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Research paper

Allatoregulatory-like systems and changes in cytosolic Ca^{2+} modulate feeding behavior in *Hydra*

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ABSTRACT

Allatotropin (AT) and allatostatin-C (AST-C) are neuropeptides originally characterized by their ability to modulate the secretion of juvenile hormones in insects. Beyond the allatoregulatory function, these neuropeptides are pleiotropic acting as myoregulators not only in insects, but also in other groups of invertebrates. We have previously proposed the existence of AT and AST-C like systems in *Hydra* sp., a member of the phylum Cnidaria, which is a basal group of Metazoa, sharing a common ancestor with Bilateria. In the present study we analyze the regulatory effects of both peptides on the activity of the hypostome during feeding in *Hydra* sp. Furthermore, the importance of changes in the cytosolic Ca^{2+} levels involved in the response of the hypostome were analyzed. Physiological assays showed that while the presence of food or treatment with AT stimulates the extrusion of the hypostome, AST-C has an inhibitory effect on the behavior induced by both, food and AT. These facts suggest that both systems participate in the regulatory mechanisms associated with feeding and, as in insects, AST-C and AT may exert opposite effects. The use of thapsigargin (TG) and nifedipine, two compounds that modify the levels of cytosolic Ca^{2+}, showed that changes in the levels of this ion are involved in the regulation of the activity of the hypostome. Indeed, these results suggest that the two basic mechanisms operating to increase the cytosolic levels of Ca^{2+} (i.e. the influx from the extracellular space and the release from endoplasmic reticulum) are relevant for the extrusion of the hypostome. Like in insects, the treatment with TG counteracted the effect of AST-C, suggesting that this peptide acts by reducing cytosolic Ca^{2+} levels. Furthermore, nifedipine prevented the myostimulatory effect of AT, showing that the effect of this peptide depends on the influx of Ca^{2+} throughout voltage-gated calcium channels. Altogether, these results suggest that the Allatotropin/Orexin and Allatostatin/Somatostatin regulatory systems could represent an ancestral mechanisms regulating hypostome activity and feeding behavior in Cnidaria.

1. Introduction

The coordination of physiological mechanisms requires precise communication between cells. On the basis of these cellular interactions, tissues and organs are functionally related, allowing the organisms the accomplishment of integrated functions. Peptidic molecules acting as messengers for intercellular communication, are widely distributed in Metazoa, playing regulatory roles in a variety of physiological processes. These molecules are ubiquitous and pleiotropic, acting as neurotransmitters and neuromodulators in the nervous system, and as hormones in endocrine and neuroendocrine ways. Moreover, it has been proposed that neuropeptides are the most ancient myoregulatory messengers (Grimmelikhuijzen and Hauser, 2012).

Allatotropin (AT) and allatostatin-C (AST-C) are neuropeptides originally characterized in insects, based on their regulatory function on the activity of the *corpora allata* (CA), modulating the synthesis of juvenile hormones (JH) (Kataoka et al., 1989; Kramer et al., 1991). In addition to their allatoregulatory function, both neuropeptides proved to be pleiotropic being secreted not only by the nervous system, but also by endocrine epithelial cells (Riccillo and Ronderos, 2010; Santini and Ronderos, 2007, 2009a, b; Sterkel et al., 2010). In fact, it has been proposed that AT is involved in midgut ion transport and digestive enzymes synthesis regulation in lepidopterans (Lee et al., 1998; Lwalaba et al., 2009) and more recently with the immune response in mosquitoes (Hernández-Martínez et al., 2017). Beyond these functions, AT has been

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widely characterized as a myostimulatory and cardiostimulatory peptide (Veenstra et al., 1994; Duve et al., 1999, 2000; Rudwall et al., 2000; Koladich et al., 2002; Matthews et al., 2007; Sterkel et al., 2010; Villalobos-Sambucaro et al., 2015), while AST-C showed myo- and cardioinhibitory functions in several insect species (Duve et al., 1999, 2000; Matthews et al., 2007; Price et al., 2002; Villalobos-Sambucaro et al., 2016).

AT and AST-C exert their functions by binding to receptors pertaining to the family of rhodopsin-like G protein-coupled receptors (GPCRs), causing the activation of different signaling cascades. The AT receptor (ATr) has been characterized in several insect species (Horodyski et al., 2011; Lismont et al., 2015; Nouzova et al., 2012; Verlinden et al., 2013; Villalobos-Sambucaro et al., 2015; Yamanaka et al., 2008) and is considered as orthologue of the orexin (Ox) receptor of Chordata (Horodyski et al., 2011). Similarly to ATr, the AST-C receptor was characterized in several holo and hemimetabolous insect species (Mayoral et al., 2010; Vuerinckx et al., 2011; Audsley et al., 2013; Villalobos-sambucaro et al., 2016), showing homology with the somatostatin (SST) family of receptors in vertebrates (Auerswald et al., 2001; Mayoral et al., 2010). Although these peptides and their receptors were mainly studied in insects, the existence of AT and AST-C-like systems has been proposed in other groups of Arthropoda and even in other phyla of invertebrates like Annelida and Mollusca (Christie, 2015a, b; Christie et al., 2015; Veenstra, 2010, 2011). Indeed, it has been proposed that AT and AST-C-like systems have myoregulatory activity in Platyhelminthes and Cnidaria (Adami et al., 2011, 2012; Alzugaray et al., 2013, 2016).

Hydra sp. is a fresh water member of the phylum Cnidaria, which is considered a basal metazoan group, sharing a common ancestor with Bilateria. Previous studies in *Hydra* sp. using AT and AST-C conjugated with nano-crystals, showed that these two neuropeptides are recognized by different myoepithelial cell populations, suggesting the existence of distinct receptors for both peptides (Alzugaray et al., 2013, 2016). Moreover, physiological assays showed that both peptides act as myoregulators, inducing different behaviors. The application of AT caused the extrusion of the hypostome, resembling the behavior observed during feeding (Alzugaray et al., 2013). On the other hand, AST-C caused changes in the shape and length of the tentacles, peduncle and gastrovascular cavity (Alzugaray et al., 2016). Furthermore, *in silico* search of the putative AT and AST-C receptors in *Hydra vulgaris* showed that the genome predicts the existence of GPCRs sharing homologies with both, AT/Ox and AST-C/SST receptors families respectively (Alzugaray et al., 2013, 2016).

The cnidarian myoepithelial cells are considered the most primitive type of contractile cell; they share the actin-myosin machinery with bilaterians groups (Chapman et al., 2010). It is also known that muscle contraction depends on the increment of the cytosolic Ca^{2+} levels. This increment occurs by the release from the sarcoendoplasmic reticulum and/or the influx through voltage-dependent calcium channels located in the plasma membrane. It has been proposed that in striated muscle of the jellyfish *Polyorchis penicillatus*, both mechanisms are involved (Lin and Spencer, 2001a,b), but the role of these processes in *Hydra* are not completely understood. AT and AST-C might be acting by modifying the cytosolic concentration of this ion, and activating different signaling cascades. In fact, it has been proposed that the activation of both AT and Ox receptors generate increments in the levels of cytosolic calcium (Rachinsky et al., 2003; Wu et al., 2013). Although there is no information about the *in vivo* mechanism of AST-C, it is known that SST receptors act mainly decreasing the cytosolic levels of calcium (Barbieri et al., 2013; Farrell et al., 2014).

In the present study we further analyzed the evolutionary origin and roles of AT and AST-C-like systems in Cnidaria, as well as the involvement of calcium on the mechanisms regulating the activity of the hypostome during feeding in *Hydra* sp.

2. Materials and methods

2.1. Animals

Individuals of *Hydra* sp. were obtained from a colony originated from wild hydroids collected in Argentina. The individuals are maintained in dechlorinated water at 20 ± 2 °C with a 12:12 h light/dark period. Animals were fed with *Artemia salina* and the water was replaced every day.

2.2. Physiological assays

The specimens selected for experiments were placed in *Hydra* medium (HM) ($NaHCO_3$ 0.5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.1 mM, $MgSO_4$ 0.08 mM, KNO_3 0.03 mM) and starved during 48 h. The hydroids were then placed individually in HM and acclimated for 10 to 15 min. Once the hydroids were acclimated, the saline solution was replaced by fresh saline HM (control), and then by medium containing the different treatments assayed. Each experiment was performed on 6 to 7 individuals. Each specimen was kept isolated throughout the entire experiment. The same hydroid was used for both control (saline) and posterior treatment. The experimental specimens were examined individually under a binocular microscope, and their activity recorded with a digital video camera. For each treatment a time-lapse was recorded, taking a picture every 3 s during 15 min. The length of the hypostome was evaluated as the number of pixels measured at different times during the 15 min of exposition (i.e. 0.25, 0.50, 1, 3, 5, 8, 10, 12 and 15 min) by using the GNU Image Manipulation Program (GIMP) software (Alzugaray et al., 2016; Kulkarni and Galande, 2014).

2.3. Analysis of feeding behavior

Starved hydroids were maintained in HM, and the behavior of the hypostome was evaluated. Then the medium was replaced by fresh saline + eggs of *A. salina*.

2.4. Response of the hypostome to AT and AST-C

In previous studies we showed that individually assayed peptides induced a maximum response at a concentration of 10^{-6} M (Alzugaray et al., 2013, 2016). The same concentration was selected to analyze the response of the hypostome. After 15 min in saline the hydroids were treated with 10^{-6} M of *Aedes aegypti* AT (APFRNSEMMTARGF) or 10^{-6} M of *A. aegypti* AST-C (QIRYRQCYFNPISCF) in the presence of food (Alzugaray et al., 2013, 2016; Li et al., 2003, 2004).

2.5. Effect of simultaneous treatment with AST-C and AT on the hypostome extrusion in Hydra sp

To study the existence of an inhibiting effect of AST-C on the extrusion of the hypostome induced by AT, another group of hydroids was treated simultaneously with both neuropeptides using a concentration of 10^{-6} M for each peptide.

2.6. Effect of changes on the cytosolic calcium levels on the extrusion of the hypostome

To study how modulation of cytosolic calcium levels affects hypostome extrusion in the presence of food or the two allatoregulatory peptides we used two compounds: the inhibitor of the

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