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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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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-

paraformaldehyde in PBS and subjected to flow cytometry analysis on a FACScan flow cytometer (Coulter EPICS Elite, Coolten Corp., Hialeah, FL).

### 2.9. Peptide synthesis

The AKH peptides (Table 1) were prepared by solid-phase synthesis using the Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perceptive Biosystems, Cambridge, MA) and purified by preparative reverse-phase high-performance liquid chromatography using a Dynamax-300 Å C18 25 cm × 21.4 mm ID column with a flow rate of 9 ml/min and two solvent systems of 0.1% TFA/$H_2O$ and 0.1% TFA/acetonitrile.

## 3. Results

### 3.1. Expression and cellular localization of AKHR

We cloned the adipokinetic hormone receptor (AKHR) cDNA from the fat body of pupae of the silkworm *B. mori* by RT-PCR, and constructed two vectors to express AKHR with either a Flag-tag at the N-terminus or enhanced green fluorescent protein (EGFP) at the C-terminus. After transfection of HEK293 cells with Flag-AKHR and AKHR–EGFP, stably expressing cells were selected by the addition of 800 μg/ml G418, and confirmed by FACS analysis and fluorescent microscopy (Fig. 1A and B). As shown in Fig. 1A, significant cell surface expression was detected by fluorescent microscopy with minimal intracellular accumulation in the absence of AKH1.

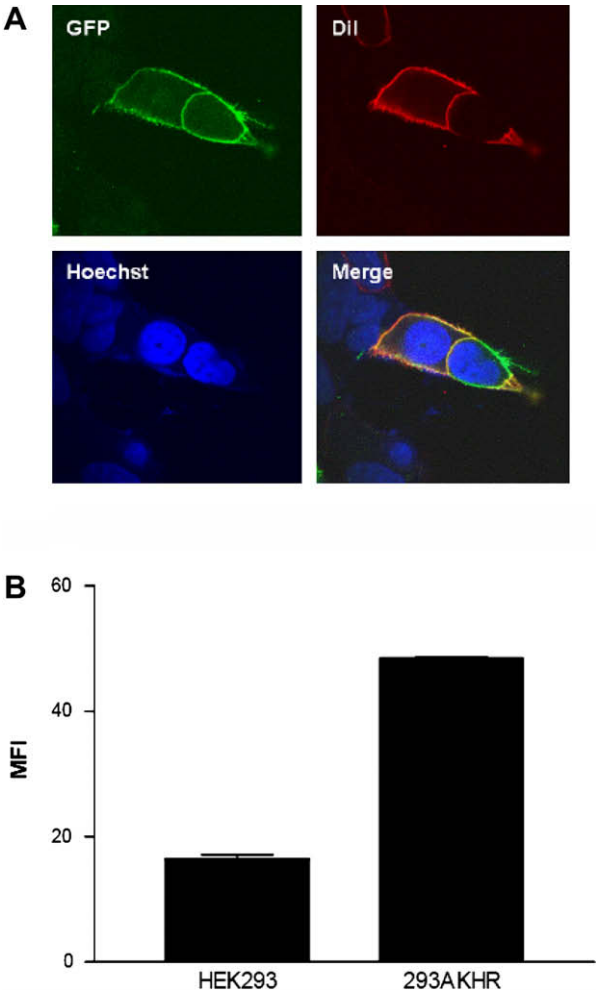


**Fig. 1.** Expression of AKHR in stably transfected HEK293 cells. (A) HEK293 cells stably expressing AKHR–EGFP (GFP) were stained with a membrane plasma probe (DiI) and a nuclei probe (Hoechst 33258). (B) The cell surface expression of the stably transfected HEK293 cells was analyzed by FACS. Stable 293 cells were analyzed for cell surface expression of Flag-AKHR by flow cytometry using the anti-Flag mAb M2. *Bars* represent the mean fluorescence intensity for cells expressing Flag-AKHR. All data are shown as means ± S.E. from at least three independent experiments.

### 3.2. cAMP accumulation and intracellular calcium mobilization in AKHR expressing cells stimulated by AKH peptides

cAMP accumulation depends upon the coupling of AKHR to G proteins. To evaluate the role of AKHR in stimulating cAMP production, a stable cell line co-transfected with Flag-AKHR and pCRE-Luc was established. Upon stimulation with different concentrations of AKH1 peptides, the cAMP inside of the cells accumulated in a dose-dependent manner with an $EC_{50}$ of 6.4 nM. As a control, no change in the cAMP level was detected in parental HEK293 cells (Fig. 2A and C). Pre-treatment with 100 ng/ml PTX was found to have no effect on cAMP generation in Flag-AKHR-expressing cells stimulated by AKH1, whereas stimulation with CTX led to a remarkable increase in the cellular levels of cAMP (Fig. 2B), suggesting that coupling of the Gs protein was involved in the AKHR signaling pathway in 293 cells.

*Bombyx* AKHR was further evaluated by an assay that is dependent upon ligand activation of the phospholipase C signaling pathway resulting in mobilization of intracellular $Ca^{2+}$ from the ER pool to the cytoplasm. We then examined the effects of AKH1 peptides on the intracellular $Ca^{2+}$ change in the AKHR-expressing cells using the calcium probe fura-2. As indicated in Fig. 2D, AKH1 peptides did not affect the $Ca^{2+}$ fluxes in the parental HEK293 cells, but, in parallel, elicited a rapid increase of $Ca^{2+}$ in the Flag-AKHR-expressing cells in a dose-dependent manner, as demonstrated by previous reports [15,19].

### 3.3. AKH1 mediates activation of MAPK pathway in AKHR-expressing cells

To investigate whether the activation of AKHR in stably transfected cells stimulates the phosphorylation of ERK1/2, the cells seeded in six-well plates were treated with AKH1 peptides, and assessed using a phospho-specific antibody known to bind only to the phosphorylated and activated forms of these kinases [20]. Fig. 3 shows that activation of AKHR elicited transient phosphorylation kinetics of ERK1/2 with maximal phosphorylation evident at 2–5 min and a return to almost basal levels by 15 min. By contrast, treatment with AKH1 did not provoke any appreciable effects on ERK1/2 in the parental 293 cells or transiently mock-transfected 293 cells. Fig. 3C illustrates the concentration dependence of AKH-mediated ERK1/2 phosphorylation and activation, with ERK1 and -2 phosphorylation increased significantly by nanomolar concentrations of AKH1 and a maximal ERK1/2 phosphorylation of at least three times the basal level.

### 3.4. Rapid internalization of AKH receptors upon activation by AKH1

In order to visualize the internalization and trafficking of AKHR, we constructed a vector to express a chimeric protein in which enhanced green fluorescent protein (EGFP) is fused to the C-terminal end of AKHR (AKHR–EGFP) and established a stable HEK293 cell line expressing AKHR–EGFP. Observation of stable AKHR–EGFP-expressing HEK293 cells with fluorescence microscopy revealed that the fluorescence of AKHR–EGFP was mainly localized in the plasma membrane, and to a lesser extent in intracellular vesicles.

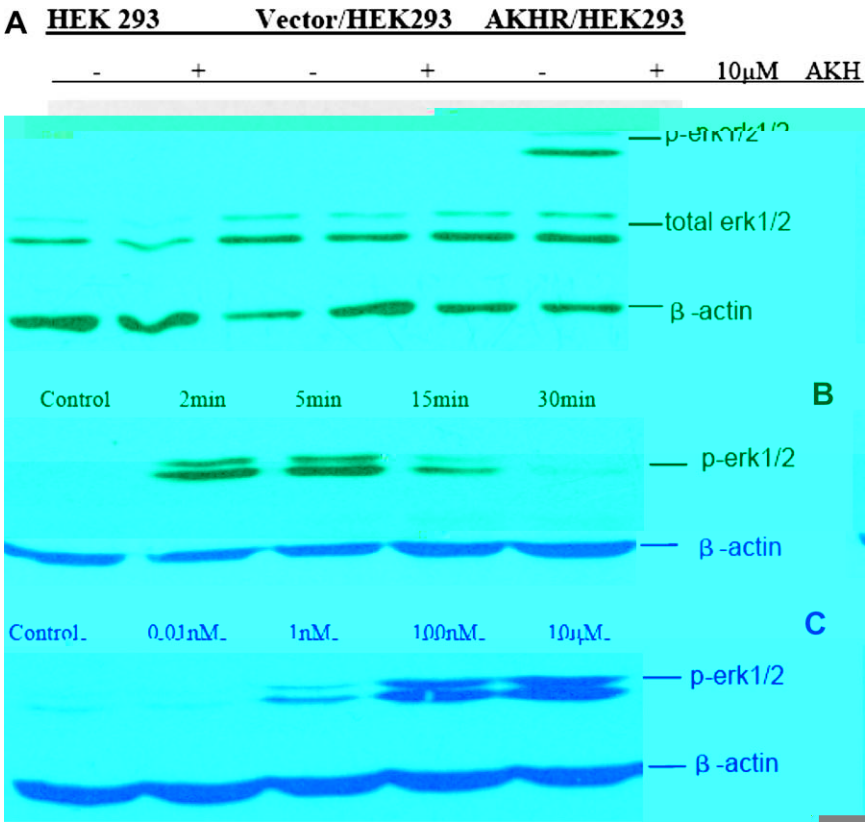


**Fig. 3.** Activation of ERK1/2 by AKH1. (A) AKH1 induce pERK1/2 only in transfected cells, not in controls of the experiment. (B) Time course of AKH-stimulated phosphorylation of ERK1/2 in stable AKHR-expressing HEK293 cells, cells were incubated with 10 μM AKH1 for the indicated times. (C) Concentration-dependent activation of ERK1/2 phosphorylation by AKH1 in HEK293 cells stably expressing Flag-AKHR. Cellular lysates were immunoblotted with phospho-specific (top lane) and non-specific (bottom lane) anti-ERK1/2 antibody, as described in Section 2. The results are representative of at least three independent experiments.

by fusion expression in different systems. In this study, an expression vector of *Bombyx* AKHR fused with EGFP at its C-terminus was constructed and expressed stably in HEK293 cells for easy visualization of receptor localization, internalization and trafficking. Compared to the wild-type AKHR, AKHR–EGFP was found to be expressed and function normally. We showed for the first time that, upon binding and activation by AKH peptide, AKHR was rapidly internalized in a dose- and time-dependent manner. Further investigation of receptor trafficking and recycling is under way in our lab.

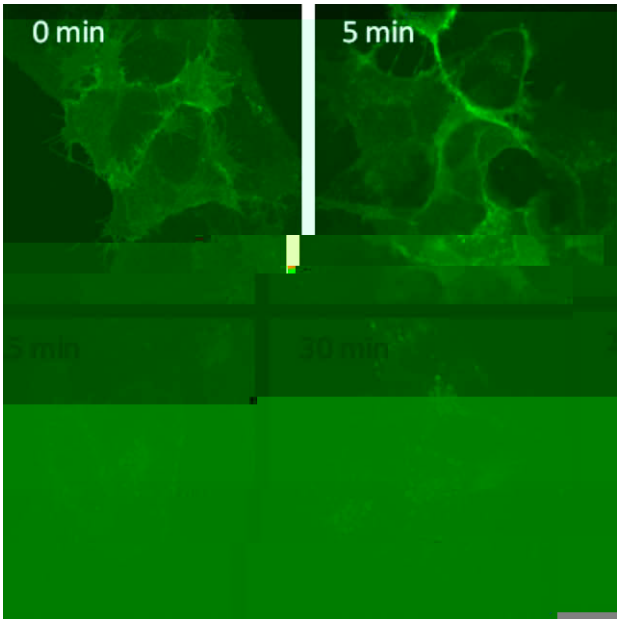


**Fig. 4.** Time course of AKHR–EGFP internalization induced by AKH1. Cells were incubated with 10 nM AKH at 37 °C for the indicated times, and after washing fixing, were examined by fluorescence microscopy as described in Section 2. The results are representative of three independent experiments.

In *Bombyx*, a non-apeptide AKH1 has been first identified [10], and quite recently Roller et al. identified another two distinct cDNAs encoding the prepro-*Bombyx* AKH2, and 3. *Bombyx* AKH1 is identical to non-apeptides found only in moths, while *Bombyx* AKH2 is closely related to many other AKH/HrTH decapeptides. Although previous studies indicated that the *Heliothis zea* hypertrehalosaemic hormone (Hez-HrTH) activated *Bombyx* AKHR with a higher affinity than that of AKH1 [12], in our research, we demonstrated that AKH2 exhibit comparable activities in intracellular cAMP accumulation to AKH1, but much lower activities in phosphorylation of ERK1/2 and receptor internalization than that of AKH1. In structure, *Bombyx* AKH2 is closely related to Hez-HrTH, but with three amino acids different, these three amino acids may be responsible for the differences of activation on *Bombyx* AKHR between *Bombyx* AKH2 and Hez-HrTH. *Bombyx* AKH3 is more closely related to non-lepidopteran AKH peptides, and was much less effective in activating *Bombyx* AKHR we tested in this study, strongly implying that it is more likely that a second intrinsic AKHR exists as a high affinity receptor for AKH3 in Bombyx. The identification of AKHR-mediated signaling pathways is of importance to obtain a better understanding of the role of AKH/AKHR in the regulation of the molecular events responsible for sugar homeostasis and energy mobilization.

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