General and Comparative Endocrinology 243 (2017) 15–21

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Molecular characterization of an adipokinetic hormone-related neuropeptide (AKH) from a mollusk

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ARTICLE INFO

Article history:
Received 7 June 2016
Revised 31 October 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
Mollusk
Bivalve
Oyster
Neuroendocrinology
AKH

ABSTRACT

Adipokinetic hormones (AKH) are key regulators of energy mobilization in insects. With the growing number of genome sequence available, the existence of genes encoding AKH related peptides has now been established in protostomes. Here we investigated the occurrence of a mature AKH-like neuropeptide (Cg-AKH) in the oyster *Crassostrea gigas*. We unambiguously elucidated the primary structure of this neuropeptide by mass spectrometry from peptidic extracts of oyster visceral ganglia. Cg-AKH mature peptide (pQVSFSTNWGS-amide) represents an additional member of the AKH family of peptides. The organization of Cg-AKH encoding gene and its corresponding transcript is also described. Cg-AKH gene was found to be expressed in the nervous system though at extremely low levels compared to other neuropeptide encoding genes such as the oyster GnRH gene. Although both reproduction and feeding are known to affect the energy balance in oysters, no significant differential expression of Cg-AKH gene could be evidenced in relation with the nutritional status or along the reproductive cycle. The possible involvement of Cg-AKH in the regulation of energy balance in oyster remains an open question.

1. Introduction

Adipokinetic hormones (AKH) were first identified as insect metabolic neuropeptides involved in the mobilization of energy substrates during energy-consuming activities such as flight and locomotion (Gäde, 2009). With the initial discovery that *Drosophila* AKH specifically binds receptors structurally related to vertebrate GnRH receptors (Staubli et al., 2002) it has become clear that insect AKH and GnRH signaling systems share a common evolutionary origin. This family of neuropeptides also includes the insect neuropeptides corazonin (CRZ) and AKH/corazonin-related peptides (ACP). Both the peptides and their cognate receptors display sequence homology and phylogenetic proximity (Hauser and Grimmelikhuijzen, 2014). GnRH and AKH-like signaling systems were well characterized in Deuterostomes (Roch et al., 2014) and Ecdysozoan Protostomes respectively (Cazzamali et al., 2002; Hansen et al., 2010; Park et al., 2002; Staubli et al., 2002). In Lophotrochozoa, the ecdysozoan protostome sister clade, data mining of genomes and transcriptomes led to the identification of GnRH and AKH-like signaling components (Hauser and Grimmelikhuijzen, 2014; Roch et al., 2014,) but till recently, only GnRH related peptides or their receptors were molecularly and functionally characterized (Bigot et al., 2012; Iwakoshi et al., 2002; Kanda et al., 2006). In the Pacific oyster *Crassostrea gigas*, the occurrence of a gene encoding an AKH precursor was mentioned (Bigot et al., 2012) and the existence of genes encoding the components of a complete AKH signaling system was demonstrated in this species (Hauser and Grimmelikhuijzen, 2014). Intriguingly, screening of *C. gigas* comprehensive transcriptomic databases (Fleury et al., 2009; Riviere et al., 2015) failed to detect any transcript encoding this precursor. To gain insight into the existence of a functional AKH signaling system in oyster, we examined the occurrence of an AKH mature peptide and investigated the pattern of expression of its encoding gene using quantitative RT-PCR.

2. Materials and methods

2.1. Animals

Two years old adult oysters *Crassostrea gigas* purchased from a local oyster farm (Normandie, France) were used for peptide characterization and transcription analyses. Stages of reproduction (Stage 0: resting undifferentiated stage, Stage I: gonial multiplication

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http://dx.doi.org/10.1016/j.ygcen.2016.11.002
0016-6480/

stage, Stage II: maturation stage, Stage III: sexual maturity) were determined by histological analysis of gonad sections as described in (Rodet et al., 2005). To study the influence of trophic conditions, one year old adult oysters were reared in water tanks either in absence of food or in presence of *Isochysis galbana* maintained at a concentration of 6 million of cells/mL during 3 weeks.

2.2. Extraction of tissues for peptide analysis

Twenty animal equivalents of visceral ganglia were extracted in 0.1% Trifluoroacetic acid (TFA) at 4 °C and centrifuged for 30 min at 35,000*g* at 4 °C. The supernatants were concentrated on Chromafix C18 solid phase extraction cartridges (Macherey-Nagel). Samples were evaporated for nano LC purification.

2.3. Nano-LC purification of visceral ganglia extract

The chromatography step was performed on a nano-LC system (Prominence, Shimadzu). Peptides were concentrated on a Zorbax 5 × 0.3 mm C18 pre-column (Agilent) and separated onto a Zorbax 150 × 75 μm C18 (Agilent). Mobile phases consisted of 0.1% trifluoroacetic acid (TFA), 99.9% water (v/v) (A) and 0.1% TFA, 99.9% ACN (v/v/v) (B). The nanoflow rate was set at 300 nL/min, and the gradient profile was as follows: constant 2% B for 6 min, from 2 to 5% B in 4 min, from 5 to 32% B in 160 min, from 32 to 60% B in 5 min, from 60 to 80% B in 5 min, and return to 2% B in 20 min. The 300 nL/min volume of the peptide solution was mixed with 1.2 μL/min volume of a mix of 5 mg/mL CHCA matrix prepared in a diluting solution of 50% ACN with 0.1% TFA. Twenty-six second fractions were spotted by an Accuspot spotter (Shimadzu) on a stainless steel Opti-TOF™ 384 targets.

2.4. Mass spectrometry analysis

MS analyses were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeam™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of Angiotenin I, Angiotenin II, Neurotensin, ACTH ⟨1 −17⟩, ACTH (18–39) and mass precision was above 50 ppm.

All acquisitions were taken in automatic mode. A laser intensity of 3400 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 700 to 4000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 4300. For the tandem MS experiments, the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as settings. The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the Mascot 2.4.0 program (Matrix Science) with the "oyster predicted proteins" file of the latest version of *C. gigas* genome (http://www.oysterdb.com) (Zhang et al., 2012). The variable modifications allowed were as follows: C-terminal amidation, N-terminal pyroglutamate, N-terminal acetylation, methionine oxidation and dioxidation. Mass accuracy was set to 300 ppm and 0.6 Da for MS and MS/MS mode respectively.

2.5. Molecular cloning of the full length Cg-AKH cDNA

The full-length cDNA sequence was obtained by (5′ and 3′) primer walking by PCR using 50 ng of a *C. gigas* "all development stages and adult central nervous system" directional and normalized cDNA library inserted into the Pal 17.3 vector (Evrogen) (Fleury et al., 2009) in 50 μl reaction volume containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM each of the primer couples Q_{A2} (5′-CCGATGCAAATCGTCGTT-3′)/Pal17D (5′-CCAGGGTTTTCC CAGTCACGA-3′) or Q_{S1} (5′-CTGTATTCTGGCTGTAGTAG-3′)/Pal17R (5′-CACAGGAAACAGCTATGACCA-3′) respectively for 5′ and 3′ amplification, 1,25 units of Go-Taq polymerase and the appropriate buffer (Promega) in nuclease-free water. Samples were submitted to the following cycling parameters (95 °C, 2 min; 30 cycles of: 95 °C 45 s, annealing temperature 30 s, 1 min 72 °C followed by 5 min 72 °C). Each PCR reaction was diluted 10-fold and 1 μL was used as template for nested PCR run under the same conditions using the following primer couples Q_{A1} (5′-CTATGAATGAAGATGAA GAA-3′)/T7 or Q_{S2} (5′-ATGCCTGCTGGACGAAAT-3′)/SP6 respectively for 5′ and 3′ amplification.

2.6. Reverse transcription and quantitative PCR

Real time RT-qPCR analysis was performed using the iCycler iQ© apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Recovered RNA was then further purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment during 20 min at 37 °C with 1U of DNase I (Sigma) to prevent genomic DNA contamination, 1 μg of total RNA was reverse transcribed using 1 μg of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200 U MMuLV Reverse Transcriptase (Promega) at 37 °C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70 °C for 10 min. iQ™ SYBR Green Supermix PCR kit (Biorad) was used for real time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95 °C/15 s, 60 °C/15 s) with the following primers: Q_{S2} (5′-ATGCCT GCTGGACGAAAT-3′) and Q_{A2} (5′-CCGATGCAAATCGTCGTT-3′) as sense and antisense primers respectively. Accurate amplification of the target amplicon was checked by performing a melting curve. Using Qs-*Cg*-EF (5′-ACCA CCCTGGTGAGATCAAG-3′) and Qa-*Cg*-EF (5′-ACGACGATCG CATTTCTCTT-3′) primers, a parallel amplification of oyster Elongation Factor 1α (EF1 α) transcript (BAD15289) was carried out to normalize the expression data of *Cg*-AKH transcript. EF1 α was found as a reliable normalization gene as no significant difference ($p < 0.05$) of Ct values was observed between the different samples compared. Coefficient of variation of EF1 α was less than 5%. The relative level of each gene expression was calculated for one copy of the EF α 1 reference gene by using the following formula: $N = 2^{(Ct\ \ EF1\ -\ Ct\ \ Cg\text{-}AKH)}$. Cg-GnRH transcript quantification was carried out using the same protocol with the following primers: Qs-Cg-GnRH (5′-AGCGTATTTCTGGCCGTACA-3′) and Qa-*Cg*-GnRH (5′-CCATGCCCAAAATTACCACT-3′) (Bigot et al., 2012). The PCR amplification efficiency (E; $E = 10^{(-1/slope)}$) for each primer pair was determined by linear regression analysis of a dilution series to ensure that E ranged from 1.98 to 2.02. The specificity of the primer pairs was confirmed by melting curve analysis at the end of each qPCR run and each amplicon was verified by gel electrophoresis.

2.7. Statistical analysis

Gene expression levels between different tissues and between samples at different reproduction stages were compared using one-way ANOVA followed by a Bonferroni post hoc test. Expression levels between fed and starved animals and between Cg-AKH and GnRH were compared using unpaired Student's t tests. Significance was set at $p < 0.05$.