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The multiple expression of Ca^{2+}-activated Cl^- channels via homo- and hetero-dimer formation of TMEM16A splicing variants in murine portal vein

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ABSTRACT

Ca^{2+}-activated Cl^- channel (CaCC) often plays substantial roles in the regulation of membrane excitability in smooth muscle cells (SMCs). TMEM16A, a member of the TMEM16 family, has been suggested as the molecular entity responsible for CaCC in several types of SMCs. In this study, the expression of TMEM16A splicing variants and their contribution to CaCC activity were examined in murine portal vein SMCs (mPVSMCs). Four transcripts of TMEM16A splicing variants, which include four alternatively spliced segments ("*a*" and "*b*" in N-terminus and "*c*" and "*d*" in the first intracellular loop), were identified; the expression ratio of four transcripts of "*abc*", "*acd*", "*abcd*" and "*ac*" was 64.5, 25.8, 4.8 and 4.8%, respectively. The immunostaining of isolated mPVSMCs with anti-TMEM16A antibody indicates the abundant expression of TMEM16A on the cell membrane. CaCC currents recorded in mPVSMCs were markedly reduced by $T16A_{inh}$-A01, a specific TMEM16A inhibitor. When the two major TMEM16A splicing variants, *abc* and *acd* isoforms, were expressed separately in HEK293 cells, the CaCC currents, which possess similar electrophysiological characteristics to those in mPVSMCs were observed. The single-molecule photobleaching analyses using total internal reflection fluorescence (TIRF) microscope indicated that the distribution of stepwise photobleaching events was fit well with a binomial distribution for homodimer. Additionally, the heterodimer formation was suggested by fluorescence resonance energy transfer (FRET) analyses in HEK293 cells co-expressing CFP- or YFP-tagged variants. In conclusion, alternatively spliced variants of TMEM16A *abc* and *acd* in mPVSMCs are two major molecular entities of CaCC and may form hetero-/homo-dimers to be functional as CaCC in the regulation of membrane excitability and contractility in mPVSMCs.

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1. Introduction

Ca^{2+}-activated Cl^- channels (CaCCs) are expressed ubiquitously in various types of cells and play wide variety of physiological roles including fluid secretion, neuronal excitation, and smooth muscle (SM) contraction. Particularly in vascular SM cells (SMCs), the activation of CaCC contributes to the shift of resting membrane potential to positive direction and regulates Ca^{2+} influx through voltage-dependent Ca^{2+} channels, resulting in the increase in muscle tone in several arteries and veins [1]. Portal vein shows spontaneous contractions, which substantially contribute to the blood flow from mesenteric vascular beds to liver and the application of CaCC blockers markedly reduces the portal vein contractions [2]. Therefore, the identification of molecular entity of CaCC in portal vein, is one of the emergent issues for obtaining the comprehensive understanding of mechanisms underlying the physiological and pathophysiological regulations of gastro-liver circulation.

Recently, TMEM16A and TMEM16B, which belong to TMEM16 family, have been identified as the counterpart of classic CaCC [3–5]. TMEM16A is expressed in various tissues including vascular SMs and expected to regulate their functions [6–8]. The pharmacological blockade of TMEM16A leads to muscle relaxation in thoracic aorta and mesenteric artery [9]. TMEM16A has eight putative transmembrane domains and is presumed to form dimers as a functional CaCC [10,11]. In addition, TMEM16A has at least four alternatively spliced segments (named segments: *a*, *b*, *c*, and *d*). The inclusion or skipping of these segments alters electrophysiological properties of TMEM16A CaCC activity, while the details have not been elucidated yet. The segment *b* includes putative calmodulin binding domain and segment *c* affects voltage dependence [12,13]. Moreover, skipping of segment *d* changes activation kinetics [14]. Thus, TMEM16A can produce multiple protein isoforms and may differentially contribute to CaCC activity.

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However, the proportional expression of each TMEM16A splicing variant and the interaction between different variants have been totally unknown regardless of cell types.

The present study was undertaken to elucidate the molecular entities of CaCC in murine portal vein smooth muscle cells (mPVSMCs). We found that TMEM16A splicing variants were expressed and substantially responsible for CaCC activity in mPVSMCs. Results suggest that the mixed expression of homodimers and heterodimers may determine the features of CaCC activity in mPVSMCs.

2. Materials and methods

2.1. Cell isolation

All experiments were approved by the Ethics Committee of Nagoya City University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society. The portal veins were dissected from male mice (C57BL/6, 8–12 weeks; Japan SLC, Hamamatsu, Japan) and incubated in Ca^{2+}/Mg^{2+}-free Hanks solution for 10 min at 37 °C. Then, the tissues were incubated in Ca^{2+}/Mg^{2+}-free Hanks solution containing 0.3% collagenase type IA, 0.2% trypsin inhibitor, 0.2% bovine serum albumin, and 0.01% protease type XIV (Sigma–Aldrich, St. Louis, USA) for 10 min at 37 °C. After incubation, myocytes were isolated by gentle agitation with a glass pipette.

2.2. Cell culture

HEK293 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma–Aldrich) at 37 °C.

2.3. Plasmid constructs

The cDNAs encoding mouse TMEM16A spliced variants were cloned from portal veins and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, USA), pEYFP-N1, pECFP-N1, and pEGFP-N1 (Clontech Laboratories, Mountain View, USA). HEK293 cells were transiently transfected with cDNA using LipofectAmine 2000 (Invitrogen). Experiments were preformed 24–72 h after transfection.

2.4. Electrophysiological recordings

Electrophysiological studies were performed using a whole cell voltage-clamp technique with an EPC-7 amplifier (HEKA Electronics, Darmstadt, Germany), an analog–digital converter (Digidata 1440A), and pCLAMP software (version 10.2; Axon Instruments, Foster City, USA), as described previously [15,16].

2.5. RT-PCR and real-time PCR

Total RNA was extracted from murine portal veins and used for reverse transcription (RT). Following PCR primer set was used for spliced variant analysis of mouse TMEM16A (GenBank Accession No. NM_178642): (+) 5′-ATG CAG GAC GCG CAG GAC AGC GA-3′ and (−) 5′-AAG ATG ATG GAG ACA AGA TTG GT-3′. Quantitative real-time PCR analysis was performed using the SYBR Green assay (SYBR Premix Ex Taq; Takara Bio, Otsu, Japan) on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, USA). Specific PCR primers were designed as follows: mouse TMEM16A (NM_178642, 2649–2781), TMEM16B (BC033409, 2374–2494), TMEM16C (NM_001128103, 2575–2686), TMEM16D (NM_178773, 2109–2221), TMEM16E (NM_177694, 2286–2386), TMEM16F (NM_175344, 2388–2493), TMEM16G (NM_207031, 2179–2283), TMEM16H (NM_001164679, 2221–2328), TMEM16J (NM_178381, 2009–2112), TMEM16K (NM_133979, 1601–1702), and GAPDH (NM_008084, 730–833).

2.6. Immunocytochemistry

Immunocytochemical experiment was performed using anti-TMEM16A antibody (ab16293, 1:50 dilution, Abcam, Cambridge, USA) and Alexa Fluor 488 anti-chicken IgG (A11039, 1:1000 dilution, Molecular Probes, Eugene, USA) [15]. Confocal images were obtained using a laser scanning confocal fluorescent microscope (A1R) equipped with a fluorescent microscope (ECLIPSE Ti), an objective lens (Plan Apo 60× 1.40 NA, oil immersion) and NIS Elements software (version 3.10; Nikon, Tokyo, Japan).

2.7. TIRF imaging and FRET analysis

Single-molecule imaging was performed with total internal reflection fluorescence (TIRF) imaging system, which consisted of a fluorescent microscope (ECLIPSE TE2000-U; Nikon), an objective lens (CFI Apo TIRF 60×/1.45, oil immersion; Nikon), an EM-CCD camera (C9100-12; Hamamatsu Photonics, Hamamatsu, Japan), and AQUACOSMOS software (version 2.6; Hamamatsu Photonics) [15]. The resolution of images was 178 nm/pixel (x–y). Efficiency of fluorescence resonance energy transfer (FRET) was evaluated based on the acceptor photobleaching method, as previously reported [15]. The fluorescence of YFP was photobleached for 2 min. TIRF images were acquired for 4650 ms. FRET efficiency (E_{FRET}) was calculated using the following equation: $E_{FRET}\ (\%) = [(CFP_{after} - CFP_{before})/CFP_{after}] \times 100$, where CFP_{after} and CFP_{before} are CFP emissions after and before YFP photobleaching, respectively.

2.8. Single-molecule GFP bleaching

The oligomerization of TMEM16A was examined by the single-molecule photobleaching method [15,17]. TMEM16A-trasnfected HEK293 cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. TIRF images with the resolution of 178 nm/pixel were scanned every 100 ms. Fluorescent intensity in a region of interest (ROI, 3 × 3 pixels) was calculated by subtracting the background in 16 pixels around the ROI.

2.9. Solutions

Ca^{2+}/Mg^{2+}-free Hanks solution contained (in mM) 137 NaCl, 5.4 KCl, 0.17 Na_2HPO_4, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, and 5.6 glucose. For electrophysiological recordings, the bath solution contained (in mM) 137 NaCl, 5.9 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, 20 TEA-Cl, and 10 HEPES. The pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 120 CsCl, 20 TEA-Cl, 2.8 $MgCl_2$, 10 HEPES, 2 $ATPNa_2$, 5 EGTA, and 4.25 $CaCl_2$ (pCa 6.0). The pH was adjusted to 7.2 with CsOH.

2.10. Chemicals

Pharmacological reagents were obtained from Sigma–Aldrich except for $T16A_{inh}$-A01 (Tocris Bioscience, Bristol, UK). $T16A_{inh}$-A01 was dissolved in dimethyl sulfoxide at the concentration of 10 mM as a stock solution.

2.11. Statistics

Data are expressed as mean ± SE. Statistical significance between two groups was determined using Student's t-test.

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