Neuroscience 156 (2008) 89–98

LEPTIN REGULATED CALCIUM CHANNELS OF NEUROPEPTIDE Y AND PROOPIOMELANOCORTIN NEURONS BY ACTIVATION OF DIFFERENT SIGNAL PATHWAYS

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Abstract—The fat-derived hormone leptin regulates food intake and body weight in part by modulating the activity of neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus (ARC). To investigate the electrophysiological activity of these neurons and their responses to leptin, we recorded whole-cell calcium currents on NPY and POMC neurons in the ARC of rats, which we identified by morphologic features and immunocytochemical identification at the end of recording. Leptin decreased the peak amplitude of high voltage–activated calcium currents (I_{HVA}) in the isolated neurons from ARC, which were subsequently shown to be immunoreactive for NPY. The inhibition was prevented by pretreatment with inhibitors of Janus kinase 2 (JAK2) and mitogen-activated protein kinases (MAPK). In contrast, leptin increased the amplitude of I_{HVA} in POMC-containing neurons. The stimulations of I_{HVA} were inhibited by blockers of JAK2 and phosphatidylino 3-kinase (PI3-k). Both of these effects were counteracted by the L-type calcium channel antagonist nifedipine, suggesting that L-type calcium channels were involved in the regulation induced by leptin. These data indicated that leptin exerted opposite effects on these two classes of neurons. Leptin directly inhibited I_{HVA} in NPY neurons via leptin receptor (LEPR) –JAK2–MAPK pathways, whereas evoked I_{HVA} in POMC neurons by LEPR–JAK2–PI3-k pathways. These neural pathways and intracellular signaling mechanisms may play key roles in regulating NPY and POMC neuron activity, anorectic action of leptin and, thereby, feeding. © 2008 Published by Elsevier Ltd on behalf of IBRO.

Key words: leptin, patch-clamp, calcium current, POMC, NPY.

Leptin, the protein encoded by the obese (*ob*) gene (Zhang et al., 1994), is secreted from adipose tissue and is thought to act in the CNS to regulate food intake and energy expenditure (Campfield et al., 1995; Schwartz et al., 1996a). Defects in leptin signaling cause severe obesity in rodents and humans. It is well established that the weight reducing effects of leptin are mediated in part by its ability to modulate hypothalamic functions (Schwartz et al., 1996b). Neuroanatomical studies have suggested that the majority of leptin's anti-obesity effects are mediated by leptin receptors (LEPRs) in the arcuate nucleus (ARC) of hypothalamus (Saper et al., 2002; Schwartz and Porte, 2005; Spiegelman and Flier, 2001). The signaling form of LEPR is coexpressed with neuropeptide Y (NPY) and agouti-related peptide (AgRP) in a group of orexigenic neurons and with proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) in a group of anorexigenic neurons (Mercer et al., 1996; Erickson et al., 1996; Schwartz et al., 1997; Hahn et al., 1998; Baskin et al., 1999). Increased NPY activity and reduced POMC activity appear to promote feeding and fat deposition, whereas reduced NPY activity and increased POMC activity inhibit feeding and body mass (Lin et al., 2000; Clark et al., 1984; Zarjevski et al., 1993; Huszar et al., 1997; Fan et al., 1997). After its binding to LEPR in the hypothalamus, leptin stimulates a specific signaling cascade that results in the inhibition of NPY neurons (Cowley et al., 2001; van den Top et al., 2004), while stimulating POMC neurons (Cowley et al., 2001; Elias et al., 1999).

Despite the well-known differential effects of leptin on NPY and POMC neurons, relatively little is known about the mechanisms especially the electrophysiological mechanisms for actions of leptin. Spanswick et al. (1997) observed that leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels (K_{ATP}) using single-channel recording techniques from hypothalamic slices and acutely dissociated neurons. It is well-established that inhibition of K_{ATP} channels leads to membrane depolarization and facilitating electrical activity, generating the opening of voltage-gated calcium channels (VGCCs) and accelerating Ca^{2+} influx, and then initiating the release of neurotransmitter (Dunne et al., 1997; Chapman et al., 1999), but the opening of K_{ATP} channels leads to membrane hyperpolarization and diminished electrical activity. Previously, Cowley et al. (2001) observed that leptin induces a slow, progressive membrane hyperpolarization

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Abbreviations: AgRP, agouti-related peptide; ARC, arcuate nucleus; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; DMEM, modified Dulbecco's Eagle's medium; DMSO, dimethysulfoxide; HVA, high voltage–activated calcium channel; I_{HVA}, high voltage–activated calcium currents; JAK2, Janus kinase 2; K_{ATP}, ATP-sensitive potassium; LEPR, leptin receptor; MAPK, mitogen-activated protein kinases; NPY, neuropeptide Y; PI3-k, phosphatidylino 3-kinase; POMC, proopiomelanocortin; STAT, signal transducers and activators of transcription; VGCCs, voltage-gated calcium channels.

0306-4522/08 © 2008 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2008.04.079

associated with a decrease in membrane resistance and a cessation of all activity of NPY/AgRP neurons in ARC through activation of K_{ATP} channel, however, leptin increases the frequency of action potentials in POMC neurons by activating a nonspecific cation channel and reduced inhibition through local orexigenic NPY/GABA neurons. It is widely accepted that the cation channels are voltage- and Ca^{2+}-sensitive and, upon patch excision, can be found in several distinguishable modes of gating (Wilson et al., 1998). These observations supported that Ca^{2+} may play a possible role in the effects of leptin on the activities of NPY and POMC neurons.

Calcium plays a critical role in mediating neuronal excitability and neuroplasticity. Regulations of calcium contents in NPY and POMC neurons by leptin have been reported in different experimental systems. For instance, Muroya et al. (2004) observed by calcium imaging technique that leptin decreases cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the isolated NPY neurons, but induces long-lasting increases of $[Ca^{2+}]_i$ in POMC neurons. Similarly, it has been reported that leptin could suppress $[Ca^{2+}]_i$ rise in NPY neurons induced by ghrelin and orexins (Muroya et al., 2004; Kohno et al., 2003, 2007). VGCCs play an important role in the regulation of $[Ca^{2+}]_i$ (Catterall, 1995), however, the direct effects of leptin on VGCCs in NPY and POMC neurons have not yet been reported.

Here, we tested the possible modulations of VGCCs by leptin in primary cultured ARC neurons using patch-clamp techniques, and if so, to specify the signal transduction mechanisms in this modulated actions of leptin.

EXPERIMENTAL PROCEDURES

Chemicals

Leptin was purchased from ProSpec (Rehovot, Israel), anti-NPY serum and anti-α-MSH were obtained from Chemicon International (Temecula, CA, USA), AG490 and PD98058 were purchased from Calbiochem (San Diego, CA, USA), wortmannin and nifedipine were obtained from Sigma (St. Louis, MO, USA) and B27 supplement was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Other common agents were purchased from commercial suppliers. Leptin was prepared freshly with distilled water. AG490, PD98059, wortmannin, and nifedipine were dissolved in dimethysulfoxide (DMSO) and stored at −20 °C. They were diluted to the final concentrations before application. The final concentration of DMSO was $<0.05\%$.

Preparation of single neurons from ARC

The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. All efforts were made to minimize the number of animals used and their suffering. Primary cultures of ARC neurons were prepared as previously described (Muroya et al., 2004) with some modifications. Briefly, brain slices containing the entire ARC were prepared from the brains of neonatal Sprague–Dawley rats (days 5–7), 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 min at 37 °C and mechanically dissociated using a fire-polished Pasteur pipettes. Cell suspension was centrifuged for 7 min at 1000×*g* and the cell pellets were re-suspended in the modified Dulbecco's Eagle's medium (DMEM) and F-12 supplement (1:1) with 10% fetal bovine serum. For whole-cell patch-clamp recording, cells (20,000–40,000) were seeded on poly-D-lysine-coated coverslips and kept at 37 °C in 5% CO_2 incubator. After 24 h, the culture medium was changed to DMEM medium supplemented with 2% B27 and the ARC neurons were fed with fresh medium twice weekly. Microscopically, glial cells were not apparent in ARC neurons employing this protocol. The neurons were maintained for 7–10 days in primary culture until used for whole-cell patch-clamp recording.

Immunocytochemical identification of single ARC neurons

The immunocytochemical identification of ARC neurons was prepared as previously reported with slight modifications (Muroya et al., 2004). The ARC neurons were fixed with 4% paraformaldehyde in 0.1 mol/L PBS overnight. They were pretreated with H_2O_2 in methanol for 1 h. Nonspecific binding sites were then blocked with 10% goat serum in 0.1 mol/L PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with primary antiserum to NPY or α-MSH diluted 1:1000 in PBS containing 1.5% normal goat serum. Cells were subsequently incubated with biotinylated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The secondary antibody was then rinsed, and the sections were labeled with avidin–peroxidase complex reagent (ABC kit; Vector) for 1 h. The sections were developed with 3,3′-diaminobenzidine (DAB). 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 as that described in our previous reports with minor modification (Chen et al., 2002; Yermolaieva et al., 2001). The bath solution for recording high-voltage activated calcium current (I_{HVA}) contained (in mmol/L): choline-Cl 110, $MgCl_2$ 2, $CaCl_2$ 10, TEA–Cl 20, Hepes 10, glucose 20, 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 mmol/L): CsF 64, CsCl 64, $CaCl_2$ 0.1, $MgCl_2$ 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, the adjustment of capacitance compensation and series resistance compensation were done 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, Southboro, 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 the recordings were made at room temperature (20–22 °C). All experiments were repeated three times using different batches of cells and at least three to four dishes with cells were used for recording in different batches of cells.

Data analysis

Dose-response curve was fitted with the Hill equation: $I/I_{max}=1/[1+(EC_{50}/C)^n]$, where I is the current amplitude after administration of leptin, I_{max} is the control current amplitude, C is the concentration of leptin, and n is 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_{rev} is the Ca^{2+} reversal potential and V_m is the membrane potential at which the current was recorded.

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