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SUPPRESSION OF K_V7/KCNQ POTASSIUM CHANNEL ENHANCES NEURONAL DIFFERENTIATION OF PC12 CELLS

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Abstract—Membrane potential shift driven by electrical activity is critical in determining the cell fate of proliferation or differentiation. As such, the ion channels that underlie the membrane electrical activity play an important role in cell proliferation/differentiation. K_V7/KCNQ potassium channels are critical in determining the resting membrane potentials in many neuronal cells. However, the role of these channels in cell differentiation is not well studied. In the present study, we used PC12 cells as well as primary cultured rat cortical neurons to study the role and mechanism of K_V7/KCNQ in neuronal differentiation. NGF induced PC12 cell differentiation into neuron-like cells with growth of neurites showing typical growth cone-like extensions. The Kv7/KCNQ blocker XE991 promoted NGF-induced neurite outgrowth, whereas Kv7/KCNQ opener retigabine (RTG) inhibited outgrowth. M-type Kv7 channels are likely involved in regulating neurite growth because overexpression of KCNQ2/Q3 inhibited neurite growth whereas suppression of KCNQ2/Q3 with shRNA promoted neurite growth. Membrane depolarization possibly underpins enhanced neurite growth induced by the suppression of Kv7/KCNQ. Additionally, high extracellular K^+ likely induced membrane depolarization and also promoted neurite growth. Finally, T-type Ca^{2+} channels may be involved in membrane-depolarization-induced neurite growth. This study provides a new perspective for understanding neuronal differentiation as well as K_V7/KCNQ channel function.

Keywords: KV7/KCNQ potassium channel, neuron, differentiation, neurite.

INTRODUCTION

Selective gene expression and gradients of signaling molecules are important for promoting cell differentiation, but ion channels involved in membrane potential control are also critical for promoting proliferation or differentiation of a cell. Generally speaking, immature cells (such as glia progenitor cells or immature neurons) that rarely enter mitosis often have a positive resting membrane potential (RMP) compared to differentiated cells (such as oligodendrocytes, astrocytes or differentiated neurons). In contrast, dividing cells often have a negative RMP (Cone, 1970; Sontheimer et al., 1989; Bordey and Sontheimer, 1997; Root et al., 2008). Furthermore, the RMP can be a factor in the differentiation process. For example, the RMP of mouse embryonic stem cells (mESCs) becomes more depolarized during the initial phase of differentiation but gradually repolarizes at the mid and later stages of differentiation. The differentiated cells are also more hyperpolarized than undifferentiated mESCs (Ng et al., 2010).

Voltage-gated K channels (K_V channels) reportedly play a key role in determining the fate of mESCs through regulation of RMP (Ng et al., 2010). In these mESCs, blocking the K_V channels leads to the loss of pluripotency and to differentiation. K_V7/KCNQ is a family of K_V channels with 5 members. $K_V7.2$–$K_V7.5$ are expressed in the nervous system and are M-type K channels (Wang et al., 1998; Lerche et al., 2000; Brown and Yu, 2000; Jentsch, 2000; Delmas and Brown, 2005; Brown and Passmore, 2009). M channels are activated at sub-threshold potentials and provide a profound dampening effect on repetitive or burst firing as well as the general excitability of neurons (Gutman et al., 2003; Spitzer, 2006). M channels are the key component in determining the RMP of neurons (Delmas and Brown, 2005; Du et al., 2014).

PC12 is a cell model widely used to study neuronal differentiation (Greene, 1978). PC12 cells express K_V7/M currents (Villarroel et al., 1989; Villarroel, 1996; Dai et al., 2013) and high and low threshold type Ca^{2+} currents (L, T and N type Ca^{2+} currents) (Takahashi et al., 1985; Kongsamut and Miller, 1986; Plummer et al., 1989). Nerve Growth Factor (NGF) induces neuronal differentiation (Colombo et al., 2014) and modulates K_V7/M currents (Jia et al., 2008) and T type Ca^{2+} currents (Garber et al., 1989). In light of these findings, we investigated the role of the K_V7/KCNQ channel in cell differentiation in this study using both the neurons differentiated from PC12 cells induced with NGF and primary cultured cortical neurons.

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Abbreviations: ESCs, embryonic stem cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MP, membrane potentials; NGF, Nerve Growth Factor; qPCR, quantitative real-time PCR; RMP, resting membrane potential; RTG, retigabine; VGCC, voltage-gated Ca2+ channels.

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EXPERIMENTAL PROCEDURES

PC12 cell culture and differentiation

The pheochromocytoma cell line PC12 was maintained in Ham's F12K medium with 1.5 g/l L-glutamine, 10% horse serum, and 2.5% fetal bovine serum at 37 °C. Neuronal differentiation was induced based on a protocol reported previously (Greene and Tischler, 1976). Briefly, PC12 cells were inoculated on collagen-coated cover slips at a density of 1×10^5 cells/ml and differentiation was induced by adding 50 ng/ml NGF in Ham's F12K medium with 1.5 g/l L-glutamine, 2.5% fetal bovine serum at 37.0 °C. After 48 h, the cells were examined.

Primary culture of cortical neurons

The meninges were removed from the cortical of newborn 24-h and 7-day-old Sprague Dawley rats (provided by the Experimental Animal Center of Hebei Province), washed with serum-free medium, cut into pieces and digested with collagenase and dispase for 30 min. The dispersed cells were seeded at a density of 1×10^6/ml, 100 μl/slip on a collagen-coated cover slip. The cells were cultured with Ham's F12K medium with B27, 2.5% fetal bovine serum at 37.0 °C. The medium was changed 4 h later and then changed every 3 days.

Measurement of neurite growth

For measuring neurite length, digital images of cells from a random selection of 10× fields were captured and analyzed with an Olympus X71 microscope and Olympus Excellence System (Olympus, Tokyo, Japan). The neurite was counted when it was longer than the diameter of the cell body. The average length of neurites was calculated from 500 cells randomly selected from each experimental group. Length was defined as the maximum possible distance along a neurite, i.e., the distance from the soma to the end of the longest neurite (if more than one neurite was present) and to the end of the longest branch at each branch point for neurites with branches. The average length of the neurite in each group was calculated by dividing total length by the total number of the cells. The average neurite number of each cell was calculated by the total neurite number divided by the total cell number of each group. The average number of cells with neurites was calculated by dividing the total number of cells with neurites by the total number of cells counted in each group.

Measurement of mRNA levels of KCNQ2 and KCNQ3 in PC12 cells

mRNA levels of KCNQ2 and KCNQ3 were analyzed by Quantitative Real-Time PCR (qPCR). qPCR analysis was performed in a Bio-Rad iCycler Thermal Cycler (BioRad, Cal, USA). The primer sequences used were as follows:

KCNQ2, sense 5′-GCTACGGTGTGGACAAGAAG-3′
antisense 5′-TGGAAGCAGGGAAGAAATCAG-3′;
KCNQ3, sense 5′-GCTACGGTGTGGACAAGAAG-3′
antisense 5′-TGGAAGCAGGGAAGAAATCAG-3,

The PCR conditions were the following: 95 °C, 15 s; 95 °C, 5 s, and 60 °C, 30 s for 45 cycles; 94 °C, 22 s; 62 °C, 30 s; and 72 °C, 5 min for 1 cycle. Melt curves were obtained by increasing the temperature from 55 to 95 °C in 0.5 °C steps for 30 s. Expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression of mRNA was calculated with the $2^{-\Delta\Delta CT}$ method.

Overexpression of KCNQ2 and KCNQ3

KCNQ2 and KCNQ3 cDNA were subcloned in pCDNA3.1, and the plasmids were transfected into PC12 cells using the Amaxa® Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) by electroporation with a Nucleofector® Device (Lonza, Basel, Switzerland). Briefly, 2 μg DNA was added to 100 μl of cell suspension, and the cell/DNA suspension was transferred into a certified cuvette and electroporated with an appropriate Nucleofector® Program (U-029). Then, the suspension was immediately added to 500 μl of pre-equilibrated culture medium and gently transferred into a 12-well plate and incubated in a humidified incubator in 37 °C with 5% CO_2 until analysis.

Lentivirus-mediated KCNQ2 and KCNQ3 suppression

Lentiviral vectors (GV112) encoding three distinct shRNA targeting KCNQ2 or KCNQ3 genes were designed to depress the expression of KCNQ2 or KCNQ3 in PC12 cells. For KCNQ2 (Accession: NM_133322), three distinct shRNA target sequences were as follows: GGAAGCCATTCTGTGTGAT, GCTCACAAACTCAAAC CTA, and GCAAATCTGGACTCACCTT. For KCNQ3 (Accession: NM_031597), three distinct shRNA target sequences were as follows: GGATGTGCCAGA GATGGAT, GCTGTCAGAATTCTACAAT, and GCAGTATTCTGCTGGACAT. PC12 cells were plated in a 96-well plate and exposed to the lentivirus shRNA for 12 h at 37 °C. At 5 days post-transfection, the efficiency of shRNA was determined with the qPCR method.

Immunofluorescence

After three washes with PBS for 5 min, PC12 cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X100 for 30 min at room temperature and subsequently blocked with 5% BSA in TPBS (Tween 20 in PBS) for 1 h at room temperature. Cells were incubated at 4 °C overnight in the presence of mouse anti-rat β III Tub (1:400) primary antibodies. In the following day, after three washes with TPBS, goat anti-mouse FITC-IgG (1:500) secondary antibodies were added at room temperature for 1 h. Photomicrographs were taken with a Olympus X71 fluorescence microscope (Olympus, Tokyo, Japan). For primary cultured cortical neuron, cells were also labeled with DAPI to distinguish individual neurons. For this 1 μg/μl DAPI working solution was added to the neurons and stained for 10 min. The neurons were inspected after

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