An. coluzzii, Ae. aegypti* and *Cx. quinquefasciatus* by stably expressing the receptors in a Chinese hamster oocyte cell line (CHO) also stably expressing a promiscuous G-protein (G16), and challenged them with the endogenous isoforms of AstA from the three mosquito species. In the culicine mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*, the AstARs demonstrated differential sensitivity to AstA, with the AstAR2s displaying a higher sensitivity than the AstAR1s, suggesting a divergence of functional roles for these AstARs. In contrast, both *An. coluzzii* AstARs demonstrated a similar sensitivity to the AstA ligands. We discuss our findings in the light of AstA acting as a regulator of blood feeding in mosquitoes. A better understanding of the regulation of host seeking and blood feeding in vector mosquitoes will lead to the rational development of novel approaches for vector control.

1. Introduction

Allatostatin-A (AstA) or FGLamide neuropeptides are involved in various physiological and behavioral processes, including the neural control and modulation of feeding in insects [1,2]. In *Drosophila melanogaster*, activation of AstA-expressing neurons increases the preference for a protein-rich diet and decreases the responsiveness to sugar [3,4], and in sugar starved flies, AstA neuron activation decreases sleep-like behavior [5]. In addition, inactivation of AstA increases food intake [3–5], whereas RNAi knockdown of its receptor reduces foraging behavior [6]. Moreover, AstA has significant effects on ion transport and gut motility [7], which is attributed, at least in part, to its expression in gut endocrine cells [8,9]. Taken together, this suggests that AstA signaling is directly involved in regulating a digestive energy-saving state in flies.

The regulation of feeding through effects on foraging, food intake, gut motility and digestive enzyme release appears to be conserved in a broad range of insects, including mosquitoes (reviewed e.g. by [10–15]). Recent semi-quantitative mass spectrometric analysis in the yellow fever mosquito, *Aedes aegypti* [16] and transcriptome together with physiological analyses in the kissing bug, *Rhodnius prolixus* [17,18] implies that the function of AstA may also be conserved in blood feeding insects. Feeding to completion on protein-rich blood in mosquitoes and *R. prolixus* induces a transient inhibition of the odor-mediated host seeking behavior [19–21]. Coinciding with the peak of this behavioral inhibition, the AstA neuropeptide level in the primary

Abbreviations: AstA, allatostatin-A; N-terminus, amino-terminus; AstAR, AstA receptor; C-terminus, carboxy-terminus; cDNA, complementary deoxyribonucleic acid; CHO, chinese hamster oocyte; ECL, extracellular loop; EC_{50}, Half maximal effective concentration; ICL, intracellular loop; PCR, Polymerase chain reaction; TM, transmembrane

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olfactory center, the antennal lobes, of *Ae. aegypti* changes and systemic injection of AstA reduces host-seeking [16]. Similarly, there is a reduction in the abundance of *AstA* transcripts in the central nervous system of *R. prolixus*, shortly after blood feeding [17], correlating with the reduction in foraging for a protein-rich food source. Blood feeding, however, does not seem to affect the *AstA* receptor transcript levels in the heads of neither *Ae. aegypti* [22] nor the malaria mosquito, *Anopheles coluzzii* [23]. To gain further insight into the putatively retained regulatory function of AstA, we here analyze the evolution of AstA receptors (AstARs) and their peptide ligands in *Ae. aegypti, An. coluzzii* and the southern house mosquito, *Culex quinquefasciatus,* as well as determine whether evolution has modified receptor function.

The AstA peptides in insects are cleaved from a common precursor peptide encoded by a single gene [17,23–27]. The number of neuropeptide isoforms encoded by the precursor is, however, highly variable between species [24]. Of the five peptides identified in *Ae. aegypti*, *An. coluzzii* and *Cx. quinquefasciatus*, only AstA-2 shares a high amino acid identity with the AstA-2 described in *D. melanogaster* [23,24]. The remaining peptides, besides sharing the conserved C-terminal Y/FXFGL/I-amide motif, generally differ in length and show amino acid substitutions across species [24].

Allatostatin-A receptors were first identified and functionally characterized in *D. melanogaster* (Drome-AstAR1 and Drome-AstAR2; [28–31]). While not unique, a characteristic feature of flies and mosquitoes is the existence of two *AstAR* genes [32,33]. The sequences of these genes in *Ae. aegypti* (*Aedae-AstAR1* and *2*), *An. coluzzii* (*Anoco-AstAR1* and *2*), and *Cx. quinquefasciatus* (*Culqu-AstAR1* and *2*), were first identified by Félix et al., who found *Aedae-AstAR1* and *Culqu-AstAR2* to be pseudogenes. To date, AstARs have also been functionally characterized in *An. coluzzii* [23], *R. prolixus* [34], the moth *Bombyx mori* [35], the honey bee *Apis mellifera* [36], as well as in the cockroaches*, Periplaneta americana* [37,38] and *Diploptera punctata* [39,40]. Together, these studies show that the conserved C-terminus Y/FXFGL/I-amide of the AstA peptide is sufficient for AstAR activation, and that the FXGLF motif generally has the highest affinity in most species [2]. Moreover, AstARs are activated by the synthetic pentapeptide YDFGL-amide, but with a lower affinity compared to the endogenous peptides, indicating that the length, and the extended amino acid sequence, of the neuropeptide C-terminus play a role in receptor binding [34,38]. Interestingly, even in receptors with very dissimilar sequences, the same affinity for AstAs can be observed. For example, both Drome-AstAR1 and 2 have a high affinity to all *Drosophila* AstAs, although these are highly divergent in protein sequence [28,31]. To date, however, the ligand binding sites of AstARs and most other neuropeptide receptors remain enigmatic.

2. Material and methods

2.1. Animals

Aedes aegypti (Rockefeller strain), *An. coluzzii* (Suakoko strain; formerly *An. gambiae* M-molecular form) and *Cx. quinquefasciatus* (Thai strain) were reared at 27 ± 1 °C, 70% relative humidity, and at a 12 h:12 h light: dark cycle, as previously described [41].

2.2. Nomenclature

The nomenclature used here was adopted from [42] with a few modifications. A five-letter code was used for interspecific neuropeptide isoforms, and for the intraspecific isoforms, the numbering was based on the order of the isoforms in the peptide precursor. For neuropeptide isoforms conserved in all three species, we omitted the five-letter code.

2.3. Phylogenetic and structural analysis of AstARs

Previous annotations of the mosquito AstA receptor and AstA precursor genes were identified in the genomes available in Vectorbase (https://www.vectorbase.org/; AaegL3.3; AgamP4.3; CpipJ2.2; Supplementary Table 1) and compared with those published by Félix et al [23]. The *AstAR* genes were cloned from cDNA (see 2.4) by PCR using specific primers (Supplementary Table 2) based on the available sequences, and their identity confirmed by DNA sequencing. In the case of *Anoco-AstAR1*, sequencing yielded an isoform with a three amino acid deletion at the intron-exon boundary in the carboxy (C)-terminus compared with that previously published and currently annotated in Vectorbase [23]. The *Anoco-AstAR1* sequence was manually curated to confirm this isoform. Full-length open reading frames of *Ae. aegypti* and *Cx. quinquefasciatus AstAR2* were predicted using BLAST search with the complete *An. gambiae/coluzzii* sequences and identification of potential splice sites using the NetGen2 server: (http://www.cbs.dtu.dk/services/NetGene2/). Phylogenetic analysis was performed using homologues of other species obtained by BLAST search from Vectorbase (https://www.vectorbase.org/Blast) and NCBI (https://www.ncbi.nlm.nih.gov/Blast) (Supplementary Table 1). The phylogenetic tree was constructed in MEGA7 [43], based on a CLUSTALW alignment of full length predicted AstAR protein sequences, using the neighbor-joining method and 2000 bootstrap replicates.

For structural analysis of the mosquito AstARs, sequences were aligned and conserved amino acids determined in GeneDoc [44]. Transmembrane domains were determined based on those described in Mirabeau and Joly [51], and modified according to TOPCONS [45] as well as motifs identified in PROSITE [46]. In addition, two-dimensional representations of the receptor three-dimensional structure were created in TOPO2 (Johns S.J., TOPO2, Transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO2/). Sequence similarities between AstARs were determined using the sequence identity and similarity tool (SIAS; http://imed.med.ucm.es/Tools/sias.html).

2.4. Cloning of the allatostatin-A receptors

To clone the *AstARs*, 6–12 days post-emergence non-blood fed mosquitoes were transferred into RNAlater (Thermo Fisher Scientific, Roskilde, Denmark), and total RNA was then extracted and purified using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany). Total RNA was used as the template for first strand cDNA synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd., Copenhagen, Denmark), according to the manufacturer's standard protocol.

Primers were designed using the Primer Design tool (http://www.clontech.com/) to enable the use of In-Fusion® technology (Clontech, Saint-Germain-en-Laye, France; Supplementary Table 2). Receptor gene sequences were amplified using Q5® Hot Start High-Fidelity 2 × Master Mix (New England Biolabs, Herlev, Denmark), and the following cycle program: denaturation at 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 60 or 62 °C for 30 s, and 72 °C for 50 s, then a final elongation step at 72 °C for 2 min (Supplementary Table 2). The PCR product was purified from a 1% agarose gel using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), and then cloned into the pIRES2-ZsGreen1 expression vector using the In-Fusion® HD EcoDry™ cloning kit (Clontech). Expression vectors were transformed into One Shot® TOP10 chemically competent *Escherichia coli* cells (Thermo Fisher Scientific), and grown on LB agar plates with 10 mg/ml kanamycin (Thermo Fisher Scientific). Vector inserts were sequenced using the pIRES Forward and pIRES Reverse primers (Supplementary Table 2). Colonies with inserts of the correct size were grown overnight in LB medium, and plasmids harvested using NucleoBond® Xtra Midi EF (Macherey-Nagel).

2.5. Bioluminescence assay

For the heterologous expression, *AstARs* were stably expressed in CHO cells, already stably expressing the human G-protein G16 (CHO/G16), and