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MOLECULAR CHARACTERIZATION OF T-TYPE Ca^{2+} CHANNELS RESPONSIBLE FOR LOW THRESHOLD SPIKES IN HYPOTHALAMIC PARAVENTRICULAR NUCLEUS NEURONS

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Abstract—The hypothalamic paraventricular nucleus (PVN) is composed of functionally heterogeneous cell groups, possessing distinct electrophysiological properties depending on their functional roles. Previously, T-type Ca^{2+} dependent low-threshold spikes (LTS) have been demonstrated in various PVN neuronal types, including preautonomic cells. However, the molecular composition and functional properties of the underlying T-type Ca^{2+} channels have not been characterized. In the present study, we combined single cell reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry and patch-clamp recordings to identify subtypes of T-type Ca^{2+} channels expressed in PVN cells displaying LTS (PVN-LTS), including identified preautonomic neurons. LTS appeared at the end of hyperpolarizing pulses either as long-lasting plateaus or as short-lasting depolarizing humps. LTS were mediated by rapidly activating and inactivating T-type Ca^{2+} currents and were blocked by Ni^{2+}. Single cell RT-PCR and immunohistochemical studies revealed Cav3.1 (voltage-gated Ca^{2+} channel) as the main channel subunit detected in PVN-LTS neurons. In conclusion, these data indicate that Cav3.1 is the major subtype of T-type Ca^{2+} channel subunit that mediates T-type Ca^{2+} dependent LTS in PVN neurons. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: preautonomic neurons, single cell RT-PCR, retrograde labeling, slice patch clamp, Cav3.1, nickel.

The hypothalamic paraventricular nucleus (PVN) is composed of heterogeneous cell groups, including magnocellular neuroendocrine, parvocellular neuroendocrine and parvocellular preautonomic neurons. These cell groups are involved in hormone secretion from the pituitary gland and regulation of the autonomic nervous system (Swanson and Sawchenko, 1980, 1983; Liposits, 1993). Among these cell groups, parvocellular preautonomic neurons project to autonomic centers in the brain stem and spinal cord, including the rostral ventrolateral medulla (RVLM), a key center of sympathetic regulation (Armstrong et al., 1980; Swanson and Kuypers, 1980; Porter and Brody, 1985, 1986; Kannan et al., 1988; Coote, 1995; Patel and Zhang, 1996; Blair et al., 1996).

Electrophysiological studies demonstrated that a low-threshold spike (LTS) is one of the common properties of parvocellular PVN neurons (Luther and Tasker, 2000; Luther et al., 2002) including preautonomic ones (Stern, 2001; Sonner and Stern, 2007). The LTS is generated by low voltage-activated T-type Ca^{2+} channels, which are activated by small depolarization of the plasma membrane. T-type Ca^{2+} channels modulate various aspects of neuronal function through regulating Ca^{2+} influx, leading to direct depolarization of the membrane, typically resulting in a burst of Na^{+} action potentials (Huguenard, 1996; Perez-Reyes, 2003). Biophysical and pharmacological studies indicated that PVN neurons show prominent bursts of spikes upon depolarization from hyperpolarized membrane potentials, presumably due to the activation of voltage dependent T-type Ca^{2+} currents (Luther and Tasker, 2000; Stern, 2001; Sonner and Stern, 2007).

Three subtypes of T-type voltage-gated Ca^{2+} channels (Cav): Cav3.1, Cav3.2, and Cav3.3, have been cloned and functionally characterized in neuronal tissues (Perez-Reyes et al., 1998; Lee et al., 1999; Talley et al., 1999; McRory et al., 2001). These subtypes possess different biophysical properties and regulate action potential generation, including pacemaking and bursting behavior (Wang et al., 1991; Huguenard and McCormick, 1992; Perez-Reyes, 1999). Widespread distributions of these three Cav3 subtypes have been demonstrated in distinct brain nuclei with different signal intensities (Craig et al., 1999; Kase et al., 1999; Talley et al., 1999; McKay et al., 2006). Furthermore, it has been demonstrated that the different expression pattern of Cav3 subtypes and different Ca^{2+} responses exist, depending on the subcellular location of the channels (Magee et al., 1995; Cavelier and Bossu, 2003; McKay et al., 2006).

Even though the electrophysiological and pharmacological properties of the LTS in PVN preautomonic neurons has been previously reported (Stern, 2001; Sonner and Stern, 2007), the molecular identification of ion channels responsible for LTS in those neurons remains to be determined. In the present study, we performed a molecular and functional characterization of the LTS and its underlying T-type Ca^{2+} current in PVN neurons using combined whole cell recordings and single cell reverse transcription-

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Abbreviations: ACSF, artificial cerebrospinal fluid; Cav, voltage-gated Ca^{2+} channel; CTB, cholera toxin b; LTS, low-threshold spikes; PBS, phosphate-buffered saline; PVN, paraventricular nucleus; RT-PCR, reverse transcription–polymerase chain reaction; RVLM, rostral ventrolateral medulla; TTX, tetrodotoxin.

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polymerase chain reaction (RT-PCR) in identified PVN neuronal populations.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (3–5 wk old) weighing 80–100 g were purchased from Samtacho (Kyonggi-do, Korea). The rats were maintained under a 12-h light/dark cycle (lights on at 8:00 A.M.) and given free access to food and water. For the surgical procedure, anesthesia was induced by injecting the xylazine 2% and ketamine 5% (1:3, 2 ml/kg i.p.) mixture. All animal experiments were carried out in agreement with the protocol for the care and use of animals approved by the Laboratory Animal Care Advisory Committee of Seoul National University and the University of Cincinnati. All experiments were conducted to conform to international guidelines on the ethical use of animals and minimized the number of animals used and their suffering.

Hypothalamic slice preparation

Hypothalamic brain slices were prepared according to the methods described previously (Stern, 2001; Han et al., 2002). Briefly, the brains were pulled out 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, St. Louise, MO, USA). The slices were incubated in oxygenated ACSF for at least 1 h at 32 °C until recordings. The recordings were made at 30~33 °C. The composition of the ACSF for current clamp experiments was (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. For voltage clamp experiments of isolated T-type Ca^{2+} currents, the ACSF consisted of (in mM): 92 NaCl, 2.5 KCl, 1 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 20 D-glucose, 5 $CaCl_2$, 0.5 μM tetrodotoxin (TTX), 30 tetraethylammonium and 5 4-aminopyridine.

Electrophysiological recording

The location of PVN was determined by both the third ventricle and the shape or location of the optic chiasm and the fornix under the upright microscope (BX50WI, Olympus, Tokyo, Japan) with differential interference contrast. The identified PVN neurons in the coronal hypothalamic slices were recorded in the whole cell configuration. 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. For the recording of RVLM-projecting PVN neurons, the labeled neurons with fluorescent microspheres were identified using the same microscope equipped with an Hg-lamp and a 'green' filter cube (WG, Olympus). After a healthy looking neuron was selected as a target for recording, selected neurons 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 Axopatch 200B (Axon Instruments, Foster City, CA, USA). Current or voltage signals were filtered at 1 kHz and digitized at 10 kHz with using an analog-digital converter (Digidata 1200B, Axon Instruments) and a pClamp software (version 8.0, Axon Instruments). Membrane input resistance was obtained from the relations of the hyperpolarizing pulses (~$-$60 pA) applied to classify the cell types. For current clamp recordings, the resting membrane potentials were corrected for the liquid junction potential (~14.3 mV). For voltage clamp recordings, all protocols were run with an output gain of 2, a Bessel filter of 2 kHz, were leak subtracted (P/4), and the series resistance was electronically compensated at least 60% throughout the recordings. The voltage clamp data were corrected for liquid junction potential (6.5 mV), which was experimentally determined using a 2 M KCl agar bridge. T-type Ca^{2+} currents were activated using a series of depolarizing command pulses (from -70 mV to -20 mV, in 5 mV increments), from a hyperpolarizing conditioning pulse (-90 mV). At voltages more positive than -20 mV, a slower activating and non-inactivating Ca^{2+} current component (likely representing high-threshold voltage-activated Ca^{2+} currents, HVA) was generally observed. Thus, to prevent contamination of T-type Ca^{2+} currents with HVA Ca^{2+} currents, the maximum command pulse was restricted to -20 mV. T-type Ca^{2+} currents activation threshold was defined as the membrane potential at which a transient current $\geq$10 pA was detected. The rate of activation of T-type Ca^{2+} currents was determined by measuring the 10–90% rise time from the baseline to the peak of the current at a command pulse of -25 mV. The time constant (τ) of inactivation of T-type Ca^{2+} currents was determined by fitting a single exponential function to the decay phase of the current activated at a command pulse of -25 mV.

Retrograde labeling of RVLM projecting PVN neurons

Fluorescent microspheres (F-8793, red fluorescent, Molecular Probe Inc., Eugene, OR, USA) were used for injection of retrograde tracing into RVLM to label the RVLM projecting PVN neurons (PVN-RVLM). The skull of the rat was fixed into a stereotaxic frame after anesthesia. After exposing the surface of the skull by incising the skin of the head, a small hole was made with a dental drill. The injection point was 1.6 mm lateral to the midline, -11.0 mm from the bregma, and 8.0 mm below the dorsal surface. Fluorescent microspheres solution (100 nl) was injected with the pneumatic picopump (PV820-G, World Precision Instruments Inc., Sarasota, FL, USA). The radius of the tip of the pulled capillary was adjusted to 20–25 μm with 20 psi for 30 ms. If the injection site was outside of the RVLM, the experiment was excluded.

Single cell RT-PCR

Single cell RT-PCR was carried out as previously described with minor modification (Glasgow et al., 1999; Di et al., 2003; Shin et al., 2007). The cytoplasm of the neuron was pulled into a patch pipette with negative pressure under the visual control, taking care not to contain the nucleus. The cytoplasm in the pipette was again dissipated into a prepared microtube containing (in μl): 4 of nuclease free water, 1 of RNaseOUT (40 U/μl) and stored at -70 °C. After recording, the microtube was heated to 65 °C for 5 min and incubated on ice for at least 1 min. The mixture of (in μl) 1 of random hexamer (100 ng/μl), 4 of 5$\times$ first-strand buffer, 2 of dithiothreitol (0.1 M), 1 of mixed deoxy NTPs (dNTPs) (10 mM), 1 of SuperScript III reverse transcriptase was subsequently added and incubated at 50 °C for 50 min. The reaction was terminated by heating at 70 °C for 15 min and stored at -20 °C. All reagents except random hexamer (Promega, Madison, WI, USA) were purchased from Invitrogen (Carlsbad, CA, USA). PCR amplification was induced by using a fraction of the single cell cDNA as a template. Mixture of PCR reaction contained (in μl): 1 of 25 μM each primer, 12.5 of 2$\times$ master mix buffer (Go *Taq* Green Master Mix, Promega), 1 of dimethyl sulfoxide and 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 were 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, Valencia, CA, USA) and the purified prod-

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