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INWARDLY RECTIFYING POTASSIUM CHANNEL KIR4.1 IS LOCALIZED AT THE CALYX ENDINGS OF VESTIBULAR AFFERENTS

T. UDAGAWA,[a,b] N. TATSUMI,[b] T. TACHIBANA,[b] Y. NEGISHI,[b] H. SAIJO,[b,c] T. KOBAYASHI,[a] Y. YAGUCHI,[a] H. KOJIMA,[a] H. MORIYAMA[a] AND M. OKABE[b]*

[a] *Department of Otorhinolaryngology, The Jikei University School of Medicine, Tokyo 105-8461, Japan*

[b] *Department of Anatomy, The Jikei University School of Medicine, Tokyo 105-8461, Japan*

[c] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan*

Abstract—Inwardly rectifying potassium (Kir) channel Kir4.1 (also called Kcnj10) is expressed in various cells such as satellite glial cells. It is suggested that these cells would absorb excess accumulated K^+ from intercellular space which is surrounded by these cell membranes expressing Kir4.1. In the vestibular system, loss of Kir4.1 results in selective degeneration of type I hair cells despite normal development of type II hair cells. The mechanisms underlying this developmental disorder have been unclear, because it was thought that Kir4.1 is only expressed in glial cells throughout the entire nervous system. Here, we show that Kir4.1 is expressed not only in glial cells but also in neurons of the mouse vestibular system. In the vestibular ganglion, *Kir4.1* mRNA is transcribed in both satellite cells and neuronal somata, whereas Kir4.1 protein is expressed only in satellite cells. On the other hand, in the vestibular sensory epithelia, Kir4.1 protein is localized at the calyx endings of vestibular afferents, which surround type I hair cells. Kir4.1 protein expression in the vestibular sensory epithelia is detected beginning after birth, and its localization gradually adopts a calyceal shape until type I hair cells are mature. Kir4.1 localized at the calyx endings may play a role in the K^+-buffering action of vestibular afferents surrounding type I hair cells.

Key words: inwardly rectifying potassium channel, Kir4.1, synapse, calyx ending, sensation of balance, inner ear.

INTRODUCTION

Vestibular afferents transmit essential information for the balance into the brain. In the vestibular ganglion, these neuronal somata are surrounded by satellite glial cells. In the vestibular sensory epithelia, vestibular afferents form synapses with vestibular hair cells. These hair cells differ in terms of their innervation. Type I hair cells are innervated by vestibular calyx afferents, the endings of which have a unique morphology with a calyceal shape, whereas type II hair cells are innervated by vestibular bouton afferents (Eatock et al., 1998, 2008). It is known that type I hair cells gradually mature with changes in K^+ conductances until 3 weeks after birth (Hurley et al., 2006).

In the vestibular sensory epithelia, the flow of K^+ ions plays a major role in the transmission of information related to the sensation of the balance. Head movement and tilt causes vibration of endolymph, which contains high concentrations of K^+. This mechanical stimulation opens mechanoelectrical channels on the apical membranes of hair cells and K^+ flows from the endolymph into hair cells. K^+ is released from type I hair cells into the calyceal synaptic cleft (Lim et al., 2011). It is considered that the accumulation of K^+ in the calyceal synaptic cleft plays a role in calyceal synaptic transmission, except for that mediated by glutamic acid, and can lead to depolarization of the calyx ending (Goldberg, 1996; Soto et al., 2002; Holt et al., 2007; Lim et al., 2011). Although it has not yet been revealed where this accumulated K^+ goes, it is assumed that K^+ channels expressed in the calyx ending allow passage of K^+ into peripheral vestibular afferents (Kharkovets et al., 2000).

Inwardly rectifying potassium (Kir) channel Kir4.1 (also called Kcnj10) is expressed in various glial cells such as brain astrocytes, retinal Müller cells, cochlear Deiters' cells and satellite glial cells (Hibino et al., 1997, 1999; Ishii et al., 1997; Higashi et al., 2001). It is suggested that these cells would absorb excess accumulated K^+ from intercellular space which is surrounded by these cell membranes expressing Kir4.1 (Hibino and Kurachi, 2006; Hibino et al., 2010). Mutations in the *KIR4.1* cause epilepsy, ataxia, sensorineural deafness, and tubulopathy (EAST) syndrome (Bockenhauer et al., 2009). Although it is considered that ataxia such as gait disturbance of EAST syndrome is caused by developmental disorder of the central nervous system (Bockenhauer et al., 2009), *Kir4.1* knockout mice also show hypertrophic degeneration of the peripheral vestibular calyx afferents (Rozengurt et al., 2003). It has been thought that degeneration of these neurons occurs secondary to a developmental disorder of satellite glial cells, because Kir4.1 is only expressed in satellite cells in the vestibular system (Rozengurt et al., 2003). *Kir4.1* knockout mice show

*Corresponding author. Address: Department of Anatomy, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan. Tel: +81-3-3433-1111x220; fax: +81-3-3433-2065.
E-mail address: maokabe@jikei.ac.jp (M. Okabe).

Abbreviations: EAST, epilepsy, ataxia, sensorineural deafness, and tubulopathy; Kir, inwardly rectifying potassium; P, postnatal day; PBS, phosphate buffer saline; PCR, polymerase chain reaction; RT, room temperature.

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another unique developmental disorder in vestibular sensory epithelia; that is, type I hair cells selectively show apoptotic nuclei but type II hair cells develop normally (Rozengurt et al., 2003). To understand the mechanisms underlying these developmental disorders, we carefully observed the localization of *Kir4.1* mRNA and protein in mouse pups.

EXPERIMENTAL PROCEDURES

All experimentation on animals was approved by The Jikei University School of Medicine's Animal Care and Use Committee.

Tissue preparation

C57BL/6J mice at post-natal day 0, 4, 8 and 21 (referred to as P0, P4, P8 and P21, respectively) were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The inner ears were dissected, further perfused through the round window with the same fixative, postfixed in the same solution at 4 °C for 12 h, washed three times for 5 min in 0.1 M PBS, decalcified P8 and P21 samples only in 0.5 M EDTA/PBS, pH 8 (24 h for P8 or 48 h for P21 at 4 °C), washed three times for 5 min in 0.1 M PBS, dehydrated in 30% sucrose in PBS at 4 °C and then embedded in Tissue Tek OCT compound (Sakura, Tokyo, Japan) and frozen. Mid-modiolar sections (30 μm thickness) were prepared using a cryostat (CM3050 S, Leica, Bensheim, Germany).

Isolation of cDNA encoding Kir4.1

Total RNA was isolated from a whole mouse embryo at embryonic day 16.5 using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was reverse transcribed from total RNA using the PrimeScript® II 1st strand cDNA Synthesis Kit (Takara Bio, Tokyo, Japan). cDNA for mouse *Kir4.1* (Genbank accession No. NM_001039484) was isolated using specific primers (forward primer, GGACAAACCCTTATCTGATTCCA; reverse primer, TGCGCAATAAGAAGCACGAT), and amplified by polymerase chain reaction (PCR) using Blend Taq plus (Toyobo, Osaka, Japan). PCR was performed under the following conditions: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, for 30 cycles. PCR products were ligated into pGEMT-easy plasmid vector (Promega, Madison, WI, USA) and sequenced.

In situ hybridization

A digoxigenin-labeled antisense RNA probe was synthesized using the DIG RNA Labeling Kit (SP6, Promega, Madison, WI, USA) with plasmids containing the genes for *Kir4.1*. Section *in situ* hybridization was performed as previously described (Riddle et al., 1993) with some modifications. Briefly, slides were permeabilized with protein K (Roche, Mannheim, Germany, 5 μg/ml) in PBS with 0.1% Tween 20 for 10 min at 37 °C, incubated with 1 μg/ml digoxigenin-labeled riboprobe in hybridization buffer for 16 h at 70 °C, blocked with 10% heat-inactivated sheep serum in Tris-buffered saline containing 0.1% Tween 20 for 30 min at room temperature (RT), incubated with anti-digoxigenin antibody-conjugated alkaline phosphatase (1:2000, Roche, Mannheim, Germany) in Tris-buffered saline containing 0.1% Tween 20 and 1% heat-inactivated sheep serum for 2 h at RT. Antibody detection was performed by incubating slides with 0.2% nitroblue tetrazolium and 0.2% 5-bromo-4-chloro-3-indolyl-phosphate toluinium in detection solution (0.1 M NaCl, 0.1 M Tris–HCl [pH 9.5], 50 mM $MgCl_2$, 1% Tween 20) for 40–48 h at RT.

Immunohistochemistry

Slides were blocked for 30 min with 10% normal goat or donkey serum with 1% bovine serum albumin in PBS containing 0.1% Triton X-100. The following primary antibodies were used: rabbit anti-Kir4.1 (1:200, APC-035, lot number AN-05, Alomone Labs, Jerusalem, Israel or AB5818, lot number JC1668357, Chemicon, Temecula, USA), mouse anti-nestin (1:200, LV1797294, Millipore, Temecula, USA), mouse anti-βIII tubulin (1:1000, MMS-435P, Covance, Princeton, NJ, USA), mouse anti-KCNQ4 (1:100, ab84820, Abcam, Cambridge, UK), goat anti-calretinin (1:500, AB1550, Millipore, Bedford, MA, USA). Primary antibody treatments were applied overnight in a humidified chamber at 4 °C. For monoclonal mouse antibodies, an additional IgG blocking step was performed using the Mouse-on-Mouse kit (BMK-2202, Vector Laboratories, Burlingame, CA, USA). Primary antibodies were detected using the following fluorescent secondary antibodies: Alexa Fluor 488 goat or donkey anti-rabbit IgG (1:1000, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 555 streptavidin or donkey anti-goat IgG (1:1000, Molecular Probes, Eugene, OR, USA). Fluorescent secondary antibodies were applied for 2 h at RT, and sections were examined on a laser scanning confocal microscope (LSM510, Zeiss, Jena, Germany).

Immunoelectron microscopy

Tissue preparation and blocking of pre-embedding immunohistochemistry was performed as described above except the fixative used was 4% paraformaldehyde plus 0.1% glutaraldehyde. The primary antibody, rabbit anti-Kir4.1 (1:100, Alomone Labs, Jerusalem, Israel APC-035, lot number AN-05), was applied overnight at 4 °C. The secondary antibody, 1.4 nm gold-conjugated Fab' fragment of goat anti-rabbit IgG (1:40, Nanoprobes Inc., Stony Brook, NY, USA), was applied overnight at 4 °C and sections were postfixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.2 for 30 min at RT. Furthermore, and HQ silver-enhancement kit (Nanoprobes Inc., Stony Brook, NY, USA) was used to visualize the gold particles in the dark for 5 min at RT. Reactions were stopped by three rinses in distilled water, and sections were postfixed in 2.5% glutaraldehyde in phosphate buffer for 30 min at RT, dehydrated through an ascending ethanol series (70%, 90%, 2 × 100% ethanol), immersed in *n*-butyl glycidyl ether and then embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). The blocks were trimmed to an area of approximately 0.5 mm and serially sectioned at a thickness of 100 nm using a Reichert OMU4 ultramicrotome (Leica UK Ltd., Milton Keynes, UK). The thin sections were stained with uranyl acetate and lead citrate, and examined at 60 kV using an electron microscope (JEM-1200EX-II, JEOL, Tokyo, Japan).

RESULTS

Kir4.1 mRNA is transcribed in the neuronal somata of the vestibular ganglion

It has not been shown where in the vestibular system *Kir4.1* mRNA is transcribed. To identify the expression pattern of *Kir4.1* in the mouse vestibular system, we performed *in situ* hybridization at P21 when the vestibular sensory epithelia ear is mature (Hurley et al., 2006). We observed high expression of *Kir4.1* throughout the entire vestibular ganglion (Fig. 1A). In a close-up view of the vestibular ganglion, *Kir4.1* expression was detected in both satellite cells and neuronal somata (Fig. 1B, arrowheads and arrow). Expression of Kir4.1 protein has not been reported in any neurons, but it is generally well known to be expressed in glial cells including satellite