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Molecular characterization, tissue distribution, and ultrastructural localization of adipokinetic hormones in the CNS of the firebug *Pyrrhocoris apterus* (Heteroptera, Insecta)

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ABSTRACT

Adipokinetic hormones (AKHs) are a group of insect metabolic neurohormones, synthesized and released from an endocrine retrocerebral gland, the corpus cardiacum (CC). Small amounts of AKH have also been identified in the brain, although their role in this organ is not clear. To address this gap in the knowledge about insect brain biology, we studied the nucleotide sequence, tissue distribution, and subcellular localization of AKHs in the brain and CC of the firebug *Pyrrhocoris apterus*. This insect expresses two AKHs; the octapeptides Pyrap-AKH and Peram-CAH-II, the presence of which was documented in the both studied organs. In situ hybridization and quantitative reverse-transcription (q-RT)-PCR revealed the expression of the genes encoding for both AKHs not only in the CC, but also in brain. Electron microscopy analysis of the brain revealed the presence of these hormones in specialized secretory granules localized predominantly in the cellular bodies of neurons. The hormones might be transported from the granules into the axons, where they could play a role in neuronal signaling. Under acute stress induced by the injection of 3 μmol KCl, the level of AKHs in the brain increased to a greater extent than that in the CC. These results might indicate an enhanced role of brain-derived AKHs in defence reaction under acute stress situations.

1. Introduction

The actions of neuropeptides in the insect body are complex, and they involve a number of effector signals of humoral or electric nature, and are organized in negative or positive feedback loops, or in a functional hierarchy. The adipokinetic peptides, members of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) arthropod peptide family, are a typical example of neuropeptides with complex functions. Chemical nature, synthesis, cell signaling, and functions of those hormones are well characterized (Gäde et al., 1997; Van der Horst et al., 2001; Kodrík, 2008; Nässel and Winther, 2010). Major functions of AKHs are control of insect metabolism and preservation of homeostasis, and AKHs predominantly mobilize different energy reserves, such as lipids, carbohydrates, and/or selected amino acids (Gäde et al., 1997; Gäde and Goldsworthy, 2003). Thus, AKHs act as typical stress hormones, stimulating catabolic reactions and inhibiting synthetic reactions to combat stress and to suppress the processes of secondary importance. Such processes, if allowed to continue, could potentially even draw on the mobilized energy, e.g., for synthesis of storage nutrients (Kodrík, 2008). AKHs exhibit pleiotropic functions related to their metabolic role, such as stimulation of heart beat, increase of muscle tone, stimulation of general locomotion, enhancement of immune response, and/or other physiological actions (reviewed by Kodrík, 2008).

AKHs are synthesized, stored, and released predominantly by the neurosecretory cells from the corpora cardiaca (CC), a neuroendocrine gland connected to the brain. However, in some insect species, such as *Locusta migratoria* (Schooneveld et al., 1985; Moshitzky et al., 1987a; Bray et al., 1993), *Carausius morosus* and *Sarcophaga bullata* (Clottens et al., 1989), *Pyrrhocoris apterus* (Kodrík et al., 2003), or *Aedes aegypti* (Kaufmann et al., 2009), presence of AKHs was documented in the brain. For example, in *P. apterus* (macropterous morph), AKHs were detected in bodies

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and axons of about 140 brain neurons involving also neuropilar regions (Kodrík et al., 2003). Hormones were identified in the brain either by immunohistochemistry or by radioimmunoassays (Schooneveld et al., 1985; Bray et al., 1993), or (Locmi-AKH-I) by high-performance liquid chromatography coupled with amino acid sequence analysis (Moshitzky et al., 1987a,b). On the other hand proofs of the AKH gene expression in brain neurones are missing, thus, the origin of the hormones is ambiguous. Further, the function of AKHs in the insect brain is also not clear despite AKHs being identified as mediators of neuronal signaling (Milde et al., 1995). AKH immunoreactivity was observed also in the central nervous system (CNS) outside of the CC–brain complex. Several groups of immunoreactive cells were identified in the suboesophageal ganglion of *P. apterus* (Kodrík et al., 2003) and in the thoracic ganglia of several mosquito species (Kaufmann and Brown, 2006; Kaufmann et al., 2009). Conversely, in insect species such as *Drosophila melanogaster*, AKH immunoreactivity is restricted just to CC cells (Noyes et al., 1995; Nässel, 2002).

AKHs are synthesized in the endoplasmic reticulum residing in the cellular bodies of adipokinetic cells, transported to the Golgi complex, and transferred into the secretory granules of the Golgi network (Jansen et al., 1989; Harthoorn et al., 1999; Van der Horst et al., 2001). AKH pre-pro-hormones from different species exhibit a conserved structure characterized by a hydrophobic signal peptide followed by one copy of an active AKH and a peptide called AKH-associated peptide (Noyes et al., 1995). The latter peptide might facilitate the transformation of the precursor peptides, assist transferring of the active AKH into the secretory granules (Noyes et al., 1995; Van der Horst et al., 2001), and/or contribute to degradation of incorrectly assembled secretory products (Bassetti et al., 1995). Northern blot, PCR, and in situ hybridization analyses revealed that AKH genes are expressed mainly in the CC cells. However, in the moth *Spodoptera frugiperda*, quantitative-reverse transcription (q-RT)-PCR revealed that Spofr-AKH transcripts are expressed in CC–brain complexes, ovaries, midgut, fat body, accessory glands, and muscles (Abdel-latief and Hoffmann, 2007). These findings might explain the role of AKH in the regulation of oocyte formation. Accordingly, expression of AKH mRNA in ovaries was reported by Jedlička et al. (2012). These authors demonstrated Acypi-AKH mRNA expression in the ovaries of viviparous parthenogenetic aphid (*Acyrthosiphon pisum*), where the endocrine system of embryos might have been responsible for AKH production. Nevertheless, a clear proof of AKH mRNA transcription in insect brain remains elusive.

The goal of the present study was to characterize the synthesis and putative role of two AKHs in the brain of the firebug *P. apterus*, namely, Pyrap-AKH (Kodrík et al., 2000) and Peram-CAH-II (*Periplaneta americana* cardioaccelerating hormone II) (Kodrík et al., 2002), and to assess their role in a stress situation. This study focussed on the cloning and characterization of complementary (c)DNA encoding for both neuropeptides and the investigation of structural distribution and ultrastructural immunolocalization of AKH in secretory granules in neuron bodies and axons.

2. Materials and methods

2.1. Experimental animals

A stock culture of the firebug, *P. apterus* (L.) (Heteroptera, Insecta), established from wild populations collected at České Budějovice (Czech Republic, 49° N), was used in the present study. Larvae and adults of a common reproductive brachypterous morph were kept in 0.5 l glass jars in a mass culture (approximately 40 specimens per jar) and reared at constant temperature of 26 ± 1 °C under long-day conditions (18 h light: 6 h dark). They were supplied with linden seeds and water ad libitum, which were replenished twice a week. Freshly ecdysed adults were transferred to small 0.25 l glass jars (females and males separately) and kept under the same photoperiod, food and temperature regimes in which they developed. Females from 3 to 10-day old (however, exactly the same age for each experimental group; for reasons see Socha and Kodrík, 1999) were used for the experiments.

2.2. CNS dissection and AKH extraction

The head was cut off from the *P. apterus* body under the Ringer saline, and the following parts of CNS were dissected: (1) brain with corpora cardiaca (CC) and corpora allata (occasionally also with suboesophageal ganglion) attached, (2) brain from which the CC were carefully removed, and (3) CC alone. The organs were then processed as described below or the AKHs were extracted from them in 80% methanol with sonication. The supernatant of the methanolic extract was then evaporated to dryness, the residue taken up in corresponding buffer with sonication, and the solution used for the competitive ELISA (see below).

2.3. Molecular characterization of Pyrap-AKH and Peram-CAH-II

2.3.1. Nucleic acid isolation

Total RNA from dissected CNS of *P. apterus* was isolated using TRI reagent (Molecular Research Center) following the manufacturer's protocol. RNA isolates were treated with TURBO DNase (TURBO DNA-free kit, Ambion) to remove traces of contaminant DNA.

2.3.2. 3′ RACE PCR

Reverse transcription was carried out using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) on 1 μg of template RNA with an oligo-dT primer. The resulting cDNA was amplified by a subsequent PCR reaction using a 1 μl aliquot of the RT reaction as a template, a reverse primer from the oligo-dT adapter and one of degenerate forward primers (sequences are shown in Table 1). These primers were designed based on the known amino acid structure of Pyrap-AKH (QLNFTPNW) and Peram-CAH II (QLTFTPNW) and the prediction of occurrence of processing site localized downstream to active biopeptide sequence (KR). The PCR profile included initial denaturation (2 min at 94 °C), 35 cycles of denaturation (94 °C for 1 min), primer annealing (1 min) and extension (72 °C for 2 min), followed by final

Table 1
Forward primers for 3′ RACE PCR.

Name	Sequence 5′-3′	Initial annealing temperature (°C)	Final annealing temperature (°C)
3F1	CARCTAAAYTTYACGCCNAAYTGGGGNAAGCG	65	58
3F2	CAGYTAAACTTYACNCCAAAYTGGGGNAARMG	63	54
3F3	CAGCTNACATTYACNCCAAAYTGGGGNAARMG	65	56
3F4	ACNTTYACGCCAAAYTGGGGYAA	63	54
3F5	TTYACNCCNAAYTGGGGNAARMGN	60	50