Orexin-A Activates Hypothalamic AMP-Activated Protein Kinase Signaling through a Ca$^{2+}$-Dependent Mechanism Involving Voltage-Gated L-Type Calcium Channel

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ABSTRACT

Hypothalamic AMP-activated protein kinase (AMPK) and orexins/ hypocretins are both involved in the control of feeding behavior, but little is known about the interaction between these two signaling systems. Here, we demonstrated that orexin-A elicited significant activation of AMPK in the arcuate nucleus (ARC) of the hypothalamus by elevating cytosolic free Ca$^{2+}$ involving extracellular calcium influx. Electrophysiological results revealed that orexin-A increased the L-type calcium current via the orexin receptor–phospholipase C–protein kinase C signaling pathway in ARC neurons that produce neuropeptide Y, an important downstream effector of orexin-A’s orexigenic effect. Furthermore, the L-type calcium channel inhibitor nifedipine attenuated orexin-A-induced AMPK activation in vitro and in vivo. We found that inhibition of AMPK by either compound C [6-[2-(1-piperidinyl)ethoxy]phenyl]-3-(4-pyridyl)-pyrazolo[1,5-a]pyrimidine) or the ATP-mimetic 9-$\beta$-o-arabinofuranoside prevented the appetite-stimulating effect of orexin-A. This action can be mimicked by nifedipine, the blocker of the L-type calcium channel. Our results indicated that orexin-A activates hypothalamic AMPK signaling through a Ca$^{2+}$-dependent mechanism involving the voltage-gated L-type calcium channel, which may serve as a potential target for regulating feeding behavior.

Introduction

Hypothalamic neuronal circuits that respond to hunger or satiety signals control the drive to eat (Horvath and Diano, 2004; Pinto et al., 2004). To regulate feeding behavior, orexigenic peptides such as neuropeptide Y (NPY), agouti-related protein, $\alpha$-melanocyte–stimulating hormone, and orexins are secreted by neurons from different areas of the hypothalamus. Orexin-A, a neuropeptide with a stimulatory effect on feeding via the selective orexin receptor-1 receptor (OX1R) (Sakurai et al., 1998; Edwards et al., 1999; Haynes et al., 2000; Yamada et al., 2000), is mainly secreted by the neurons in the lateral hypothalamic area that project far and wide in the hypothalamus, including the arcuate nucleus (ARC) (de Lecea et al., 1998; Date et al., 1999). It is well documented that orexin-A plays an important role in both normal energy homeostasis and abnormal control of eating behavior such as anorexia nervosa (Baranowska et al., 2008; Bronsky et al., 2011). NPY-containing neurons in the ARC, which have a strong orexigenic effect on feeding, have been shown to express orexin receptor-1 receptor (OX1R), a neuropeptide with a stimulatory effect on feeding via the selective orexin receptor-1 receptor (OX1R) (Dube et al., 2000; Ida et al., 2000; Jain et al., 2000; Baranowska et al., 2008; Bronsky et al., 2011). This work was supported by grants from the National Natural Science Foundation of China (NSFC; Grant 81222040) to W.-N.W.; the National Basic Research Program of China (973 Program, Grant 2013CB531303); the Key Project of the NSFC (Grant 30993014); and the International Science & Technology Cooperation Program of China (Grant 2011DFA32670) to J.-G.C.

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ABBREVIATIONS: ACF, artificial cerebrospinal fluid; AMPK, AMP-activated protein kinase; ara-A, 9-$\beta$-o-arabinofuranoside; ARC, arcuate nucleus; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-$\text{N,N,N',N'}$-tetraacetic acid tetrasodium acetoxymethyl ester; BayK8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[[2-fluoromethyl]phenyl]pyridine-3-carboxylic acid methyl ester; [Ca$^{2+}$]i, cytosolic free Ca$^{2+}$; CaMKKs, calmodulin-dependent protein kinase kinases; compound C, 6-[4-[2-(1-piperidinyl)ethoxy]phenyl]-3-[4-(pyridyl)-pyrazolo[1,5-a]pyrimidine; DMSO, dimethylsulfoxide; GF109203X, 3-[N-(dimethylamino)propyl]-3-indolyl]-4-[(3-indolyl)maleimide 3-[1-[3-(dimethylamino)propyl]1H-indol-3-yl]-4-[1H-indol-3-yl]1H-pyrrrole-2,5-dione bisindolylmaleimide I; L$\text{H}_{\text{VWA}}$, high-voltage activated calcium current; LKB1, liver kinase B1; NPY, neuropeptide Y; OX1R, orexin receptor-1 receptor; PBS, phosphate-buffered saline; PKC, protein kinase C; PLC, phospholipase C; SB334867, 1521-0111/84/6/876

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Yamanaka et al., 2000). However, the precise molecular mechanism that links OXIR with NPYergic function still remains largely unknown.

Recently, it has been revealed that hypothalamic AMP-activated protein kinase (AMPK) is a key sensor of energy status that underlies the orexigenic effects of many hormones. For example, adiponectin increases food intake by activating AMPK in the arcuate hypothalamus (Kubota et al., 2007; Wen et al., 2010), and ghrelin promotes feeding through a mechanism involving the short-term activation of hypothalamic AMPK (Lopez et al., 2008). Accumulated evidence indicates that AMPK in NPY neurons senses hunger signals and shifts energy homeostasis toward increased food intake (Murphy et al., 2009; Kohno et al., 2011). For instance, decreased leptin and glucose after fasting activated AMPK in glucose-inhibited NPY neurons and increased neuron excitation, which led to NPY release and food intake (Murphy et al., 2009). A novel finding has revealed that a hunger signal results in a persistent upregulation of excitatory synaptic input to NPY neurons, then increases their firing rate via an AMPK-dependent positive feedback loop (Yang et al., 2011). Therefore, AMPK signaling in NPY neurons may be a common pathway that integrates orexigenic cues to control feeding behavior. AMPK can be indirectly activated by cytosolic free Ca$^{2+}$ (Ca$^{2+}_{i}$) via Ca$^{2+}$-sensitive calmodulin-dependent protein kinase kinases (CaMKks) (Anderson et al., 2008; Bair et al., 2009; Fogarty et al., 2010). It has been demonstrated that appetite-regulating hormones such as ghrelin and orexin-A increase [Ca$^{2+}_{i}$] in many native neurons, including NPY neurons (Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2008). Thus, calcium-dependent activation of AMPK may couple orexin-A’s action with the NPYergic system. Voltage-gated calcium channels (VGCCs) are the main route of calcium entry in neurons (Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2008). However, there are few reports about the role of VGCCs in AMPK activation. Here, we demonstrated for the first time that VGCC-dependent AMPK activation was also involved in orexin-A-mediated feeding behavior.

Materials and Methods

**Chemicals.** Orexin-A was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Anti-NPY serum H was obtained from Chemicon International (Temecula, CA). SB334867 (N-(2-methyl-6-benzoxazolyl)-N′-[1-17-ylamino]hexyl-[1H]-pyrrole-2,5-dione), GF10920X (3-[N-[dimethy lamino]propyl]-3-indolyl)-4-(3-indolyl)maleimide 3-[1-[3-[1-(3-dimethylam inopropyl)]-1H-indol-3-yl]-4-[2-(indol-3-yl)]-1H-pyrole-2,5-dione bisindolylmaleimide I], STO609 (7-oxo-7H-benzimidazo[2,1-a]benz[e]iso quinoline-3-carboxylic acid-acetic acid), BayK8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-2-[tri fluoromethyl]phenyl]pyridine-3-carboxylic acid methyl ester), BAPTA-AM (1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)), EGTa, thapsigargin (TG), SKG96365 (1-[3-(4-methylphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazo hydrochloride), 9-B-9-arabinofuranoside (ara-A), and nifedipine were obtained from Sigma-Aldrich (St. Louis, MO). Compound C (6-[4-(2-piperidinyl)ethoxy]phenyl)-3-(4-pyridyl)pyr azolol[1,5-a]pyrimidine) and B27 supplement were obtained from Gibco/Invitrogen (Carlsbad, CA). α-Conotoxin GVIA and α-agatoxin TK were obtained from Alomone Labs (Jerusalem, Isreal). AMPK antibody and liver kinase B1 (LKB1) antibody were obtained from Cell Signaling Technology (Danvers, MA). Other general agents were purchased from commercial suppliers. Orexin-A was prepared fresh with distilled water, and others were dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. Agents were diluted to the final concentrations before application. The final concentration of DMSO was <0.05%.

**Intracerebroventricular Cannulation and Injection.** Male Sprague-Dawley (SD) rats weighing 250–300 g were housed in a controlled environment with a temperature of 22 ± 1°C, humidity of 70%, and a 12-hour light/dark cycle from 9:00 AM to 9:00 PM. Laboratory standard food and water were available ad libitum. Care of the rats was conducted according to the requirements of the Guide for the Care and Use of Laboratory Animals by the National Research Council. The cannula was implanted according to Paxinos et al. (1980) with some modifications. Rats were anesthetized with 350 mg/kg chloral hydrate by intraperitoneal injection and placed in a stereotaxic apparatus with the bregma and posterior on the same level. Body temperature was maintained at 37.0 ± 0.2°C by an electric incandescent lamp. To fix the cannula, three small holes were drilled into the bone to place a jeweler screw. A fourth hole was made to insert a cannula into the lateral ventricle (0.8 mm posterior to the bregma, 1.4 mm lateral to the midline, 3.5 mm from the cranial theca). A stainless steel cannula 7 mm long and with 0.6-mm outside diameter was implanted into the place according to the previously-mentioned coordinates. The cannulae were fixed to the skull with the aid of jeweler screws and dental acrylic resin and cranioplastic dental cement. To maintain patency, a stylos 0.5 mm longer than the guide cannula was inserted into the guide. Cannula placement was confirmed by injection of black ink. The experiments were started after a 7-day period of recovery from surgery. After removal of the stylos from the cannula, the drugs were injected into the lateral ventricle with a microsyringe (10 μl) connected by a PE-10 polyethylene tubing (10 cm) to a needle (outside diameter = 0.3 mm), 0.5 mm longer than the guide cannula, which was introduced into the brain through the cannula fixed to the head of the rat. The injection volume was set at 5 μl within a period of 5–10 minutes.

**Western Blotting.** After treatment, animals were sacrificed by decapitation under 10% chloral hydrate anesthesia, and the entire ARC tissue was isolated from the brain slice. Culture cell or isolated ARC tissue was washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed on ice in extraction buffer containing 50 mM Tris base (pH 7.4), 100 mM NaCl, 1% NP40, 10 mM EDTA, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 3 mM Na3VO4, and protease inhibitors. The homogenates were centrifuged at 12,000g for 15 minutes at 4°C. Supernatant was separated and stored at −80°C until use. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Protein samples (30 μg) were separated by 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature, transferred membranes were incubated overnight at 4°C with different primary antibodies (phospho-AMPK and AMPK 1: 800 dilution; phospho-LKB1 and LKB1 1:800 dilution). Following three washes with TBST, membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (1:3000) in TBST with 1% nonfat milk for 1 hour at room temperature. Repeated washes, membranes were reacted with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for 5 minutes, and visualized with X-ray films (X-Omat; Kodak, Rochester, NY). The films were scanned and the optical density of the bands was determined using Optiquant software (Packard Instrument, Downers Grove, IL). Results are expressed as the percentage of control signals in each blot to correct for variations between blots.

**Assay of AMP/ATP Ratio.** The procedure for assay of the AMP/ATP ratio in the ARC of male SD rats was executed as described by Coolen et al. (2008) with some modifications. Samples dissected from ARC were homogenized and vortex mixed for 5 minutes with 0.5 ml of ice-cold 8% perchloric acid (v/v) in a 1.5-ml Eppendorf tube (Eppendorf, Hamburg, Germany). After precipitation of the protein fraction (centrifuged at 12,000g, 10 minutes, 4°C), the supernatant was removed and neutralized to pH 6.5 with 0.65 ml of 6 M KOH and

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40 μl of 2 M K₂CO₃. The mixture was vortex mixed and centrifuged for 5 minutes, and the supernatant was collected in a 5-ml test tube. The volume was made up to 5 ml with 0.05 M NH₄H₂PO₄ (pH 5.70). A sample from the solution was drawn into a pipette and filtered through a filtering cartridge with a 0.45-μm nylon membrane using a disposable syringe set before high-performance liquid chromatography analysis. A Thermo-Finnigan high-performance liquid chromatography system, containing a Surveyor MS pump, a Surveyor photodiode array detector, an autosampler, and the Xcalibur TM 1.3 software (Thermo-Finnigan Corporation, San Jose, CA) was used. A reversed-phase column (Agilent TC-C18, 250 × 4.6 mm, 5 μm; Agilent Technologies, Santa Clara, CA), maintained at 30°C, was also used. The standards and samples were separated using a gradient mobile phase consisting of 0.05 M NH₄H₂PO₄ (pH 5.7; A) and acetonitrile/water (60:40, v/v, B). The linear gradient conditions were as follows: 0–6.5 minutes, 100% A; 6.5–12.5 minutes, 100% B; 12.5–25 minutes, 100% A. The flow rate was set at 0.8 ml·min⁻¹, and the injection volume was 20 μl. The detection wavelength was set at 257 nm.

Preparation of Cultured Neurons from ARC. All experiments conformed to local and international guidelines for the use of animals, and all experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. Primary cultures of ARC neurons were prepared as previously described (Muroya et al., 2004; Wang et al., 2008) with some modifications. Brain slices containing the entire ARC were prepared from the brains of neonatal Spagne-Dawley rats (days 2–3), and the entire ARC was excised from the left and right sides. The dissected tissues were treated with 0.125% trypsin in Hanks’ balanced salt solution for 25 minutes at 37°C and mechanically dissociated using a fire-polished Pasteur pipette. Cell suspension was centrifuged for 7 minutes at 800g, and the cell pellets were resuspended in Dulbecco’s modified Eagle’s medium and F-12 supplement (1:1) with 10% fetal bovine serum. For whole-cell patch-clamp recording, cells (20,000–50,000) were seeded on poly-D-lysine-coated coverslips and kept at 37°C in a 5% CO₂ incubator. After 24 hours, the culture medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 2% B27, and the ARC neurons were fed with fresh medium twice weekly. Microscopically, glial cells were not apparent in ARC neuron cultures using this protocol. The neurons were maintained for 7–10 days in primary culture until used for experiments.

Immunocytochemical Identification of Single ARC Neurons. Single ARC neurons were immunocytochemically identified as previously reported, with slight modifications (Muroya et al., 2004; Wang et al., 2008). In the voltage-clamp experiments, the cells with typical characteristics of NPY-positive neurons were selected to record, photograph to mark position, and sequence. Then the cells were fixed with 4% paraformaldehyde in 0.1 M PBS overnight. They were pretreated with H₂O₂ in methanol for 1 hour. Nonspecific binding sites were then blocked with 10% goat serum in 0.1 M PBS for 1 hour at room temperature. Cells were incubated overnight at 4°C with primary antiserum to NPY diluted 1:1000 in PBS containing 1.5% normal goat serum. Cells were subsequently incubated with biotinylated goat antirabbit IgG secondary antibody for 1 hour at room temperature. The secondary antibody was then rinsed, and the sections were labeled with avidin-peroxidase complex reagent (ABC kit; Vector, Burlingame, CA) for 1 hour. The sections were developed with 0.05% 3,3’-diaminobenzidine. In control sections, the primary antibodies were replaced by the corresponding nonspecific IgG and processed in parallel.

Whole-Cell Patch-Clamp Recording. The procedure for whole-cell patch-clamp recording was executed as described in our previous reports, with minor modification (Wang et al., 2008; Ma et al., 2009). The bath solution for recording high-voltage activated calcium current (Iₜᵥₚᵥₚ) contained the following (in mM): choline-Cl 110, MgCl₂ 2, CaCl₂ 10, TEA-Cl 20, HEPES 10, glucose 10, and the pH was adjusted to 7.4 with CsOH. Glass pipettes were used with a resistance of about 2–4 MΩ when filled with the following solution (in mM): CsF 64, CsCl 64, CaCl₂ 0.1, MgCl₂ 2, EGTA 10.0, HEPES 10.0, Tris-ATP 5.0, and the pH was adjusted to 7.2 with CsOH. After establishing a whole-cell configuration (Date et al., 1999), capacitance compensation and series resistance compensation were adjusted before recording. The current signals were acquired at a sampling rate of 10 kHz and filtered at 3 kHz. Whole-cell patch-clamp recordings were carried out using an EPC-10 amplifier (HEKA, Lambrecht, Germany) driven by Pulse/PulseFit software (HEKA, Southborro, Germany). Drug actions were measured only after steady-state conditions were reached, which were judged by the amplitudes and time courses of currents remaining constant. All recordings were performed at room temperature (20–22°C). All experiments were repeated three times using different batches of cells, and at least 3–4 dishes with cells were used for recording in different batches of cells.

Calcium Imaging. Digital calcium imaging was performed as described previously (Ming et al., 2006; Lin et al., 2007). The cells were washed three times by artificial cerebrospinal fluid (ACSF) containing the following (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH 7.3), then loaded with 1 μM Fura-2/AM in ACSF for 30 minutes at 37°C to remove the excess extracellular Fura-2/AM. Coverslips were then mounted on a chamber positioned on the movable stage of an inverted microscope (TE2000; Nikon, Tokyo, Japan), which was equipped with a calcium imaging system (Photon Technology Internation, Inc., Birmingham, NJ). The cells were superfused by ACSF at a rate of 2 ml·min⁻¹ for 10 minutes. Fluorescence was excited at wavelengths of 340 nm for 150 milliseconds and 380 nm for 50 milliseconds at 1-second interval by a monochromator (K-178-S; Photon Technology Internation, Inc.), and the emission was imaged at 510 nm with a video camera (CoolSNAP HQ2; Photometrics, Tucson, AZ) through a fluor oil immersion lens (Nikon) and a wide band emission filter. F340/F380 fluorescence ratio was recorded and analyzed by MetaFluor version 6.3 software (Molecular Devices Corporation, San Francisco, CA). In each experiment, the peak 340/380 ratio after addition of control solution or orexin was averaged in the graphs. The control means the 340/380 ratio after addition of control solution. Then, on the same cell, the peak 340/380 ratio after addition of orexin was measured and averaged. All experiments were repeated at least three times using different batches of cells to ensure reproducibility.

Feeding Study. First, the effect of drugs (compound C, ara-A, nifedipine, ST0608, and U73122) on orexin-A–mediated feeding was investigated in rats fed ad libitum. Orexin-A was prepared fresh with distilled water, and other drugs were dissolved in DMSO. Before injection, the final concentration of DMSO was set at 0.5%. The male SD rats were divided into vehicle group (distilled water), orexin-A group, and other drugs combined with orexin-A group. Cumulative food intakes were measured at 1, 2, and 4 hours after injection of the drugs into the lateral ventricle by the cannula. All feeding behavior experiments were conducted in the early light phase. To test the effect of nifedipine on refeeding after fasting, all experimental rats were fasted (with free access to water) for 24 hours before being injected with nifedipine or vehicle (DMSO). Fifteen minutes after injection, rats were given ad libitum access to food, and cumulative food intakes were measured at 1, 2, and 4 hours after refeeding.

Measurement of NPY Content. The procedure for NPY analysis was executed as described by Rocha et al. (2006) with minor modifications. After treatment, animals (male SD rats) were sacrificed by decapitation under 10% chloral hydrate anesthesia, and entire ARC tissue was isolated from the brain slice. Culture cell or isolated ARC tissue was washed twice with ice-cold PBS and then lysed on ice in extraction buffer containing 50 mM Tris base (pH 7.4), 100 mM NaCl, 1% NP40, 10 mM EDTA, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 3 mM Na₂VO₃, and protease inhibitors. The homogenates were centrifuged at 12,000g for 15 minutes at 4°C. Supernatant was separated and stored at −80°C until use. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Inc.), and NPY content was measured by a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according
to the manufacturer’s protocol. NPY content was expressed as picograms per microgram of protein.

Electrophysiological Data Analysis. Only the results from NPY-immunoreactive neurons were collected into data statistics. The dose-response curve was fitted with the Hill equation:

\[ I/I_{\text{max}} = 1/[1 + (EC_{50}/C)^n] \]

where \( I \) is the current amplitude after administration of orexin-A, \( I_{\text{max}} \) is the control current amplitude, \( C \) is the concentration of orexin-A, and \( n \) is the Hill coefficient. The voltage dependence of activation was determined using standard protocols. The conductance \( G \) was calculated according to \( G = I/(V_m - V_{rev}) \), where \( V_m \) is the Ca\(^{2+}\) reversal potential and \( V_{rev} \) is the membrane potential at which the current was recorded. Normalized peak conductance \( (G/G_{\text{max}}) \) was then fitted by the following Boltzmann equation:

\[ G/G_{\text{max}} = 1/[1 + \exp((V_m - V_{1/2})/k)] \]

where \( G_{\text{max}} \) is the maximum conductance, \( V_{1/2} \) is the membrane potential of half-maximal activation, and \( k \) indicates the slope factor. To investigate the voltage-dependent inactivation, normalized currents \((I/I_{\text{max}})\) were plotted against the voltages of conditioning voltage, and fitted with a Boltzmann function:

\[ I/I_{\text{max}} = 1/[1 + \exp((V_m - V_{1/2})/k)] \]

where \( I_{\text{max}} \) is the maximal current, \( V_m \) is the conditioning voltage, \( V_{1/2} \) is the potential of half-maximal inactivation, and \( k \) is the slope factor.

Statistical Analysis. Values were presented as the mean ± S.E.M.

Data from experiments were analyzed with the statistical program SPSS (SPSS, Chicago, IL). A two-sided Student’s t test with paired comparisons was used to evaluate differences in electrophysiological data. For other data, comparison between two groups was evaluated by a two-sided and unpaired Student t test. Comparison between three groups at every test time point, respectively. Differences at the \( P < 0.05 \) level were considered statistically significant.

**Results**

Orexin-A Activates AMPK via CaMKK \( \beta \)-Dependent Pathway in the Rat Hypothalamic ARC. To test the effect of orexin-A on hypothalamic AMPK activation under an orexigenic condition, the AMPK phosphorylation in the ARC was tested at 0.5 hour after injection of orexin-A (1 nmol). As shown in Fig. 1A, orexin-A significantly increased the phosphorylation of AMPK by 69.67% \( ± \) 19.13%. We found that orexin-A did not change the AMP/ATP ratio in the ARC (Fig. 1B) and had no effect on phosphorylation of LKB1, an upstream AMPK kinase (Fig. 1C). CaMKK\( \beta \) is one of the major types of calmodulin-dependent protein kinases and activates AMPK (Anderson et al., 2008; Bair et al., 2009; Fogarty et al., 2010). STO609 is widely used to inhibit CaMKK\( \beta \) (Tokumitsu et al., 2003; Anderson et al., 2008; Pfisterer et al., 2011). Intracerebroventricular injection of STO609 inhibited AMPK activation induced by orexin-A in hypothalamic ARC (Fig. 1D), but had no effect on the phosphorylation of AMPK in hypothalamic ARC when used alone (Supplemental Fig. 1). These results suggest that, under an orexigenic condition, orexin-A regulates AMPK via CaMKK\( \beta \) activation in the hypothalamic ARC.

Orexin-A Stimulates AMPK Activation via Extracellular Calcium-Dependent Pathway in the Cultured Rat ARC Neurons. Previous studies have shown that CaMKK\( \beta \) is activated by Ca\(^{2+}\)/calmodulin, and that orexin-A increased [Ca\(^{2+}\)]\(_i\) in NPY neurons of ARC (Muroya et al., 2004). To further investigate the precise mechanism involved in hypothalamic AMPK activation, cultured ARC neurons were used. According to the results of a preliminary experiment (Supplemental Fig. 2), 100 nM orexin-A was selected to observe its effect in cultured ARC neurons. After 0.5 hour, the phosphorylation of AMPK was increased significantly by 82.64% \( ± \) 22.76% in ARC neurons (Fig. 2A). This effect was attenuated by the intracellular calcium chelator BAPTA-AM.

![Fig. 1. Orexin-A activates AMPK in the hypothalamic ARC in vivo.](https://example.com/f1.png)
(10 μM), whereas BAPTA-AM alone exhibited little effect on AMPK activation in ARC neurons (Fig. 2B). These results indicate that orexin-A–mediated hypothalamic AMPK activation is calcium-dependent. We further examined whether extracellular calcium influx or intracellular calcium release involved orexin-A–induced AMPK activation in ARC neurons. First, the effect of orexin-A on AMPK activation in Ca²⁺-free medium was observed. Preincubation with a slow Ca²⁺ chelator EGTA (5 mM) for 30 minutes alone had no effect on the activation of AMPK in ARC neurons, but it completely prevented orexin-A–induced AMPK activation (Fig. 2C). Second, the effect of intracellular calcium release on orexin-A–induced AMPK activation was investigated. Preincubation with a sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase inhibitor TG for 30 minutes did not influence the activation of AMPK in ARC neurons. Interestingly, after depolarizing the intracellular calcium store by TG, orexin-A also induced AMPK activation in hypothalamic neurons (Fig. 2D), although this effect seemed to be partially attenuated. These results suggest that extracellular calcium influx is predominant in orexin-A–induced AMPK activation in hypothalamic neurons, whereas Ca²⁺ release from the intracellular store is also involved in this effect. We further confirmed this result by a calcium imaging experiment. Orexin-A markedly increased [Ca²⁺], by 51.16% ± 7.58% in the ARC neurons. About 59% ± 8.2% of the ARC neurons responded to orexin-A, which was similar to the percentage of NPY-positive neurons (57% ± 6.4%). This effect was significantly attenuated in Ca²⁺-free medium (Fig. 2, E and F).

Orexin-A Increases High-Voltage Activated Calcium Current in NPY Neurons of ARC. Previous reports have suggested that VGCCs provide an important pathway for orexin-A–induced calcium signal (Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2008). Therefore, the effect of orexin-A on VGCCs was investigated in cultured rat hypothalamic NPY neurons, which are involved in the orexigenic effect of orexin-A. As shown in Fig. 3A, NPY neurons are typically small and medium neurons with triangular or spindle-shaped perikaryons and poorly ramified primary dendrites. The percentage of NPY-positive neurons was about 57% ± 6.4% in ARC neurons. In the voltage-clamp experiments, the cells with typical characteristics of NPY neurons were selected to record, photograph, and sequence. After whole-cell patch-clamp recording, the cells were immunocytochemically identified as in our previous reports (Wang et al., 2008; Yang et al., 2010). The cells were stepped from a holding potential of −80 to −40 mV (50 milliseconds), and then depolarized to +10 mV (200 milliseconds) after briefly hyperpolarizing the membrane potential to 10 milliseconds to −45 mV (Fig. 3C). The $I_{\text{HVA}}$ was activated by the second depolarization. The protocol was applied every 5 seconds. Among 34 neurons with characteristics of NPY neurons that responded to orexin-A, 30 neurons (88%) were proved to be NPY-immunoreactive neurons (Fig. 3B). Orexin-A (1 nM) increased the amplitude of $I_{\text{HVA}}$ by 31.28% ± 2.80%, and almost returned to control level after washout (Fig. 3C). The concentration of orexin-A producing a 50% increase in $I_{\text{HVA}}$ (EC₅₀) was 0.75 ± 0.09 nM, and the Hill coefficient was 0.72 ± 0.19 (Fig. 3D). The effect was observed 30–60 seconds after

![Fig. 2. Orexin-A induces AMPK activation via an extracellular calcium-dependent pathway in cultured rat hypothalamic ARC neurons. (A) Representative immunoreactive bands and statistical results from six independent experiments show an increase in phosphorylated AMPK (pAMPK) in cultured rat ARC neurons after treatment with orexin-A. (B) Representative immunoreactive bands and statistical results from six independent experiments showing BAPTA-AM had no effects on AMPK activation, but it inhibited orexin-A–induced AMPK activation in the ARC neurons. (C) Representative immunoreactive bands and statistical analysis from six independent experiments showing an inhibitor of sarcoplasmic reticulum Ca²⁺ ATPase, TG, had no effects on AMPK activation, but it abolished orexin-A–induced AMPK activation in the ARC neurons. (D) Representative immunoreactive bands and statistical results from six independent experiments showing a slow Ca²⁺ chelator EGTA had no effects on AMPK activation, but it abolished orexin-A–induced AMPK activation in the ARC neurons. (E) Representative traces and statistical results showing orexin-A increased [Ca²⁺], in ARC neurons (n = 10). (F) Representative traces and statistical results showing orexin-A failed to elevate [Ca²⁺] in ARC neurons after replacement with Ca²⁺-free medium (n = 12). Data are expressed as means ± S.E.M. *P < 0.05 versus control or TG; **P < 0.05 versus orexin-A.
application of orexin-A, and rapidly reached a plateau in 90–150 seconds (Fig. 3E). To study the effect of orexin-A on the activation curve of $I_{\text{HVA}}$, the currents were evoked by a series of 200-ms voltage steps between $-50$ and $+50$ mV in 10-mV increments preceded by a holding potential of $-80$ mV. The voltage-current curves in Fig. 3F indicate that the amplitude of $I_{\text{HVA}}$ was voltage-dependent. Orexin-A at 1 nM increased the peak amplitude of $I_{\text{HVA}}$ in NPY neurons, but had no effect on the reversal potential of $I_{\text{HVA}}$. At $+10$ mV, the current amplitude was increased from $238 \pm 65$ to $316 \pm 52$ pA in the presence of 1 nM orexin-A. As shown in Fig. 3G, 1 nM orexin-A shifted the activation curve of $I_{\text{HVA}}$ positively, with $V_{1/2} = 6.90 \pm 2.70$ mV and $k = 6.40 \pm 2.13$ in the control group, and $V_{1/2} = 1.47 \pm 0.67$ mV and $k = 6.54 \pm 1.30$ in the orexin-A group ($P < 0.05$). To investigate the steady-state activation properties of $I_{\text{HVA}}$, 300-millisecond conditioning prepulses from $-90$ to $20$ mV in 10-mV increments were applied before step depolarizations to the fixed potential of 10 mV. Orexin-A at 1 nM did not significantly alter the voltage dependence of inactivation kinetics of $I_{\text{HVA}}$ in NPY neurons, with $V_{1/2} = -36.34 \pm 7.50$ mV and $k = -40.8 \pm 4.00$ in the control group, and $V_{1/2} = -30.36 \pm 5.98$ mV and $k = -33.00 \pm 3.80$ in the orexin-A group (Fig. 3H). In addition, we further observed that orexin-A can increase the frequency of action potentials and cause membrane depolarization in hypothalamic NPY neurons (Supplemental Fig. 3), which may facilitate activation of VGCCs under basal conditions.

Orexin-A Mainly Increases L-Type Calcium Current via OX1R–Phospholipase C–Protein Kinase C Signaling Pathway. To further examine which type of calcium channel was regulated by orexin-A, the effects of orexin-A on $I_{\text{HVA}}$ in NPY neurons were observed in the presence of nifedipine (L-type calcium channel blocker), $\omega$-conotoxin GVIA (N-type calcium channel blocker), and $\omega$-agatoxin TK (P/Q-type calcium channel blocker), respectively. As shown in Fig. 4A, after pretreatment with 10 $\mu$M nifedipine, orexin-A failed to increase the current amplitude of $I_{\text{HVA}}$ in NPY neurons. However, $\omega$-conotoxin GVIA (1 $\mu$M) and $\omega$-agatoxin TK (200 nM) did not affect orexin-A–induced $I_{\text{HVA}}$ increase in NPY neurons (Fig. 4, B and C), suggesting that the L-type calcium channel mediates the effect of orexin-A on $I_{\text{HVA}}$. This point was confirmed by polymerase chain reaction. We found that both Ca$_{1.2}$ and Ca$_{1.3}$ channels are expressed in ARC neurons (Supplemental Fig. 4). Previous reports have shown that activation of the OX1R-phospholipase C (PLC)-protein kinase C (PKC) signaling pathway contributes to orexin-A–mediated [Ca$^{2+}$]$_i$ elevation (Uramura et al., 2001). To determine whether the OX1R-PLC-PKC signaling pathway was involved in the effect of orexin-A on the L-type calcium current, we used SB334867 (selective OX1R antagonist), U73122 (PLC inhibitor), and GF109203X (PKC inhibitor) in this experiment. Application with SB334867 (selective OX1R antagonist), U73122 (PLC inhibitor), and GF109203X (PKC inhibitor) alone exhibited no effects on $I_{\text{HVA}}$, but 10 $\mu$M SB334867, U73122, and GF109203X significantly inhibited the increase in $I_{\text{HVA}}$ produced by orexin-A (Fig. 4, D–F). These results suggest that the L-type calcium channel and OX1R-PLC-PKC signaling pathway are required for orexin-A–induced upregulation of the VGCC’s function in hypothalamic NPY neurons.

L-Type Calcium Channel Mediates Orexin-A–Induced AMPK Activation in Hypothalamic ARC. Next, we further determined whether the L-type calcium channel was involved in calcium-dependent AMPK activation induced by orexin-A in hypothalamic ARC. First, BayK8644, a selective L-type calcium channel activator that prolongs Ca$^{2+}$ channel open times and elevates cytosolic Ca$^{2+}$ via the L-type Ca$^{2+}$ channel under basal conditions (Miyaiuchi et al., 1990; Dessy and Godfraind, 1996; Mulvaney et al., 1999; Peti-Peterdi and...
Bell, 1999), was used to investigate the role of the L-type calcium channel in AMPK activation. As shown in Fig. 5A, BayK8644 (10 μM) promoted AMPK activation in the hypothalamic neurons, and this effect can be inhibited by 10 μM STO609, indicating that upregulation of L-type calcium channel function can lead to AMPK activation. Then, the effect of L-type calcium channel blocker nifedipine on orexin-A–induced AMPK activation was investigated. Preincubation with nifedipine (10 μM) in cultured ARC neurons or i.c.v. injection of nifedipine (50 μg/kg) exhibited little effect on hypothalamic AMPK activation, but largely inhibited orexin-A–induced AMPK activation (Fig. 5, B and C). Considering that activation of the PLC-PKC signaling pathway is responsible for the action of orexin-A on the L-type calcium channel, we further investigated the effect of the PLC-PKC signaling pathway on orexin-A–mediated hypothalamic AMPK activation. Intracerebroventricular injection of PLC inhibitor U73122 (10 nmol) had no effect on hypothalamic AMPK phosphorylation (data not shown), but reduced orexin-A–mediated hypothalamic AMPK activation (Fig. 5D). Our preliminary experiment demonstrated that low concentration of orexin-A (1 nM), which can increase the L-type calcium current, exhibited little effect on AMPK activation (Supplemental Fig. 2). Thus, besides VGCCs, there may exist other mechanisms. Orexin-A can also activate transient receptor potential cation channels (TRPCs). We found that i.c.v. injection of SKF96365 (25 nmol), a blocker of TRPCs, partially attenuated, but did not abolish, orexin-A–induced AMPK activation (Supplemental Fig. 5).
These results suggest that, although other calcium entry routes exist, the L-type calcium channel is largely predominant in orexin-A–mediated hypothalamic AMPK activation.

**Calcium-Dependent Hypothalamic AMPK Activation Is Involved in Orexin-A–Mediated NPY Increase and Feeding Behavior.** Hypothalamic AMPK signal is involved in the orexigenic signaling of metabolic hormones, including adiponectin and ghrelin. NPY, an important orexigenic peptide secreted by NPY neurons, is considered the downstream effector of orexin-A’s orexigenic effects. Therefore, we further investigated the effect of hypothalamic AMPK on NPY content and feeding behavior induced by orexin-A. As shown in Fig. 6, A–C, compared with the vehicle group, i.c.v. injection of orexin-A (1 nmol) significantly increased NPY content in hypothalamic ARC. Intracerebroventricular injection of AMPK inhibitor compound C, CaMKKβ inhibitor STO609, and nifedipine inhibited the increase in NPY content induced by orexin-A, but exhibited little effect on hypothalamic NPY content when used alone, indicating that orexin-A increases hypothalamic NPY content via calcium-dependent AMPK activation.

We then determined the role of hypothalamic AMPK in orexin-A–induced feeding behavior. All drugs were dissolved in DMSO, and we found that i.c.v. injection of DMSO (0.5%) exhibits little effect on orexin-A (Supplemental Fig. 6). AMPK inhibitor compound C (500 nmol) significantly attenuated orexin-A–stimulated feeding behavior (Fig. 6D), whereas it did not influence the feeding behavior at 1 and 2 hours after i.c.v. injection and slightly inhibited food intake at 4 hours after i.c.v. injection. Considering that compound C has been shown to block other protein kinases with similar potency, the ATP mimic ara-A, a precursor of ara-ATP that can decrease the AMP:ATP ratio within cells and inhibit AMPK activation (Russell et al., 1999; Wu et al., 2003; Potter et al., 2010), was used as an AMPK inhibitor to confirm the role of AMPK in orexin-A–induced food intake. Similarly, i.c.v. injection of ara-A (2 μmol) did not influence feeding behavior, but significantly inhibited orexin-A–induced food intake (Fig. 6E). Then, inhibitors of AMPK upstream kinases such as CaMKKβ inhibitor STO609 and PLC inhibitor U73122 were also used. Both STO609 and U73122 exhibited little effect on food intake, but they inhibited orexin-A–induced orexigenic effects (Fig. 6, F and G). Hence, we further tested the role of the L-type calcium channel in orexin-A–stimulated feeding behavior. Nifedipine exhibited little effect on food intake, but inhibited orexin-A–stimulated feeding behavior (Fig. 6H). Orexin-A has also been reported to be involved in the regulation of fasting-induced hyperphagia. As shown in Fig. 6I, i.c.v. administration of nifedipine (50 μg/kg) significantly alleviated the increase in food intake induced by fasting.
These results suggest that calcium-dependent hypothalamic AMPK activation, especially by the L-type calcium channel, contributes to the orexigenic effects induced by orexin-A.

**Discussion**

The present study is the first to provide evidence that the hypothalamic L-type calcium channel mediates orexin-A–induced calcium-dependent AMPK activation, which may be involved in orexin-A–induced orexigenic effects. Considering the current knowledge of the signaling pathways underlying orexin-mediated effects and the role of AMPK in food intake, our study connects the dots of understanding of the known effect of orexin-A.

Orexin-A is released from orexin-producing neurons that localize in the lateral hypothalamic area in response to fasting, and causes hyperphagia (Baird et al., 2009). Orexin-A–stimulated hyperphagia plays an important role in the abnormal control of eating behavior in patients with eating disorders (Steffen et al., 2006; Baranowska et al., 2008; Bronsky et al., 2011). However, the precise molecular mechanism underlying orexin-A–stimulated eating behavior remains largely unknown. Many anorexic and orexigenic molecules alter AMPK activity and produce effects on energy metabolism, including leptin, α-lipoic acid, fatty acid synthase inhibitor (C75), adiponectin, ghrelin, and cannabinoids (Minokoshi et al., 2002; Kim et al., 2004a,b; Kola et al., 2005; Kubota et al., 2007; Lopez et al., 2008; Wen et al., 2010). Our present study first demonstrated that orexin-A increased hypothalamic AMPK activity. Two AMPK inhibitors can prevent the orexigenic effect of orexin-A, indicating that orexin-A exerts orexigenic action via an AMPK signaling pathway. AMPK can activate NPY neurons.
in the hypothalamic ARC to increase food intake and increase NPY synthesis (Kohno et al., 2011). In our current study, orexin-A upregulated the total NPY content, which was consistent with a previous study (Martins et al., 2010). This effect was blocked by AMPK inhibitor compound C, indicating a positive correlation of AMPK activity and NPY neuron activity. Taken together, the results of our present study demonstrate that orexin-A–induced feeding behavior at least largely results from AMPK activation. Considering the nonspecificity of chemical inhibitors, a genetic approach to conditional knockdown of AMPK in NPY neurons should be used in the future.

Numerous studies indicate that the tumor suppressor protein LKB1 and the cellular AMP/ATP ratio are responsible for the activation of AMPK in pathological conditions, including hypoxia and glucose deprivation (Kemp et al., 1999). In the present study, we found that orexin-A had little influence on LKB1 phosphorylation. No changes in the AMP/ATP ratio in the hypothalamus were observed in our experimental setting. Recently, CaMKKβ was shown to function as an established AMPK upstream kinase. CaMKKβ can regulate the phosphorylation of AMPK in NPY neurons of the hypothalamus independent of the AMP/ATP ratio (Anderson et al., 2008; Bair et al., 2009; Fogarty et al., 2010). These findings highlight that the Ca2+/CaM/CaMKKβ pathway may act as a coupler linking metabolic hormone to the phosphorylation of AMPK. Intracerebroventricular injection of STO609, a widely used inhibitor of CaMKKβ (Tokumitsu et al., 2003; Anderson et al., 2008; Pfisterer et al., 2011), attenuated AMPK activation induced by orexin-A in hypothalamic ARC. We also confirmed the role of the Ca2+/CaM/CaMKKβ pathway in cultured hypothalamic neurons. Preincubation with the intracellular calcium chelator BAPTA-AM almost completely blocked orexin-A–induced AMPK activation in cultured hypothalamic neurons. In Ca2+-free medium, orexin-A–stimulated AMPK activation was also inhibited. These results demonstrate that orexin-A activates AMPK activation via the Ca2+/CaM/CaMKKβ pathway. The role of the CaMKKβ pathway in the orexigenic effect of orexin-A requires further investigation using genetic approaches.

It has been demonstrated that activation of VGCC mediates orexin-A–activated Ca2+ entry in native neurons (Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2008). Our present study showed that the L-type calcium channel blocker nifedipine significantly inhibited the increase in I_{HVA} induced by orexin-A in NPY neurons. Nifedipine also significantly inhibited orexin-A–induced AMPK phosphorylation in cultured hypothalamic neurons. Considering that there are some differences between cultured hypothalamic neurons from the ARC of neonatal rats and adult animals, we further investigated whether activation of the L-type calcium channel mediates orexin-A–induced AMPK activation in vivo. Intracerebroventricular injection of nifedipine significantly prevented orexin-A– and fasting-induced feeding behavior. Taken together, these results strongly support our hypothesis that activation of the L-type calcium channel is largely responsible for hypothalamic AMPK activation, and may affect orexin-A–induced orexigenic effects. Recently, it has been reported that antagonism of the T-type calcium channel inhibits high-fat-diet–induced weight gain in mice (Uebel et al., 2009). Hence, we speculate that the L-type calcium channel is another potential drug target for the treatment of metabolic diseases.

To understand the mechanism of action of orexin-A on the L-type calcium channel, PLC inhibitor U73122 and PKC inhibitor GF109203X were used. U73122 significantly inhibited the increase in I_{HVA} produced by orexin-A. Furthermore, U73122 attenuated orexin-A–induced hypothalamic AMPK activation and orexigenic effects. These results suggest that the OX1R-PLC signaling pathway is required for orexin-A’s effect. Previous studies have indicated that U73122 exerts several additional effects, including blockade of the L-type Ca2+ channels and a direct activation of PLC (Taylor and Broad, 1998; Klein et al., 2011). In our current study, few direct effects of U73122 on VGCC currents were observed. As a key downstream of PLC, PKC mediated orexin-A’s effect on the calcium channel. A PKC inhibitor, GF109203X, abolished orexin-A–induced increase in calcium current, which was consistent with previous studies of different neurons (Uramura et al., 2001; Kohlmeier et al., 2008). PKC activation could lead to L-type calcium channel phosphorylation and cause channels to be easily activated (Li et al., 2005). However, the regulation of L-type calcium channels by PKC activation is complex, with both stimulation and inhibition of Ca2+ current being observed (Kemp and Bell, 2000). We found both Ca1,2 and Ca1,3 expressed in ARC neurons using PCR technology (seen in Supplemental Fig. 3). It remains to be determined whether any individual phosphorylation site of Ca1,2 or Ca1,3 by PKC will contribute to orexin-A’s effect. Further investigation will be performed in our next study.

It should be noted that, besides VGCCs, there may exist other calcium entry routes involved in the effect of orexin-A. TRPC blocker SKF96365 (25 nmol) attenuated, but did not prevent, orexin-A–induced activation of hypothalamic AMPK (seen in Supplemental Fig. 5), indicating that TRPCs, which are activated by orexin-A (Larsson et al., 2005; Peltonen et al., 2009; Cvetkovic-Lopes et al., 2010), may contribute to orexin-A’s effect. In our preliminary experiment, we found that a low concentration of orexin-A (1 nM) seemed to exhibit little effect on AMPK activation, although it can increase calcium current. We found that calcium influx through VGCCs was predominant in orexin-A–induced AMPK activation, whereas Ca2+ release from intracellular store and other calcium entry routes was also involved in this effect. Thus, we speculated that orexin-A–induced Ca2+ current was the initiation factor to activate other calcium entry routes. A low concentration of orexin-A failed to increase AMPK phosphorylation, which may be due to its weak action on Ca2+ current that cannot produce enough calcium signal to activate secondary cascade reaction, including calcium-dependent calcium release and calcium-dependent calcium influx.

Our electrophysiological results revealed that orexin-A increased the frequency of action potentials and caused membrane depolarization in hypothalamic NPY neurons (seen in Supplemental Fig. 3). How L-type calcium channel potentiation affects the firing properties of NPY neurons is a very interesting question. NPY neurons are sensitive to energy status, and previous studies have revealed that AMPK activity controls excitation of NPY/agouti-related protein neurons (Murphy et al., 2009; Kohno et al., 2011). We hypothesize that L-type channel potentiation increased the frequency of action potentials via activation of AMPK. On the other hand, it can be speculated that increased action potential frequency can open more VGCCs. Some studies have also revealed that AMPK activation increases [Ca2+]i in NPY neurons (Kohno et al., 2011), but little is known about
mechanism. A synaptic AMPK-dependent positive feedback loop was involved in the sensing of hunger states (Yang et al., 2011). A positive loop including AMPK activation and selective enhancement of Ca\(^{2+}\) transients mediated by L-type calcium channels. J Neurophysiol 100:2265–2281.


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