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Bombyx Adipokinetic Hormone Receptor Activates Extracellular Signal-Regulated Kinase 1 and 2 via G Protein-Dependent PKA and PKC but β-Arrestin-Independent Pathways[†]

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ABSTRACT: Neuropeptides of the adipokinetic hormone (AKH) family are among the best studied hormone peptides. They play important roles in insect hemolymph sugar homeostasis, larval lipolysis, and storage-fat mobilization. Mechanistic investigations have shown that, upon AKH stimulation, adipokinetic hormone receptor (AKHR) couples to a Gs protein and enhances adenylate cyclase activity, leading to intracellular cAMP accumulation. However, the underlying molecular mechanism by which this signaling pathway connects to extracellular signal-regulated kinase 1/2 (ERK1/2) remains to be elucidated. Using HEK293 cells stably or transiently expressing AKHR, we demonstrated that activation of AKHR elicited transient phosphorylation of ERK1/2. Our investigation indicated that AKHR-mediated activation of ERK1/2 was significantly inhibited by H-89 (protein kinase A inhibitor), Go6983, and GF109203X (protein kinase C inhibitors) but not by U73122 (PLC inhibitor) or FIPI (PLD inhibitor). Moreover, AKHR-induced ERK1/2 phosphorylation was blocked by the calcium chelators EGTA and BAPTA-AM. Furthermore, ERK1/2 activation in both transiently and stably AKHR-expressing HEK293 cells was found to be sensitive to pretreatment of pertussis toxin, whereas AKHR-mediated ERK1/2 activation was insensitive to siRNA-induced knockdown of β-arrestins and to pretreatment of inhibitors of EGFR, Src, and PI3K. On the basis of our data, we propose that activated AKHR signals to ERK1/2 primarily via PKA- and calcium-involved PKC-dependent pathways. Our current study provides the first in-depth study defining the mechanisms of AKH-mediated ERK activation through the *Bombyx* AKHR.

Insects, the largest group of animals on earth, play very important roles in their ecosystems through plant pollination, nutrient recycling, and maintenance of plant community composition and structure. In addition, they provide us with many useful materials, such as honey, silk, and varnish. The importance of insects as biomedical models is evident by the fact that many discoveries in digestion, muscle contraction, and important metabolic and developmental pathways in insects are applicable to vertebrate systems (*1*). Adipokinetic hormones (AKHs)[1] 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 (*2*−*5*). The structure of AKH was first identified from the locusts *Locusta migratoria* and *Schistocerca gregaria* (*6*). 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 hemolymph of *Drosophila* and *Bombyx* larvae (*7*, *8*). Furthermore, it plays important roles in behavior, diapause, development, and reproduction (*9*, *10*).

The receptor of AKH was first identified as a typical G protein-coupled receptor from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* in 2002 (*11*) and later from the cockroach *Periplaneta americana* (*12*) and African malaria mosquito *Anopheles gambiae* (*13*). Previous biochemical characterization of isolated fat bodies suggested that AKH binds to its receptor and activates adenylyl cyclase via G proteins, 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 (*14*, *15*). However, the mechanistic details of AKHR signaling remain to be further elucidated. A *Drosophila* mutant with a knockout of AKHR demonstrated that the adipokinetic hormone signaling pathway is important for chronic accumulation and acute mobilization of storage fat, along with the Brummer lipase, the homologue of mammalian adipose triglyceride lipase (ATGL) (*16*). Determination of the detailed regulation of energy storage and mobilization in insects will lead to a better understanding of the dysfunction of body fat regulation that results in obesity and lipodystrophy in humans.

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[1]Abbreviations: GPCR, G protein-coupled receptor; AKH, adipokinetic hormone; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PTX, pertussis toxin; PI_3K, phosphoinositide 3-kinase; EGFR, epidermal growth factor receptor; PKA, protein kinase A; PKC, protein kinase C; MMP, matrix metalloproteinase.

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In our previous study, we demonstrated that AKH induces phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) through AKHR in a dose-dependent manner (*17*). However, the mechanistic details of AKHR signaling remain largely unknown. We have therefore in this current study investigated the mechanistic basis of AKH-mediated ERK1/2 activation using HEK293 cells stably and transiently expressing AKHRs. The results demonstrate the involvement of multiple signaling pathways in signaling of AKH receptors to ERK1/2. These findings provide a foundation for future studies of the physiological role of AKHR-mediated ERK1/2 activation in energy homeostasis and mobilization in diapause, development, and reproduction of *Bombyx*.

EXPERIMENTAL PROCEDURES

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). Fetal bovine serum was purchased from Hyclone (Beijing, China). The pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA), and Sigma (St. Louis, MO), respectively. The kinase inhibitors were purchased from Sigma (St. Louis, MO) and Calbiochem (Cambridge, MA). Pertussis toxin (PTX) was purchased from Tocris (Missouri, MA). Primary antibodies for Western blotting were purchased from Cell Signaling (Danvers, MA).

Cell Culture and Transfection. The human embryonic kidney cell line (HEK293) was maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 4 mM L-glutamine (Invitrogen). The AKHR cDNA plasmid constructs were transfected or cotransfected 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).

Molecular Cloning, Plasmid Construction, and Mutagenesis of Bombyx AKHR. Total RNA was isolated from the fat body of *B. mori* pupae using the TRIzol reagent (Keygen, Nanjing, China), according to 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. Corresponding PCR systems were performed as described previously (*17*). AKH receptor deletion mutants were constructed by using overlap extension PCR strategies. All of the constructs were constructed by ligation of the chimeric receptors or mutated receptors into the *Hin*dIII and *Xba*I or *Hin*dIII and *Kpn*I sites of the pCMV-Flag or pEGFP-N1 vectors. All constructs were sequenced to verify the sequence and orientation.

Synthesis of Small Interfering RNAs and siRNA Transfection. All small interfering RNAs (siRNAs) were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). Arrestin 2 and 3 siRNAs were purchased as a SMARTpool. The nonspecific control siRNA VIII (5′-AAACUCUAUCUGCACGCUGAC-3′) was used as the control for all siRNA experiments. The transfection protocol for arrestin siRNAs has been described previously(*18*). Forty-eight hours after transfection, cells were split for the indicated assay to take place the following day.

Immunoblot Analysis. The HEK293 cells stably or transiently expressing AKHR were seeded in six-well plates and starved in serum-free media overnight. After stimulation with AKH, cells were lysed with RIPA lysis buffer (Beyotime, Haimen, China) on ice for 30 min, and the solubilized cells were pipetted into 1.5 mL microcentrifuge tubes and centrifuged at 12500 rpm for 15 min. After denaturing at 95 °C for 10 min, the supernatants were removed and frozen at −80 °C. Equal amounts of total cell lysate were separated by Tris−glycine SDS−PAGE (12%) and transferred to a PVDF membrane (Millipore) using transfer buffer (192 mM glycine, 25 mM Tris base, 0.01% SDS, 20% methanol, and pH 8.3). Membranes were blocked in TBST (20 mM Tris, pH 8.0, 150 mM NaCl, 0.075% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature and then probed with rabbit monoclonal anti-p-ERK1/2 (1:2000; Cell Signaling) in TBST containing 5% bovine serum albumin (BSA) with agitation at 4 °C overnight. The primary antibody was removed, and the sample was washed three times for 15 min each with TBST. The membranes were then probed with anti-rabbit HRP-conjugated secondary antibody (1:3000; Chemicon) in TBST plus 5% nonfat dry milk for 1 h at room temperature. Total ERK1/2 was assessed as a loading control after p-ERK1/2 chemiluminescence detection.

Internalization Assay and Fluorescence Microscopy. For the internalization assay, HEK293 cells stably expressing AKHR−EGFP were seeded onto glass coverslips coated with 0.1 mg/mL poly-L-lysine and allowed to attach overnight under normal growth conditions. After treatment with AKH peptides at 37 °C for 60 min, cells were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. The cells were mounted in mounting reagent (DTT/PBS/glycerol, 1:8:2) and visualized by fluorescence microscopy 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.

For quantification of receptor internalization, ELISA was performed as described previously with some modifications (*19*). Briefly, 48 h after transfection, cells in 48-well plates were stimulated with agonist for 60 min, fixed with 3.7% formaldehyde for 10 min at room temperature, and blocked for 45 min with 1% bovine serum albumin in TBS (20 mM Tris, 150 mM NaCl, pH 7.5). Cells were then incubated for 1 h with a 1:5000 dilution of a mouse anti-Flag M2 monoclonal antibody. Next, cells were washed three times with TBS and blocked again in 1% BSA/TBS for 15 min followed by incubation with horseradish peroxidase- (HRP-) conjugated goat anti-mouse (1:5000 in 1% BSA/TBS) for 60 min. To each well was added 200 μL of HRP substrate (Sigma), and the samples were incubated at 37 °C for 20−30 min. Reactions were stopped by adding an equal volume of 1% SDS, and the sample absorbances were measured at 405 nm using a Bio-Rad microplate reader.

Flow Cytometry Analysis. Approximately 2×10^5 cells were washed with phosphate-buffered saline (PBS) supplemented with 0.5% BSA (FACS buffer) and incubated with 10 $\mu g \cdot mL^{-1}$ FITC-labeled anti-Flag M2 monoclonal antibody (Sigma) in a total volume of 100 μL. After incubation for 60 min at 4 °C, cells were pelleted and washed three times in FACS buffer. The cells were then fixed with 2% paraformaldehyde in FACS buffer and subjected to flow cytometry analysis on a FACScan flow cytometer (Coulter EPICS Elite; Coolten Corp., Hialeah, FL).

Peptide Synthesis. The AKH peptides were prepared by solid-phase synthesis using Fmoc chemistry on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perceptive Biosystems, Cambridge, MA). Crude peptides were purified by preparative reverse-phase high-performance liquid chromatography using a Dynamax-300 Å

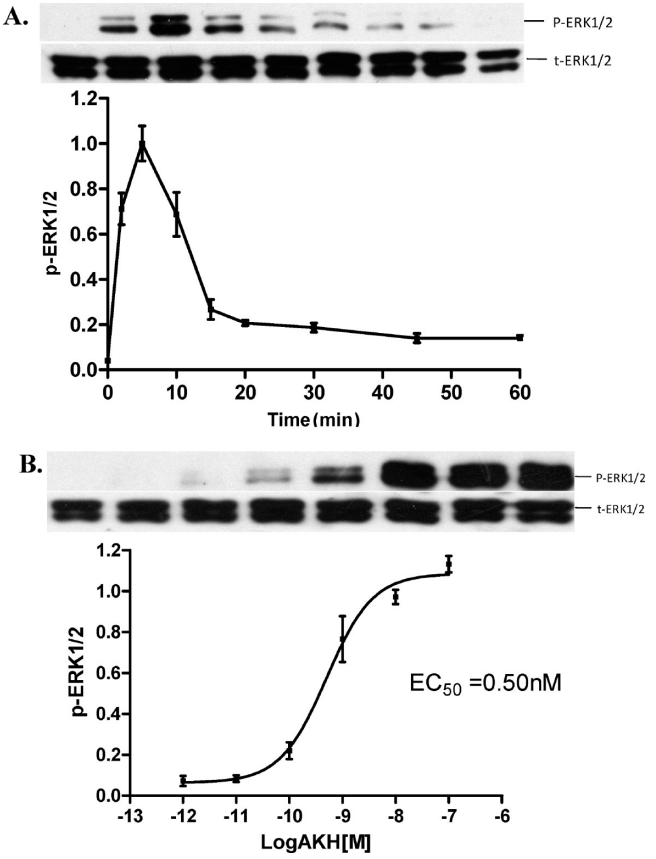

FIGURE 1: Kinetics of AKHR-induced ERK1/2 activation. (A) Time course of ERK1/2 activation following AKH stimulation in stable AKHR-expressing HEK293 cells. Cells were incubated with 100 nM AKH for the indicated time. (B) Dose-dependent curve of AKH-induced ERK1/2 activation. AKHR expressing cells were serum-starved for 1 h and then incubated for 5 min with different concentrations of AKH ranging from 0.001 to 100 nM. Cell lysates were immunoblotted with phospho-specific (top lane) and nonspecific (bottom lane) anti-ERK1/2 antibody, as described in the Experimental Procedures. P-ERK1/2 immunoreactivity was quantified by Quantity One software, and data are normalized to the maximal phospho-ERK1/2 response. Data are expressed as the mean ± SEM (n = 3).

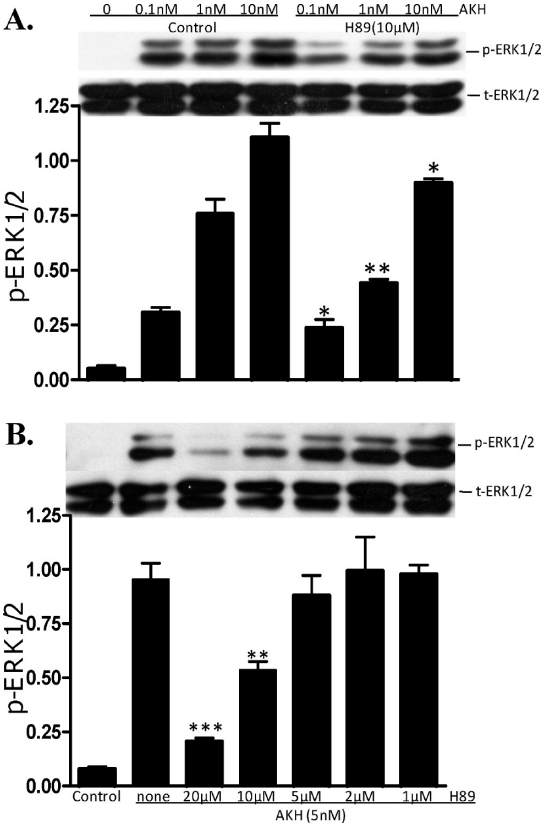

FIGURE 2: AKHR-mediated ERK1/2 phosphorylation is PKA-dependent. (A) Effect of PKA inhibitor on AKHR-induced ERK1/2 activation. HEK293 cells stably expressing AKHR were preincubated with PKA inhibitor (H89, 10 μM) or with vehicle (control) at 37 °C for 1 h followed by stimulation with AKH (0.1−10 nM) for 5 min. (B) Dose-dependent effects of PKA inhibitor on AKHR-mediated activation of ERK1/2. Stable cells were pretreated with H89 (1 μM to 20 μM) for 1 h and were then stimulated with AKH (5 nM, 5 min). Data are expressed as the mean ± SEM (n = 3). Statistical analysis was performed by a two-tailed Student's t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus counterpart control or none).

C18 25 cm × 21.4 mm i.d. column with a flow rate of 9 mL/min and two solvent systems of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. Fractions containing the appropriate peptide were pooled and lyophilized. The purity of the final product was assessed by analytical reverse-phase high-performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

RESULTS

AKHR Signals to ERK1/2 upon AKH Stimulation. To study AKH-stimulated ERK1/2 MAP kinase phosphorylation, HEK293 cells stably expressing the **Bombyx** AKH receptor were established using G418 selection. We previously reported that AKH stimulation led to a ligand concentration-dependent intracellular cAMP accumulation and Ca^{2+} mobilization, as well as an increase in ERK1/2 phosphorylation, with maximal activity at 5 min and a return to almost basal levels by 15 min. In this study, we quantitatively analyzed the ERK1/2 signaling pathway in AKHR-expressing HEK293 cells. As shown in Figure 1, AKH produces a dose-dependent increase in levels of ERK1/2 phosphorylation with an EC_{50} of 0.50 nM.

Involvement of cAMP/PKA in Activation of ERK1/2. Because cAMP plays an essential role in AKH-mediated biological function, its impact on ERK1/2 activation in HEK293 cells was investigated. AKHR couples to Gs protein and enhances adenylate cyclase activity, leading to intracellular cAMP accumulation. Increased cAMP levels lead to the activation of PKA, but whether PKA is involved in the activation of ERK1/2 is still unknown. To identify the role of PKA in AKHR-mediated ERK1/2 activation, HEK293 cells stably expressing Flag-AKHR were pretreated with or without PKA inhibitor H89 (10 μM) for 1 h and then incubated with varying concentrations of AKH (0.1−10 nM) for 5 min. The addition of H89 significantly decreased ERK1/2 phosphorylation (Figure 2A). To determine if H89-mediated inhibition of ERK1/2 activation occurs in a dose-responsive manner, AKHR expressing cells were preincubated with varying concentrations of H89 (1−20 μM) for 1 h prior to incubation with AKH (5 nM) for 5 min (Figure 2B). ERK1/2 phosphorylation was detected by Western blot. Inhibition of ERK1/2 is dependent on the concentration of H89, in agreement with Figure 2A. Collectively, these results indicate that AKHR-mediated activation of ERK1/2 is PKA dependent.

Ca^{2+}/PKC, but Not PLC or PLD, Is Required for Activation of ERK1/2. Our previous results showed that AKH

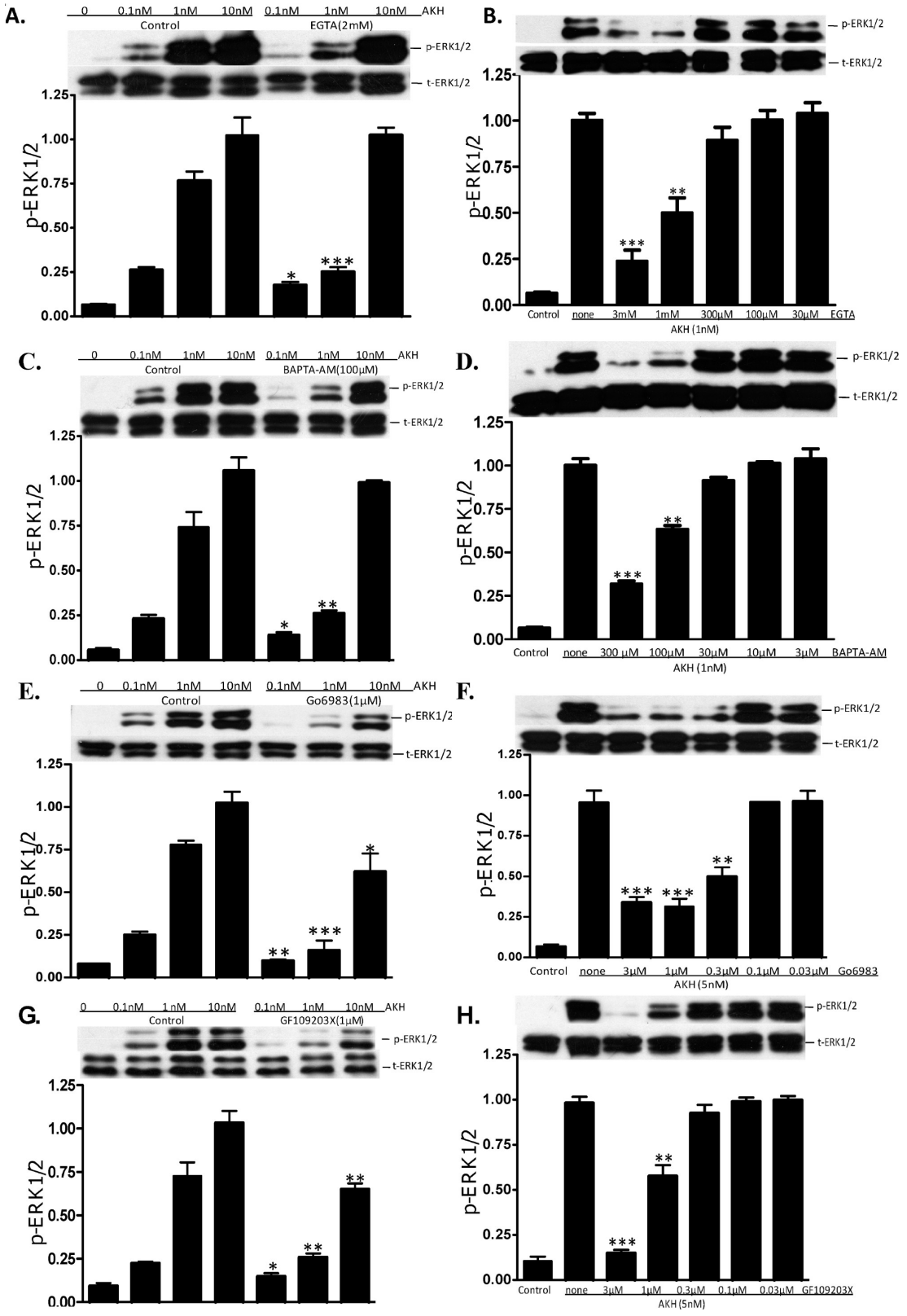

FIGURE 3: AKHR-mediated ERK1/2 phosphorylation is via a Ca^{2+}/PKC-dependent pathway. (A, B) Effects of an extracellular calcium chelator on AKHR-mediated ERK1/2 activation. Stable cells were serum-starved for 1 h and pretreated for 5 min with vehicle or the calcium chelator EGTA in serum-free medium. Cells were then stimulated with AKH for 5 min, the concentration as indicated in the figure. (C, D) Effects of an intracellular calcium sequestrant BAPTA-AM on AKHR-mediated ERK1/2 activation. Stable cells were serum-starved with BAPTA-AM for 30 min prior to stimulation with AKH for 5 min, the concentration as indicated in the figure. (E, G) HEK293 cells stably expressing AKHR were preincubated at 37 °C for 1 h with vehicle (0.1% DMSO, control) or with PKC inhibitor (Go6983, 1 μM; GF109203X, 1 μM) before stimulation for 5 min with AKH (0.1−10 nM). (F, H) Dose-dependent effects of PKC inhibitors on AKHR-mediated activation of ERK1/2. Stable cells were pretreated with Go6983 or GF109203X (0.03−3 μM) for 1 h and were then stimulated with AKH (5 nM, 5 min). The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis was performed by a two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus counterpart control or none).

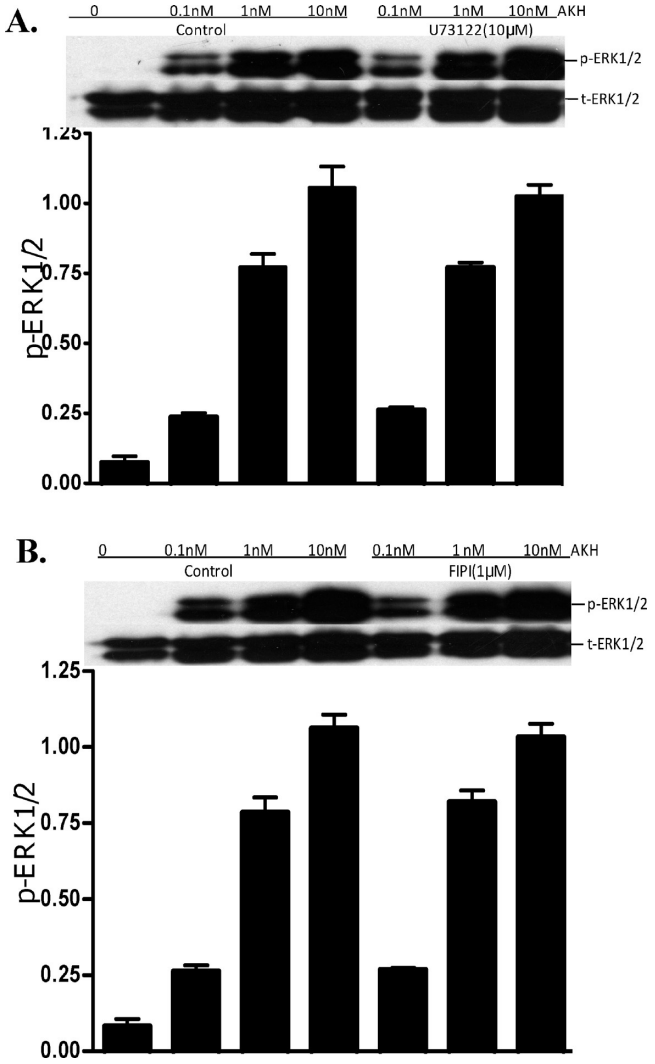

FIGURE 4: PLC and PLD are not involved in AKHR-mediated activation of ERK1/2. Stable cells were serum-starved with PLC inhibitor (U73122, 10 μM) (A) or with PLD inhibitor (FIPI, 1 μM) (B) for 1 h before stimulation with AKH (5 nM, 5 min). The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis was performed by a two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus counterpart control).

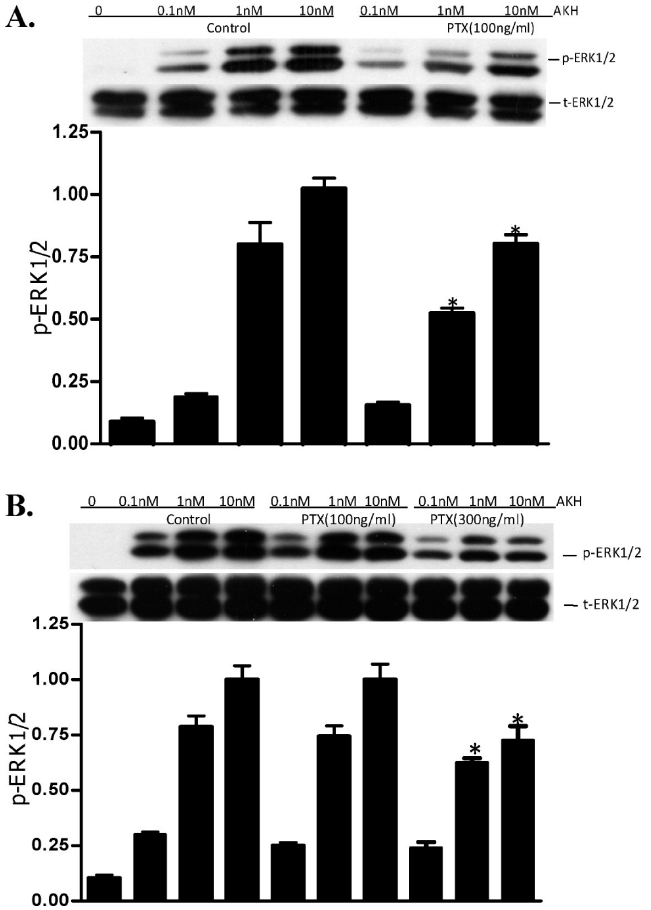

FIGURE 5: Pertussis toxin inhibits AKHR-stimulated ERK1/2 activation in transiently expressing HEK293 cells. HEK293 cells were transiently (A) or stably (B) transfected with AKHR and preincubated at 37 °C overnight with or without pertussis toxin (PTX, 100 ng/mL) before stimulation for 5 min with AKH at the indicated concentration. The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis was performed by a two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus counterpart control).

peptides did not affect Ca^{2+} fluxes in the parental HEK293 cells but elicited a rapid increase of Ca^{2+} in AKHR-expressing cells (*17*). We next determined whether or not the Ca^{2+}-dependent PKC contributed to AKH-mediated activation of ERK1/2. First, calcium chelators EGTA and BAPTA-AM were used to reduce the concentration of extracellular or intracellular calcium, respectively, as shown in Figure 3A−D; treatment of cells with EGTA and BAPTA-AM resulted in inhibition of AKH-stimulated ERK1/2 phosphorylation in a dose-dependent manner, suggesting that both intracellular and extracellular calcium are necessary for activation of ERK1/2. Next, PKC inhibitor Go6983 or GF109203X was added to cell cultures 60 min prior to stimulation with AKH. As illustrated in Figure 3E−H, inhibition of PKC did interfere with ERK1/2 phosphorylation after treatment of stably transfected cells with AKH. Addition of Go6983 or GF109203X led to a decrease in the activation of ERK1/2 stimulated by 5 nM AKH in a dose-dependent manner.

We also evaluated the effect of the upstream signaling molecules of PKC, PLC, and PLD in the AKHR-mediated ERK1/2 signaling pathway. The results showed that PLC inhibitors U73122 (10 μM) (Figure 4A and Supporting Information Figure S1) or ET-18-OCH_3 (100 μM) (Supporting Information Figure S1) and PLD inhibitor FIPI (1 μM) could not block the activation of ERK1/2 stimulated induced by 5 nM AKH (Figure 4B and Supporting Information Figure S1). Intriguingly, activated AKH receptors signal to ERK1/2 via PKC-dependent but PLC- and PLD-independent pathways, leading us to believe that calcium might play an important role in this process.

Activation of ERK1/2 in AKHR-Expressing HEK293 Cells Is PTX-Sensitive. To explore the role of G_i proteins in the AKH-stimulated ERK1/2 signaling pathway, we first examined the effect of pertussis toxin pretreatment on AKH-stimulated ERK1/2 activation in transiently expressing HEK293 cells. Cells were preincubated at 37 °C overnight with or without pertussis toxin (PTX, 100 ng/mL) before stimulation for 5 min with different concentrations (0.1−10 nM) of AKH, as shown in Figure 5A. PTX (100 ng/mL) can decrease ERK1/2 activation significantly. However, the partial but significant impairment of the AKH-stimulated ERK1/2 activation by treatment of higher concentration of PTX (300 ng/mL) was observed in stably transfected HEK293 cells (Figure 5B). To investigate the differences between transiently and stably expressed AKHR cells, we,

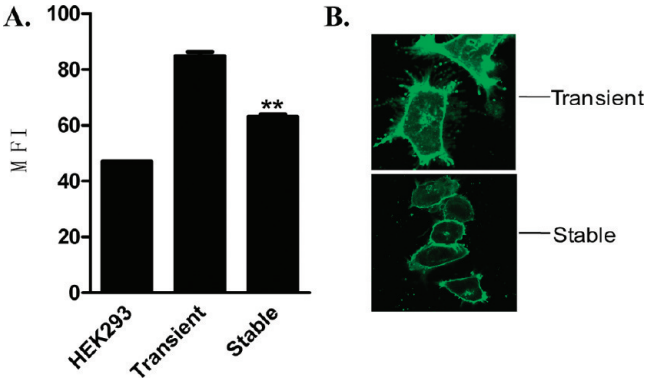

FIGURE 6: Expression of AKHR in transiently or stably transfected HEK293 cells. (A) The cell surface expression of the transiently or stably transfected HEK293 cells was analyzed by FACS. HEK293 cells were analyzed for cell surface expression of Flag-AKHR by flow cytometry using the anti-Flag mAb M2. Bars represent the mean fluorescence intensity for cells expressing Flag-AKHR. All data are shown as means ± SE from at least three independent experiments. (B) HEK293 cells transiently or stably expressing AKHR−EGFP (GFP) were detected by the confocal microscopy at the same conditions. The pictures shown are representative of most expressed cells.

then, quantitatively detected the cell surface expression of AKHR in stable and transient expressing HEK293 cells, as shown in Figure 6; the expression level of AKHR in the transient cells is significantly higher than that in the stable cells. Combined with our observation that PTX pretreatment resulted in a significant increase in AKH-induced cAMP accumulation in both transiently and stably AKHR-expressing cells (Supporting Information Figure S3), these results suggest that AKHRs are very likely to couple to G_i pathway in HEK293 cells, although AKHR is identified as a Gs-coupled receptor.

Arrestins and Other Factors Are Not Involved in Phosphorylation of ERK1/2. It is well established that GPCRs activate the ERK1/2 cascade via multiple mechanisms such as the β-arrestin-dependent pathway and the EGFR transactivation pathway (*20*). We therefore investigated whether or not additional pathways are involved in AKH-mediated ERK1/2 activation. Previous studies have shown that arrestins are involved in the ERK1/2 pathway mediated by some GPCRs(*21*). To examine whether the arrestins were required for the transient activation of ERK1/2 mediated by AKHR, we used siRNA to knock down the expression of arrestins. The siRNAs were transfected into HEK293 cells, and the cells were evaluated for arrestin expression after 3 days. The endogenous expression of arrestin was effectively and specifically knocked down by the specific siRNA treatment but was unaffected in cells treated with nonspecific or control siRNA (Figure 7A). As shown in Figure 7C, silencing of arrestin 2 or arrestin 3 had no effect on AKHR-mediated ERK1/2 activation, although knockdown of arrestin 3 significantly blocked AKH-triggered internalization (Figure 7B). To further examine the role of arrestins in AKHR-activated ERK1/2 phosphorylation, we constructed an AKHR mutant with a deletion in the C-terminal domain from residue 363 to residue 343 (AKHRΔ343−363), which is defective in AKH-induced internalization. HEK293 cells expressing wild-type AKHR or AKHRΔ343−363 mutant were treated with 100 nM AKH, as indicated in Figure 8A; AKHRΔ343−363 mutant exhibited significant impairment of internalization (Figure 8B) but showed similar activation of ERK1/2 compared to wild-type AKHR (Figure 8C). These results suggest that AKHR signals to the ERK1/2 pathway via arrestins and an internalization-independent pathway.

Previous studies have demonstrated that the EGFR tyrosine kinase, the nonreceptor RTK Src, and phosphatidylinositol 3-kinase (PI3K) are also involved in GPCR-mediated ERK1/2 activation (*20*). To assess the role of EGFR transactivation, Src and PI3K in the AKHR-induced ERK1/2 signaling pathway, MMP inhibitor (GM6001, 5 μM), EGFR inhibitor (AG1478, 1 μM), PI3K inhibitor (Wortmanmin, 1 μM), and Src inhibitor (PP2, 10 μM) were used to pretreat cells. As shown in Figure 9, treatment of cells with inhibitors U0126, H89, and Go6983 resulted in a significant decrease of AKH-induced phosphorylation of ERK1/2, whereas inhibitors GM6001, AG1478, Wortmanmin, and PP2 showed no effect on ERK1/2 activation in response to AKH (Figure 9 and Supporting Information Figure S2). These results suggest that it is unlikely for EGFR, PI_3K, MMP, and Src to be involved in AKHR-mediated phosphorylation of ERK1/2.

DISCUSSION

Since the characterization of the first AKH peptide from the locusts *S. gregaria* and *L. migratoria*, more than 30 peptide members have been isolated from various species of insects. The essential functions of AKH peptides are analogous to vertebrate glucagons (*22*). In addition to its role in hyperglycemia and hyperlipidemia, AKH has a diverse array of functions including inhibition of protein synthesis (*23*), activation of glycogen phosphorylase (*24*, *25*), and inhibition of lipid synthesis (*26*). Recent research has emphasized the value of insects as a powerful model system for the study of human lipometabolic disorders (*27*). The AKH receptor has been identified to couple to Gs protein, resulting in intracellular cAMP accumulation upon agonist stimulation. In our previous study, we have demonstrated that AKH agonist triggers phosphorylation of ERK1/2 through the AKH receptor. However, the underlying molecular mechanism of its signaling to extracellular signal-regulated kinase1/2 (ERK1/2) remains to be elucidated.

Mitogen-activated protein kinase (MAPK) pathways regulate diverse processes ranging from proliferation and differentiation to apoptosis. Although it is well established that GPCRs play important roles in the regulation of intermediary metabolism, they have only recently been recognized as important mediators of cellular growth and differentiation via the MAPK pathway (*20*). It is now known that many GPCRs regulate MAPK cascades via distinct G protein, β-arrestin-dependent, and EGFR transactivation signaling pathways, leading to activation of the extracellular signal-regulated kinases (ERKs), which function as transcriptional regulators. Therefore, characterization of the signaling pathways that stimulate MAPK activation through a particular receptor is essential to understand its role in physiology and pathology. In this study, the HEK293 cell line was selected as a model system for assessing signaling pathways involved in AKHR-mediated ERK1/2 activation because it is commonly used to characterize GPCR coupling to various signaling pathways. Using HEK293 cells either stably or transiently expressing AKHR combined with the addition of different kinase inhibitors, we demonstrated that, upon stimulation with AKH peptides, activated AKH receptors signal to ERK1/2 via PKA- and PKC-dependent, but β-arrestin-independent signaling pathways (Figure 10).

Although the first observation of Gs protein-dependent effects of β_2ARs on ERK1/2 has shown that cAMP-mediated inhibition

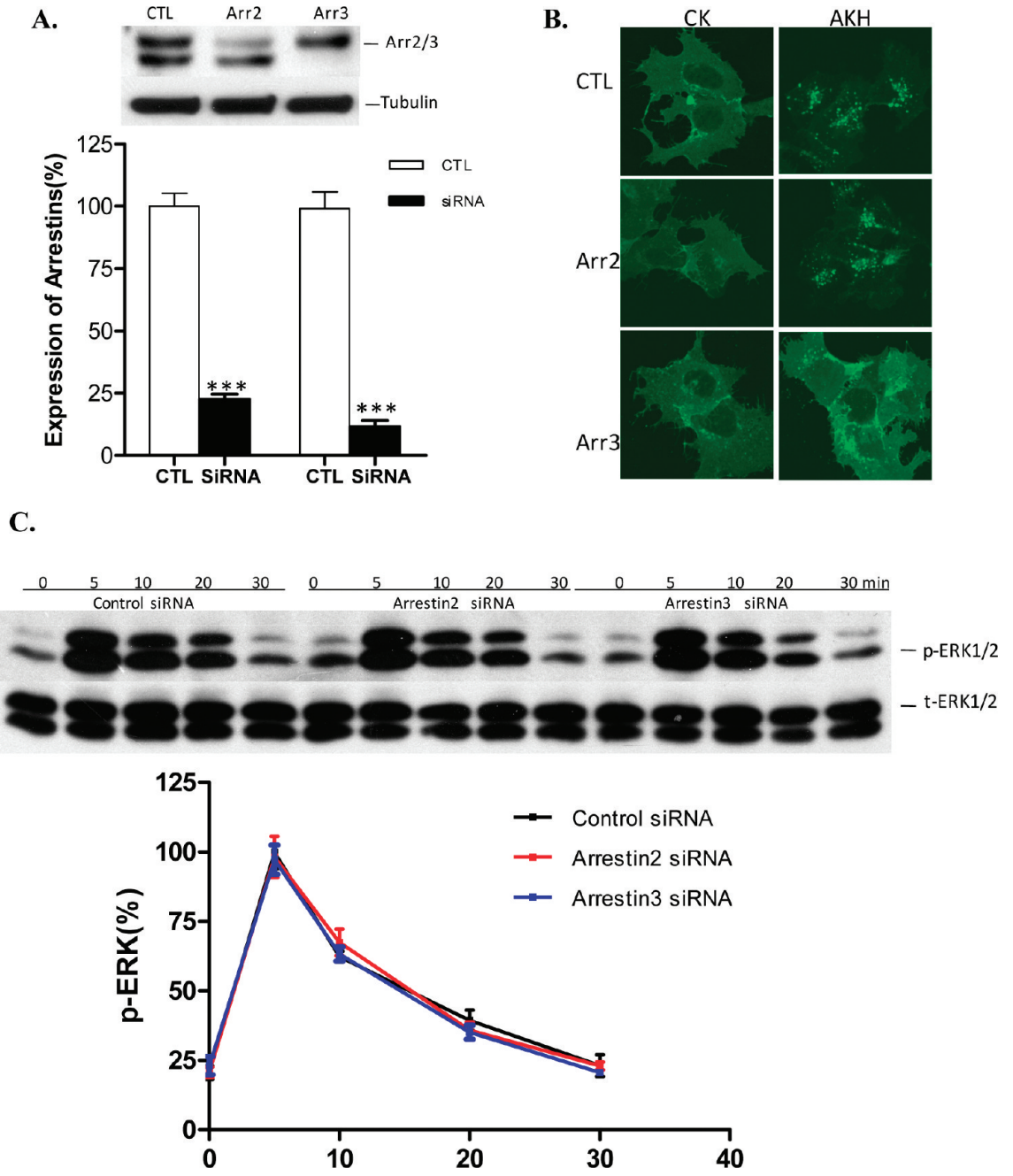

FIGURE 7: Activated AKHR signals to ERK1/2 via arrestin-independent pathways. (A) Knockdown of arrestins by siRNA. HEK293 cells stably expressing AKHR were transfected with specific arrestin siRNAs or nonspecific control siRNA. Seventy-two hours after transfection, cells were harvested, and equal amounts of total cell lysate were separated by 12% SDS−PAGE, transferred to nitrocellulose, and incubated with the indicated antibodies. Blots were stripped and reprobed for tubulin to control for loading. Shown is a representative immunoblot from five independent experiments. (B) Seventy-two hours after transfection with specific arrestin siRNAs or nonspecific control siRNA, cells stably expressed AKHR−EGFP were stimulated with 1 μM AKH for 60 min and examined with fluorescence microscopy. The pictures shown are representative of three independent experiments. (C) Effect of siRNA-induced knockdown of arrestin expression on AKHR-mediated ERK1/2 activation. Activation of ERK1/2 in HEK293 cells transfected with siRNA for 3 days followed by incubation with 1 nM AKH for different time was assessed by Western blot as described in the Experimental Procedures. Signals were quantified by densitometry and expressed as percentage of phosphorylated ERK1/2 of control obtained at 5 min. Graphs represent mean ± SEM from at least four independent experiments.

of ERK1/2 is due to the Gs-dependent activation of PKA, which leads to phosphorylation and inhibition of c-Raf1 (*28*, *29*), activation of ERK1/2 via a Gs-dependent pathway by the stimulation of the β_2AR has been demonstrated in HEK293 cells (*30*) and S49 lymphoma cells (*31*). The Gs-coupled hormone receptors β_3-adrenergic receptor (*32*), glucagon receptor (*33*), follicle-stimulating hormone receptor (*34*), and parathyroid hormone receptor (*35*) were found to mediate ERK1/2 phosphorylation via a Gs-dependent cAMP−PKA pathway. Furthermore, the dominant pathway for β_2AR activation of ERK1/2 in HEK293 cells has been shown to require activation of a Src family member that may be downstream of PKA (*36*). However, in this current study, we did not observe an inhibitory effect of Src inhibitor PP2 on AKHR-mediated ERK1/2 activation, thereby suggesting that Src is unlikely involved in AKH-mediated activation of ERK1/2. Most likely, AKHR activation evokes phosphorylation of ERK1/2 via a Ras-related small G protein, Rap1, and the downstream kinase, B-Raf (*37*).

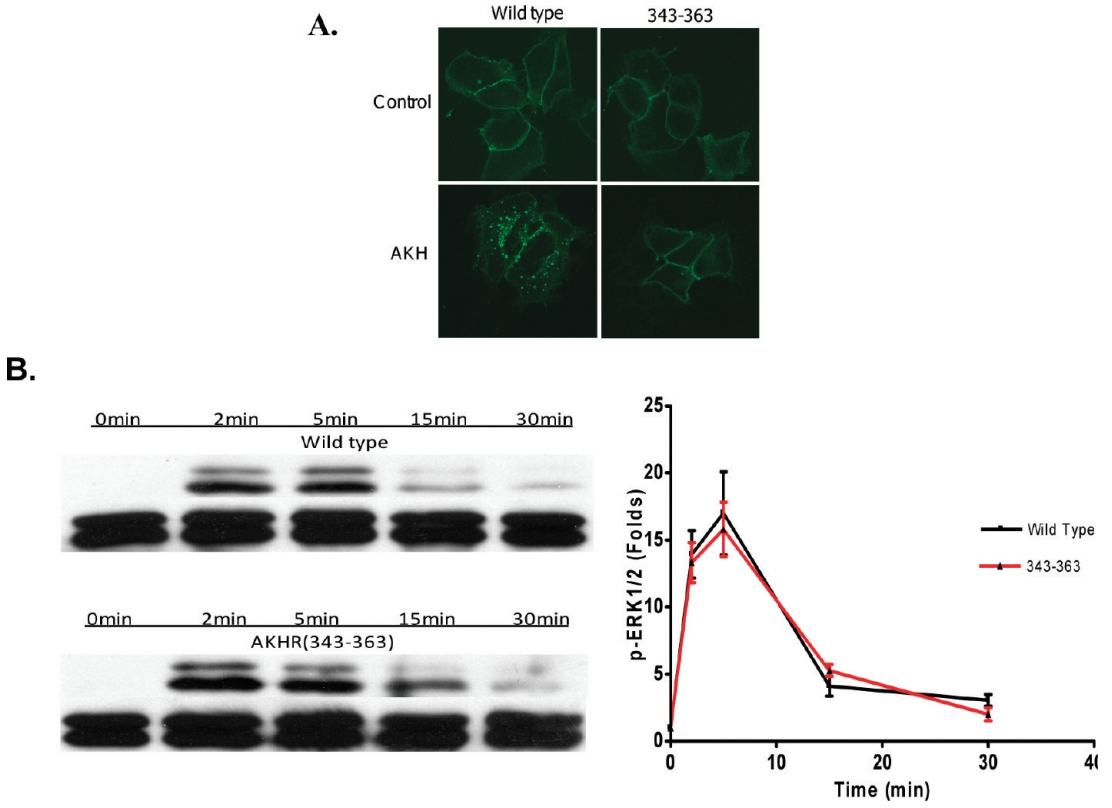

FIGURE 8: Role of receptor internalization in AKHR-mediated ERK1/2 activation in HEK293 cells. (A) Cells were transiently transfected with wild-type AKHR or AKHR Δ343−363 mutant and, 48 h after transfection, incubated with 100 nM AKH at 37 °C for 30 min. After washing and fixing, cells were examined by confocal microscopy as described in the Experimental Procedures. (B) HEK293 cells were transiently transfected with AKHR or AKHR Δ343−363. After 48 h, cells were transferred to serum-free media, incubated for 1 h, and subsequently treated with AKH (100 nM) for the indicated time. The data are expressed as fold increase over control p-ERK1/2 levels. The data shown are representative of three independent experiments (mean ± SEM).

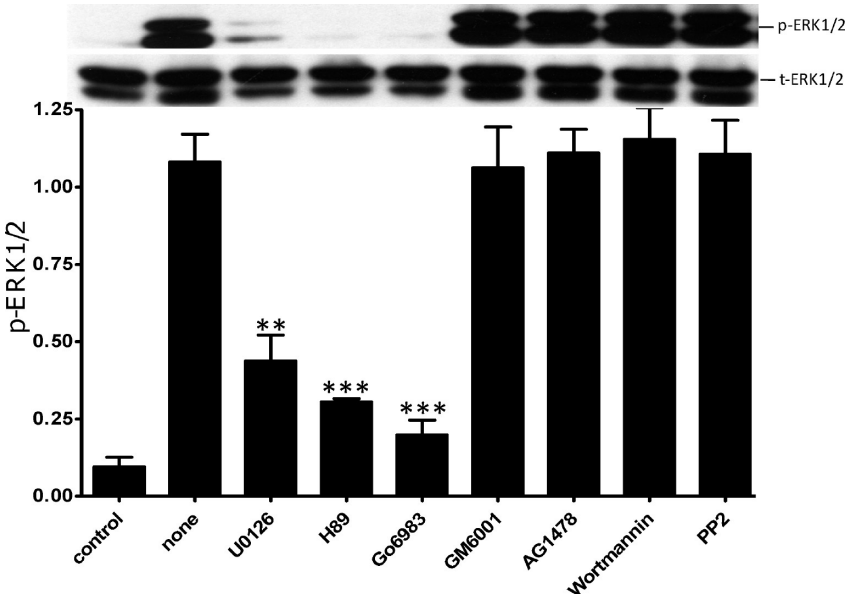

FIGURE 9: Effect of pretreatment of EGFR, Src, and PI3K inhibitors on AKHR-mediated ERK1/2 activation. Serum-starved HEK293 cells stably expressing AKHR were pretreated or not with the indicated inhibitors at 37 °C prior to stimulation with agonist. Cells were pretreated for 1 h with MEK inhibitor (U0126, 100 nM), PKA inhibitor (H89, 20 μM), PKC inhibitor (Go6983, 3 μM), MMP inhibitor (GM6001, 5 μM), EGFR tyrosine kinase inhibitor (AG1478, 1 μM), PI3K inhibitor (wortmannin, 1 μM), and Src-family tyrosine kinase inhibitor (PP2, 10 μM) and then exposed to AKH (5 nM) for 5 min. The data shown are representative of three independent experiments (mean ± SEM). Statistical analysis was performed by a two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus none).

Agonist-mediated AKHR activation elicits a rapid increase of intracellular cAMP and Ca^{2+} (*17*). The origin of the AKH-mediated increase in Ca^{2+} has been discussed in detail (*38*). AKH stimulation causes an entry of Ca^{2+} via channels into the fat body of *L. migratoria* (*39*, *40*). In addition, inhibitors of Ca^{2+} channels, verapamil and nifedipine, effectively impair the adipokinetic

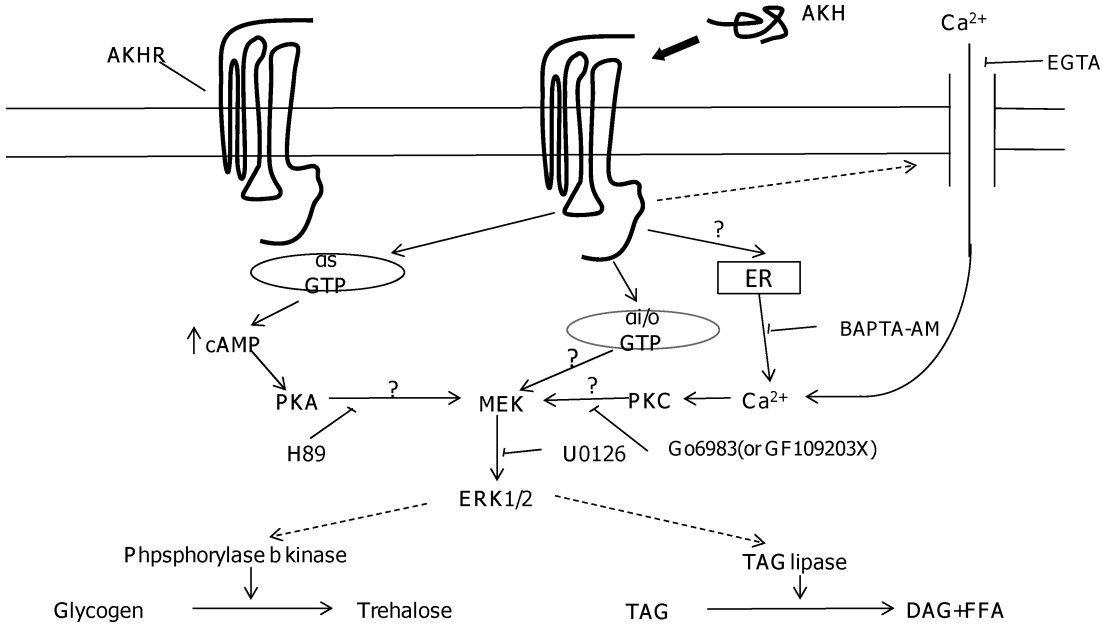

FIGURE 10: Schematic diagram of regulation of agonist-induced AKHR ERK1/2 activation in HEK293 cells. AKH binding to AKHR activates the Gs family of heterotrimeric G proteins, leading to the dissociation of G protein subunits. Gαs can enhance adenylate cyclase activity, leading to intracellular cAMP accumulation, and then cAMP will increase the activity of PKA; activated receptor may contribute to open the calcium channel in the membrane, results in increased intracellular concentration of calcium, which led to activate PKC; Gi protein inhibitor PTX partially decreased activation of the ERK1/2 mediated by AKHR. These pathways will contribute to ERK1/2 activation, but whether ERK1/2 is involved in AKHR-mediated fat lipolysis and how the ERK1/2 regulated the lipid metabolism need to be further studied.

effect of AKH in *Manduca sexta*, in vivo (*41*). The influx of extracellular Ca^{2+} plays an essential role in lipid release from fat bodies of locusts and in hyperprolinemia signaling in the fruit beetle (*42*, *43*) and exhibits a direct, positive effect on lipid mobilization in moths (*41*). These previous observations are consistent with the results presented in this report. The AKH-mediated ERK1/2 activation significantly decreased upon treatment of Go6983, a PKC inhibitor, but not with PLC inhibitor U73122 or PLD inhibitor FIPI, suggesting that PKC is involved in activation of ERK1/2 via a PLC- and PLD-independent pathway. Further investigation demonstrated that AKHR-induced ERK1/2 phosphorylation was blocked by the calcium chelators, EGTA and BAPTA-AM. Taken together, our data suggest that the Ca^{2+} influx is likely to play an important role in ERK1/2 activation.

Our previous study has demonstrated that the CB_1 receptor is capable of dually coupling to G_s-mediated cAMP accumulation and G_i-induced activation of ERK1/2 and Ca^{2+} mobilization multiple cell lines including HEK293, CHO, COS-7, and NIH3T3 cells (*44*). In the current study, the activation of ERK1/2 by AKH stimulation is PTX-sensitive (Figure 5B), and PTX pretreatment resulted in a significant increase in AKH-induced cAMP accumulation in both transiently and stably AKHR-expressing HEK293 cells (Supporting Information Figure S3), suggesting that AKHRs are very likely to dually couple to G_s and G_i pathways in HEK293 cells. Previous studies showed that several receptors such as the angiotensin II AT_1 receptor (*45*), the muscarinic acetylcholine M_4 receptor (*46*), the dog thyrotropin receptor (*47*), the prostaglandin EP_3D receptor (*48*), and the human α_2-adrenoceptor (*49*) can dually couple to G_s and G_i, although the physiological significance of a dual G_s and G_i response to CB_1 activation remains to be examined. It has been proposed that the density of receptors plays a role in governing the receptor−G protein coupling. In recombinant systems, promiscuous interaction of receptors with G proteins was observed, when receptor density increased (*49*). This is in a good agreement with our observation that the expression level of AKHR in the transient cells is significantly higher than that in the stable cells. Additional experiments are necessary to further clarify whether the AKHRs in endogenous condition dually couple to G_s and G_i pathways (Figure 10).

There is a growing body of evidence that indicates that there are two mechanisms with specialized roles in the activation ERK1/2: EGF receptor transactivation and activation of ERK bound to β-arrestin scaffolds (*20*). EGFR transactivation can mediate GPCR-induced ERK1/2 activation and therefore contribute to coupling between GPCRs and cell cycle progression (*50*, *51*). Previous studies have demonstrated that GPCR-induced EGFR transactivation is mediated by the release of precursor forms of the EGFR ligands HB-EGF, generated by activation of metalloproteinases of the zinc-dependent a disintegrin and metalloproteinase (ADAM) family (*52*, *53*). However, in HEK293 cells stably expressing *Bombyx* AKHR, AKH peptide-mediated ERK1/2 activation was not blocked by EGFR or MMP inhibitors. Moreover, we used RNA interference and an internalization-defective AKHR mutant to show that β-arrestins are not involved in AKH-induced ERK1/2 activation. It is well established that GPCRs employ multiple mechanisms to activate the ERK1/2 cascade. Depending on the receptor and cell type, one mechanism may predominate, or multiple mechanisms may be activated simultaneously (*54*). It seems likely that the signaling mechanism underlying AKHR-mediated ERK1/2 activation predominantly results from PKA- and PKC-dependent pathways.

Activated Gs-coupled hormone receptors are known to stimulate intracellular cAMP accumulation, leading to the activation of a cAMP-dependent protein kinase, PKA. Hormone-sensitive lipase (HSL) and perilipin are phosphorylated by PKA, resulting in catalysis in the breakdown of triglycerides and diglycerides (*55*, *56*). Experiments have revealed that, *in vitro*, activated ERK1/2 causes HSL phosphorylation at Ser600 (*57*), producing a hydrolytic activity similar to that observed when

HSL is phosphorylated by PKA (*58*). Recently, β_3AR-stimulated lipolysis was found to require activation of PKA as well as the ERK1/2 pathway (*59*). Moreover, magnolol-induced lipolysis was inhibited by PD98059, an inhibitor of mitogen-activated protein kinase kinase (MEK) (*60*). The details of the hormonal regulation of the lipolytic process that takes place in the insect fat body remain to be elucidated. In our preliminary experiment, we demonstrated that treatment of MEK inhibitor U0126 resulted in a blockade of lipolysis in the fat body of *Bombyx* (data not shown). Further experiments are required to dissect the role of AKH-activated ERK1/2 in mediating various physiological events (Figure 10). Therefore, the present findings will help to clarify the role of AKHR-mediated ERK1/2 activation in the regulation of the molecular events responsible for energy homeostasis and mobilization in insects and will also be helpful to better understand the dysfunction of body fat regulation that results in obesity and lipodystrophy in humans.

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SUPPORTING INFORMATION AVAILABLE

Supporting results, experimental procedures, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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