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Molecular and functional characterization of adipokinetic hormone receptor and its peptide ligands in *Bombyx mori*

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ABSTRACT

Neuropeptides of the adipokinetic hormone (AKH) family are among the best studied hormone peptides, but its signaling pathways remain to be elucidated. In this study, we molecularly characterized the signaling of *Bombyx* AKH receptor (AKHR) and its peptide ligands in HEK293 cells. In HEK293 cells stably expressing AKHR, AKH1 stimulation not only led to a ligand concentration dependent mobilization of intracellular Ca^{2+} and cAMP accumulation, but also elicited transient activation of extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. We observed that AKH receptor was rapidly internalized after AKH1 stimulation. We further demonstrated that AKH2 exhibited high activities in cAMP accumulation and ERK1/2 activation on AKHR comparable to AKH1, whereas AKH3 was much less effective.

1. Introduction

Adipokinetic hormones (AKHs) produced by the insect corpora cardiaca are among the most extensively characterized peptide hormones with almost 40 family members from most of the major insect orders [1–7]. AKH is normally 8–10 amino acids long with a pyroglutamate at the N-terminus and an amidated C-terminus. In addition to the essential role of mobilization of metabolites during energy-expensive activities such as flight and locomotion, AKH is involved in the control of carbohydrate homeostasis in the haemolymph of *Drosophila* and *Bombyx* larvae [8,9]. As shown in Table 1, in *Bombyx*, a non-apeptide identical with *Manduca* AKH (AKH1) has been chemically identified [10], and recently another two cDNAs encoding the prepro-*Bombyx* AKH2, and 3 have been annotated and identified by combining homology search with cDNA cloning [11].

The receptor of AKH was first identified as a typical G protein-coupled receptor (GPCR) from the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori* in 2002 [12], and then from the cockroach *Periplaneta americana* [13] and African malaria mosquito *Anopheles gambiae* [14]. Previous biochemical characterization with isolated fat body suggested that AKH binds to its receptor and activates adenylyl cyclase via the Gs protein, which results in an increase of intracellular cAMP levels. In addition, AKH activates phospholipase C (PLC) to induce the release of Ca^{2+} from intracellular Ca^{2+} stores [15–17]. However, the mechanistic details of AKHR signaling remain to be further elucidated.

In this present study, we cloned the AKHR from the fat body of the silkworm *B. mori* and further functionally characterized it and its peptide ligands in HEK293 cells. We conclude that after activation of AKHR, in addition to cAMP accumulation and Ca^{2+} release from Ca^{2+} stores, the mitogen-activated protein kinase (MAPK) pathway is subsequently activated and AKHRs are rapidly internalized from the plasma membrane upon agonist stimulation. AKH1 and AKH2 activated AKHR with similar affinity, but AKH3 exhibits almost much less activity on AKHR. These findings provide a

Abbreviations: GPCR, G protein-coupled receptor; AKH, adipokinetic hormone; Hez-HrTH, *Heliothis zea* hypertrehalosaemic hormone; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase1/2; PTX, pertussis toxin; CTX, cholera toxin

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Table 1
Primary structure of adipokinetic hormone peptides from *Bombyx mori*.

Adipokinetic peptides	Sequence	Ref.
Adipokinetic hormone 1 (AKH1)	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH$_2$	[10]
Adipokinetic hormone 2 (AKH2)	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-GlnNH$_2$	[11]
Adipokinetic hormone 3 (AKH3)	pGlu-Ile-Thr-Phe-Ser-Arg-Asp-Trp-Ser-GlyNH$_2$	[11]

foundation for future studies of the physiological role of AKH/AKHR signaling in the diapauses, development and reproduction of *Bombyx*.

2. Materials and methods

2.1. Materials

Larvae and pupae of the silkworm strain Feng-Yi were kindly provided by Dr. Kerong He (Zhejiang Agricultural Institute). Cell culture media and G418 were purchased from Invitrogen (Carlsbad, CA). The pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories Inc. (Palo Alto, CA) and Sigma (St. Louis, MO), respectively. The membrane probe DiI and nuclear dye Hoechst33258 were purchased from Beyotime (Haimen, China). Pertussis toxin (PTX) and cholera toxin (CTX) were purchased from Sigma and Calbiochem (Cambridge, MA), respectively. Primary antibodies for Western blotting were purchased from Cell Signaling (Danvers, MA) and Beyotime.

2.2. Cell culture and transfection

The human embryonic kidney cell line (HEK293) was maintained in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 2 mM L-glutamine (Invitrogen). The AKHR cDNA plasmid constructs were transfected or co-transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, selection for stable expression was initiated by the addition of G418 (800 μg/ml). Transfected cells were evaluated for expression of AKHR at the cell surface by flow cytometry.

2.3. Cloning of Bombyx AKHR cDNA and construction of mammalian expression vectors

Total RNA was isolated from the fat body of pupae of *B. mori* using the TRIzol reagent (Keygen, Nanjing, China) following the manufacturer's instructions. The cDNA was prepared with an AMV First Strand cDNA Synthesis Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. To amplify the full-length sequence encoding *Bombyx* AKHR, two pairs of primers were designed based on the sequence of GenBank Accession No. AF403542 and are as follows: forward primer 5′-AAGCTTATGGATATAGACGAGAAAGTGTCC-3′; reverse primer 5′-TCTAGATTAAACCATACCGTTCGTTACGTG-3′ for pCMV-Flag; and forward primer 5′-AAGCTTGCCACCATGGATATAGACGAGAAAGTGTCC-3′; reverse primer 5′-GGTACCGTAACCATACCGTTCGTTACGTGGTT-3′ for pEGFP-N1. The corresponding PCR products were inserted into the HindIII and XbaI sites of the pCMV-Flag vector and the HindIII and KpnI sites of the pEGFP-N1 vector, named these two vectors Flag-AKHR and AKHR–EGFP, respectively. All constructs were sequenced to verify the correct sequences and orientations.

2.4. cAMP accumulation measurement

After seeding in a 24-well plate overnight, 293 cells stably co-transfected with Flag-AKHR and pCRE-Luc were grown to 90–95% confluence, stimulated with the indicated concentration of AKH in DMEM without FBS and incubated for 4 h at 37 °C. Luciferase activity was detected by use of a firefly luciferase assay kit (Kenreal, Shanghai, China). When required, cells were treated overnight with PTX (100 ng/ml) or CTX (300 ng/ml) in serum-free DMEM before the start of the experiment.

2.5. Intracellular calcium measurement

Calcium mobilization was performed as described previously with slight modifications [18]. The stable Flag-AKHR-expressing 293 cells were harvested with Cell Stripper (Mediatech, Herndon, VA), washed twice with phosphate-buffered saline, and resuspended at 5×10^6 cells/ml in Hanks' balanced salt solution containing 0.025% bovine serum albumin. The cells were then loaded with 3 μM fura-2/AM (Molecular Probes, Eugene, OR) for 30 min at 37 °C. Calcium flux was measured using excitation wavelengths of 340 and 380 nm in a fluorescence spectrometer (LS55, Perkin–Elmer Life Sciences).

2.6. Immunoblot analysis

The 293 cells stably expressing Flag-AKHR seeded in six-well plates were starved by growth in serum-free media overnight. After stimulation with AKH, cells were lysed. Equal amounts of total cell lysates were size-fractionated by Tris–glycine SDS–PAGE (10%) and transferred to a PVDF membrane (Millipore). Membranes were blocked in blocking buffer TBST containing 5% nonfat dry milk for 1 h at room temperature (RT) and then probed with rabbit monoclonal anti-p-extracellular signal-regulated kinase 1/2 (ERK1/2) antibody (Cell signaling, Danvers, MA) and next probed with anti-rabbit HRP-conjugated second antibody (CHEMICON, Temecula, CA) according to protocol of the products. β-Actin (Beyotime, Haimen, China) and total ERK1/2 (Cell signaling, Danvers, MA) was assessed as a loading control after p-ERK1/2 chemiluminescence detection using HRP-substrate purchased from Cell signaling.

2.7. Internalization assay and fluorescence microscopy

For the internalization assay, 293 cells stably expressing AKHR–EGFP were seeded in cover glass-bottomed six-well plates. After treatment with AKH peptides at 37 °C for 60 min, 293 cells were stained with the membrane probe DiI (Beyotime, Haimen, China) at 37 °C for 5–10 min, fixed with 2% paraformaldehyde for 15 min, and finally incubated with Hoechst 33258 (Beyotime) for cell nuclei staining for 10 min. The cells were mounted in mounting reagent (DTT/PBS/glycerol). Fluorescence microscopy was performed on a Zeiss LSM510 laser scanning confocal microscope attached to a Zeiss Axiovert 200 microscope using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion lens.

2.8. Flow cytometry analysis

Cells (5×10^5) were washed with PBS supplemented with 0.5% BSA and incubated with 10 μg/ml FITC-labeled anti-Flag M2 monoclonal antibody (Sigma, St. Louis, MO) in a total volume of 100 μl. After incubating for 30 min at 4 °C, cells were fixed with 2%