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Orcokinins contribute to the regulation of vitellogenin transcription in the cockroach *Blattella germanica*

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ABSTRACT

Orcokinins (OKs) are neuropeptides that were first identified in crustacean through their myotropic activity. In insects, the *OK* gene gives rise to two mRNAs coding for two different families of conserved mature neuropeptides: OKA and OKB. Although OKs are conserved in many insect species, its physiological role in this animal class is not fully understood. Until now prothoracicotropic, regulatory of light entrainment to the circadian clock and "awakening" activities have been reported for these peptides in different insect species. Here we report the identification of OKA and OKB precursors in the cockroach *Blattella germanica*. OKA mRNA was detected in brain, whereas OKB mRNA was detected both in brain and midgut. In vivo silencing of OK precursors suggests the involvement of *OK* gene products in the regulation of vitellogenin expression in the fat body, an action that appears to be independent of juvenile hormone. This is the first time that a function of this kind has been reported for OKs.

1. Introduction

Sexual reproduction allows genetic recombination, promotes offspring survival and enables evolution to occur. To be able to reproduce, insects and other metazoans need to regulate multiple interrelated processes as nutrition, gonadal maturation, reproductive behavior, oogenesis and embryogenesis. To achieve the production and encounter of female and male mature gametes, biological processes occurring in different insect organs should be tightly coordinated. In this context, ovaries and fat body are crucial organs for oocyte maturation.

In insects, neuropeptides and lipophilic hormones play an important role in the regulation and coordination of reproductive biology (Belles and Maestro, 2005; Van Wielendaele et al., 2013). In the cockroach *Blattella germanica*, as in most insect species, juvenile hormone (JH) is the main gonadotrophic hormone, being synthesized in the corpora allata and released to hemolymph, thus activating vitellogenin (Vg) production in the fat body. Vg is then incorporated into growing oocytes as a storage protein for embryo growth and development (Belles, 2005; Raikhel et al., 2004). Vg production in the fat body and its uptake by oocytes is also regulated by neuropeptides in *B. germanica* and other insect species (Badisco et al., 2011; Brown et al., 2008; Martin et al., 1996).

Orcokinins (OKs) are arthropod neuropeptides that were first discovered in the spiny-cheek crayfish *Orconectes limosus* through its myotropic activity (Stangier et al., 1992). An OK neuropeptide was identified for the first time in insects in *B. germanica* (Pascual et al., 2004), and subsequently in species from different insect orders (Hofer et al., 2005; Hummon et al., 2006; Jiang et al., 2015; Ons et al., 2009; Roller et al., 2008). In insects, the OK gene is transcribed into two different mRNAs that code for two families of conserved mature neuropeptides: Orcokinin A (OKA) and Orcokinin B (OKB) (Jiang et al., 2015; Sterkel et al., 2012). Until now, few studies have analyzed the physiological functions of OKs. The results of these studies show that OKA has a prothoracicotropic effect in vitro in the lepidopteran *Bombyx mori* (Yamanaka et al., 2011), and that plays a role in the regulation of circadian locomotor activity in the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006a). In addition, in *Tribolium castaneum*, using specific depletion of OKA and OKB mRNAs, it has been reported that both OKA and OKB have what authors called "awakening" activities that may be involved in the control of circadian rhythms (Jiang et al., 2015).

In the present paper, we report the identification and characterization of OKA and OKB transcripts in *B. germanica*, and its involvement in the control of vitellogenesis and oocyte growth in adult female of this cockroach, as shown by experiments of transcript depletion mediated by RNAi.

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2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared on dry dog food (Panlab 125C3) and water in the dark at 30 ± 1 °C and 60–70% relative humidity. Virgin females were used for the study of gene expression levels during the first gonadotrophic cycle. Tissues were dissected under saline solution from carbon dioxide-anesthetized animals. After dissection, tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Cloning of BgOKA and BgOKB transcripts

Sequences corresponding to *B. germanica* A and B orcokinin neuropeptide precursor mRNAs were identified in transcriptomic databases obtained in our group, representing different organs and stages of development (data available at NCBI, BioProject PRJNA268902). Using these sequences, we designed specific primers (Supplementary Table 1) to obtain *B. germanica* cDNA fragments for OKA and OKB open reading frames by RT-PCR as described previously (Maestro and Belles, 2006), except that Transcriptor First Strand cDNA Synthesis kit (ROCHE) was used. The fragments were cloned in pSTBlue-1 vector (Novagen), following the manufacturer's protocol, and sequenced.

2.3. RNA extraction, cDNA synthesis, real-time PCR analyses and quantification of proteins in ovaries

The expression levels of the different genes studied were analyzed using quantitative real-time PCR (qRT-PCR) in cDNA prepared from different tissues. cDNA was synthesized from total RNA as described above. The absence of genomic contamination was confirmed using a control without reverse transcription. cDNA amplifications of OKA, OKB, Vg, 3-hydroxy-3-methylglutaryl coenzyme A synthase 1 (HMG-CoA synthase-1), juvenile hormone acid methyltransferase (JHAMT) and actin 5C were performed in duplicate or triplicate, in a 20 µl final volume (primers detailed in Supplementary Table 1). cDNA levels were quantified using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad). The schedule used for the amplifying reaction was as follows: (i) 95 °C for 3 min, (ii) 95 °C for 10 s; (iii) 57 °C for 1 min; (iv) steps (i) and (ii) were repeated for 50 cycles. Real-time data was collected through the iQ5 optical system software v.2.0 (BioRad). For quantification of soluble proteins, ovaries were dissected and placed at −80 °C until their use. Total soluble proteins were extracted by ultrasonication and centrifugation in NaCl 0.4 M solution and quantified according to Bradford (Bradford, 1976).

2.4. RNA interference

dsRNA for RNAi experiments were prepared as previously described (Maestro and Belles, 2006). Three different fragments were used to generate three different dsRNA: a 224 bp fragment spanning positions 1–224, encompassing the signal peptide, a part of the molecule that is common to both transcripts (dsOKs); a 210 bp fragment, exclusive for depleting BgOKA mRNA, spanning positions 416–626 (dsOKA); a 211 bp fragment in the 3′-UTR of BgOKB mRNA, exclusive for this transcript, spanning positions 1484–1695 (dsOKB). A heterologous 250-bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsMock) was used as a control. A dose of 2 µg diluted in sterile saline was injected into the abdomen of freshly emerged penultimate (fifth) nymphal instar females, followed by a second 2-µg dose injected just after molting to the last (sixth) instar. Dissections were carried out 5 days after the adult emergence.

2.5. Juvenile hormone and synthetic peptide treatments

JH treatment was performed by topical application. Four days after adult emergence, the wings of dsOKs-treated female insects were cut and 1 µl of JH III (Sigma) diluted in analytical grade acetone at a concentration of 2 µg/µl was topically applied on the abdominal tergites using a 10 µl Hamilton syringe. Controls were equivalently treated with acetone.

OKA type peptide (NFDEIDRSGFNSFV) and OKB type peptide (ALDSIGGGNLV-NH_2) were synthetized by the Protein Chemistry Laboratory at the *Centro de Investigaciones Biológicas* (CIB, CSIC), using Fmoc chemistry, and diluted in 10% DMSO to a concentration of 1.25 µg/µl. dsOKs-treated females were injected with 2 µl (2.5 µg) of peptide solution, or the equivalent solvent (controls), 2 and 4 days after adult emergence. Fat bodies were dissected one day after the second injection.

3. Results and discussion

3.1. B. germanica has OKA and OKB precursor mRNAs

Using the sequences of OKA and OKB of *Rhodnius prolixus* as queries we screened a transcriptomic database obtained in our group, representing different organs and stages of development (BioProject PRJNA268902). This search revealed the presence of a complete open reading frame (ORF) for OKA (BgOKA), and two sequences representing a portion of OKB ORF (BgOKB). We designed specific primers in order to clone the complete ORFs for both types.

The cloned cDNA sequence for BgOKA (Genbank™ accession number: KP744806) spans 626 nucleotides, with an ORF encoding a prepropeptide of 167 amino acid residues (Fig. 1). The cloned BgOKB (Genbank™ accession number: KP744807) spans 1695 nucleotides, with an ORF encoding a prepropeptide of 479 amino acid residues (Fig. 1). Both mRNAs share a 256 nt in the 5′ region, which contains the putative first Met and a total of 54 amino acids, including the predicted signal peptide. This suggests that both precursors are alternative splicing variants expressed by the same gene, as occurs in other insect species (Sterkel et al., 2012; Veenstra and Ida, 2014). Taking into account the putative monobasic or dibasic cleavage sites that would give rise to peptides showing the characteristic N-terminal OKA motif NXDEID (X = F or L), we could find the sequence coding for three OKA peptides in the BgOKA transcript, including the peptide already biochemically purified from brain extracts NFDEIDRSGFNS (Pascual et al., 2004). The BgOKB transcript shows the sequence coding for 22 putative mature peptides showing, with very few variations, the sequence X_1DSIGGGNX_2V (X_1 and X_2 = L or I), with a Gly residue (which allows amidation) or not at the C-terminus, compatible with the characteristic sequence of OKB peptides. In addition, the BgOKA mRNA encodes a sequence (LDSIGGGHLL) that is also compatible with a mature OKB peptide. Interestingly, OKB peptides from *R. prolixus* and *Drosophila melanogaster* have a His in position 8 (Sterkel et al., 2012; Veenstra and Ida, 2014), whereas in *B. germanica* this position is occupied by an Asn.

3.2. BgOKs are expressed in brain and midgut

Specific primers were designed to measure the levels of BgOKA and BgOKB transcripts in the brain, midgut, ovaries and fat body of *B. germanica* adult females. Results showed that BgOKA is expressed only in brain at moderate levels, whereas BgOKB is