Neuroscience 205 (2012) 49–62

SINGLE CELL ANALYSIS OF VOLTAGE-GATED POTASSIUM CHANNELS THAT DETERMINES NEURONAL TYPES OF RAT HYPOTHALAMIC PARAVENTRICULAR NUCLEUS NEURONS

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Abstract—The hypothalamic paraventricular nucleus (PVN), a site for the integration of both the neuroendocrine and autonomic systems, has heterogeneous cell composition. These neurons are classified into type I and type II neurons based on their electrophysiological properties. In the present study, we investigated the molecular identification of voltage-gated K^+ (Kv) channels, which determines a distinctive characteristic of type I PVN neurons, by means of single-cell reverse transcription-polymerase chain reaction (RT-PCR) along with slice patch clamp recordings. In order to determine the mRNA expression profiles, firstly, the PVN neurons of male rats were classified into type I and type II neurons, and then, single-cell RT-PCR and single-cell real-time RT-PCR analysis were performed using the identical cell. The single-cell RT-PCR analysis revealed that Kv1.2, Kv1.3, Kv1.4, Kv4.1, Kv4.2, and Kv4.3 were expressed both in type I and in type II neurons, and several Kv channels were co-expressed in a single PVN neuron. However, we found that the expression densities of Kv4.2 and Kv4.3 were significantly higher in type I neurons than in type II neurons. Taken together, several Kv channels encoding A-type K^+ currents are present both in type I and in type II neurons, and among those, Kv4.2 and Kv4.3 are the major Kv subunits responsible for determining the distinct electrophysiological properties. Thus these 2 Kv subunits may play important roles in determining PVN cell types and regulating PVN neuronal excitability. This study further provides key molecular mechanisms for differentiating type I and type II PVN neurons.

Key words: hypothalamic paraventricular nucleus, Kv channels, single-cell RT-PCR, single-cell real-time PCR.

The hypothalamic paraventricular nucleus (PVN) is an important integrating site of hormone secretion and regulation of the autonomic nervous system (Swanson and Sawchenko, 1980). PVN neurons are composed of heterogeneous cell groups, including magnocellular and parvocellular cells (Armstrong et al., 1980; Swanson and Kuypers, 1980). Magnocellular neurosecretory cells and parvocellular neurosecretory cells are responsible for hormone secretion from the posterior pituitary and the anterior pituitary gland, respectively, and parvocellular preautonomic neurons projecting to the autonomic centers in the brain stem and spinal cord underlie the regulation of autonomic nervous system outflow (Porter and Brody, 1985, 1986; Kannan et al., 1988; Coote, 1995; Blair et al., 1996; Patel and Zhang, 1996).

The PVN neurons have been well-classified based on their electrophysiological properties. Type I neurons, putative magnocellular neurons, express transient outward rectification, whereas type II neurons, putative parvocellular neurons, do not show the transient outward rectification, and some of the type II neurons exhibit low-threshold spikes (LTS) that are not found in type I neurons (Hoffman et al., 1991; Tasker and Dudek, 1991; Luther and Tasker, 2000). The transient outward rectification, which was distinguished by a delay in the onset of the first action potential, is known to be associated with the A-type K^+ current (I_A) (Luther and Tasker, 2000).

Previously, three types of K^+ currents such as I_A, slowly activating and non-inactivating delayed rectifier, and slowly activating and inactivating K^+ current have been recorded in PVN neurons (Luther and Tasker, 2000; Sonner and Stern, 2007). Particularly, I_A, transient activated and rapidly inactivated voltage-gated K^+ (Kv) current, has been reported to exert a strong influence on repetitive firing patterns by altering the interspike interval and action potential repolarization, as well as the postsynaptic responsiveness (Rogawski et al., 1985; Rudy, 1988). Also, I_A was determined to influence the action potential waveform and firing activity in PVN neurons projecting rostral ventrolateral medulla (RVLM) (Sonner and Stern, 2007).

The electrophysiological differences between type I and type II neurons may be because of the differential expression of ion channels; specifically, Kv channels encoding A-type K^+ currents. However, extensive differential identification of Kv channels in the PVN neurons has not yet been completely reported. Several subunits of Kv channels were identified in PVN neurons. Kv1.4 and Kv4.3 were identified in presympathetic PVN neurons (Sonner and Stern, 2007), and Kv1.2 immunoreactivity was detected in the PVN (Chung et al., 2001). However, the direct comparison of Kv channel expression profiles between type I and type II neurons was not performed.

Using the combined method of current-clamp recording with single-cell reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, we exam-

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Abbreviations: ACSF, artificial cerebrospinal fluid; Cav, voltage-gated calcium; CRH, corticotropin-releasing hormone; C_t, threshold cycle; I_A, A-type K^+ current; Kv channel, voltage-gated potassium channel; LTS, low-threshold spikes; OXY, oxytocin; PBS, phosphate-buffered saline; PVN, paraventricular nucleus; RT-PCR, reverse transcription-polymerase chain reaction; RVLM, rostral ventrolateral medulla; TRH, thyrotropin-releasing hormone; VP, vasopressin; 4-AP, 4-aminopyridine.

0306-4522/12 $36.00
doi:10.1016/j.neuroscience.2011.12.031

ined the differential expression profiles of Kv1.x and Kv4.x, which are known to be involved in generating I_A (Shibata et al., 2000; Jerng et al., 2004) between type I and type II PVN neurons. Additionally, we analyzed the relationship of Kv subunit expression with a delay in the onset of the first action potential and performed single-cell real-time RT-PCR to compare the expression density of each Kv channel subunit between type I and type II PVN neurons.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (n=61, 4–5 weeks old) were purchased from Orient Bio Inc. (Kyonggi-do, Korea). The rats were maintained under a 12 h light/dark cycle (lights on at 9:00 AM) and given free access to food and water until sacrifice. For the surgical procedure, anesthesia was induced by an intraperitoneal injection a mixture of tiletamine and zolazepam (Zolatil 50, 50 mg/kg; Virbac Lab., Carros Cedex, France) and xylazine (Rompun, 12.5 mg/kg; Bayer Korea, Ansan, Korea). All efforts were made to minimize the number of animals and their suffering. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Seoul National University and approved by the Institute of Laboratory Animal Resources of Seoul National University (SNU-100713-7).

Hypothalamic slice preparation

Hypothalamic brain slices were prepared according to the methods described previously (Stern, 2001; Han et al., 2002, 2010). The brains were immediately removed under anesthesia and immersed in oxygenated (95% O_2; 5% CO_2) ice-cold artificial cerebrospinal fluid (ACSF). Two or three coronal hypothalamic slices (300 μm) were caudally cut to the optic chiasm with a vibrating tissue slicer (Vibratome 1000 plus, Vibratome Company, St. Louis, MO, USA). The slices were incubated in oxygenated ACSF for at least 1 h at 32 °C until recordings. The recordings were made at 30~32 °C. The ACSF for current clamp experiments contained (in mM): 126 NaCl, 26 $NaHCO_3$, 5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose.

Electrophysiological recording

The PVN neurons were identified under the upright microscope (BX50WI, Olympus, Tokyo, Japan) with differential interference contrast and recorded in the whole cell current clamp mode. Patch pipettes were pulled from borosilicate glass capillaries of 1.7 mm diameter and 0.5 mm wall thickness. The pipette internal solution contained (mM): 135 K-gluconate, 5 KCl, 20 HEPES, 0.5 $CaCl_2$, 5 EGTA, and 5 MgATP. The pH was adjusted to 7.2 with KOH. Healthy-looking neurons were selected for recording and were approached with the aid of a three-dimensional hydraulic micromanipulator (Narishige Co., Tokyo, Japan). The open resistance of the pipette ranged from 3 to 6 MΩ, and a tight giga-ohm seal was obtained in the selected neuron. Electrical signals were recorded by an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA). Current or voltage signals were filtered at 1 kHz and digitized at 10 kHz using an analog-digital converter (Digidata 1320A, Axon Instruments) and pClamp software (version 9.0, Axon Instruments). Membrane input resistance was obtained from the relations of the hyperpolarizing pulses (~60 pA). The resting membrane potentials were corrected for the liquid junction potential (~14.3 mV). The current clamp recording was used to classify cell types of PVN neurons. The serial depolarizing current pulses for 250 ms were applied after a hyperpolarizing pre-pulse to near −100 mV for 250 ms in a whole cell configuration (Fig. 1A). In response to depolarizing from a hyperpolarizing membrane potential, PVN neurons displaying a delay in onset of the first action potential were classified as type I neurons, whereas PVN neurons not displaying a delay as type II neurons.

4-aminopyridine (4-AP), an A-type K^+ channel blocker (Rudy et al., 1988), was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was applied to the bath solution (ACSF) at the appropriate concentration.

Single-cell RT-PCR

Single-cell RT-PCR was carried out as previously described with minor modifications (Glasgow et al., 1999; Di et al., 2003; Lee et al., 2008). We first classified PVN neurons into two cell types as type I or type II neurons by whole cell recording, and performed post hoc PCR analysis by extracting the cytoplasm of the classified cell. The cytoplasm of the neuron that was identified was pulled into a patch pipette with negative pressure under visual control, taking care not to contain the nucleus.

The cytoplasm in the pipette was again dissipated into a prepared microtube containing 5 μl of nuclease-free water (Qiagen, Valencia, CA, USA) and then immediately moved into −70 °C deep freezer. The RT for single-cell cDNA preparations carried out with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual.

PCR amplification was induced with a fraction of the single-cell cDNA as a template. The mixture of PCR reaction contained (in μl): 1 of 10 μM each primer, 10 of 2× Taq master mix buffer (GoTaq Green Master Mix, Promega, Madison, WI, USA), and 2~4 of the cDNA template. The annealing temperature in the thermal cycler was 60 °C, and 50 cycles were performed. The primer pairs used in the experiments are presented in Table 1. All primers were synthesized by Bioneer (Daejeon, Korea). Final PCR products were detected by electrophoresis in 1.8% agarose gels with Ethidium Bromide staining. All the PCR products were purified using a PCR purification kit (Qiagen) and the purified products were sequenced to confirm the amplified sequences. Negative controls for checking contamination from the genomic DNA were ascertained by running without the reverse transcriptase for every set.

Single-cell real-time RT-PCR

All procedures before real-time PCR amplification were carried out in the same way as described in single-cell RT-PCR. Single-cell real-time PCR was performed using single cells harvested from the slices by means of the StepOnePlus System (Applied Biosystems) with SYBR Green detection. Primers for rat Kv1.2, Kv1.3, Kv4.2, and Kv4.3 subunits were designed using Primer Express 2.0 (Applied Biosystems) and synthesized in Cosmo Genetech (Kyonggi-do, Korea). Primer sequences are presented in Table 2. The real-time PCR reactions contained (in μl): 2 of cDNA template, 0.4 of 10 μM of each forward and reverse primer, 0.4 of 50× Rox dye and 10 of 2× SYBR master mix (SYBR premix Ex Taq, Takara Bio Inc., Shiga, Japan), and 6.8 of nuclease-free water. The thermal protocol followed was a pre-denaturation at 95 °C for 10 s, amplification with 50 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 31 s, and dissociation stage programmed in the system for melt curve analysis for PCR product specificity. Negative controls to check contamination from the genomic DNA were ascertained by running RT without the reverse transcriptase. The threshold cycle (Ct) values for transcripts were calculated using StepOne Software 2.0 (Applied Biosystems). All samples were run in duplicate. Ct values that showed a different Tm or two peaks were all discarded.

Quantifying the relative difference in target gene expression between type I and type II neurons was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer efficiencies for each target and reference gene were calculated using the equation $E=10^{[-1/slope]}$ to apply the $2^{-\Delta\Delta Ct}$ method to the relative quantification in gene expression (Livak and Schmittgen, 2001; Pfaffl, 2001; Zhang et al., 2009; Sonner et al., 2011).