

TMEM16A contributes to angiotensin II-induced cerebral vasoconstriction via the RhoA/ROCK signaling pathway

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Abstract. Calcium activated chloride channels (CaCCs) are critical in vascular smooth muscle function as they regulate proliferation/apoptosis of smooth muscle cells (SMCs) and vascular tone. Transmembrane protein 16A (TMEM16A) was demonstrated to encode CaCCs in basilar artery SMCs (BASMCs) and participate in basilar artery remodeling during hypertension. In addition, TMEM16A has recently been illustrated to contribute to pressure-induced myogenic response in cerebral vasculature. However, whether TMEM16A is involved in cerebral vasoconstriction that is stimulated by other vasoconstrictors remains unclear. The aim of the present study was to establish whether TMEM16A is involved in the progression of angiotensin II (Ang II)-induced basilar artery constriction and elucidate its potential role during hypertension. The study demonstrated that the specific inhibitor of TMEM16A, T16A-inhA01 attenuated Ang II-induced constriction in rat basilar arteries, and that this effect was weakened in parallel with the decline of TMEM16A expression in basilar arteries of 2-kidney, 2-clip hypertensive rats. Furthermore, it was found that 100 nM Ang II evoked a chloride current in cultured BASMCs with a basal 100-nM intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) level. In addition, the current could be abolished by TMEM16A small interfering RNA pretreatment and Ang II receptor type 1 (AT1) receptor blocker, losartan, while Ang II failed to cause a further increase to Ca^{2+} -dependent Cl^- currents activated by 500 nM $[\text{Ca}^{2+}]_i$. In addition, in cultured BASMCs, Ang II

induced phosphorylation of myosin phosphatase-targeting subunit 1, and myosin light chains were significantly enhanced by TMEM16A overexpression, which were reversed by Rho-associated protein kinase (ROCK) inhibitor, Y-27632, while TMEM16A silencing demonstrated an opposing result. Furthermore, Ang II-induced RhoA activation was enhanced by TMEM16A overexpression. In conclusion, the present study revealed that Ang II elicited a TMEM16A-mediated current and TMEM16A participated in Ang II-induced basilar constriction via the RhoA/ROCK signaling pathway.

Introduction

Hypertension is currently one of the most serious public health challenges worldwide. It is a major risk factor for cardiovascular diseases, myocardial infarction, stroke and chronic renal failure (1). Vascular tone, which represents the contractile activity of vascular smooth muscle cells (VSMCs) in the small arteries and arterioles, is the major determinant of vascular resistance to blood flow through the circulation, rendering it a particularly important component of hypertension (2). Enhanced vascular contractility contributes to the long-term control of systemic blood pressure (3), and impaired cerebral vasoconstriction during hypertension affects brain regional blood flow, which makes it a risk factor for stroke (4). Ion channels have been demonstrated to be critical in vascular tone and the development of hypertension, and numerous investigations into the role of ion channels in arterial smooth muscle contractility have been conducted. In these studies, cation channels, including calcium channel, voltage-dependent, L type, $\alpha_1\text{C}$ subunit, various K^+ channels (5) and nonselective transient receptor potential (TRP) channels (6) have been found to regulate vascular contraction (1). Anion channels, such as chloride channels are also involved. Chloride channels within the plasma membrane of VSMCs cause Cl^- efflux and membrane depolarization (7), and Ca^{2+} -activated chloride currents (CaCCs) may be involved in increasing vascular contractility. CaCC-dependent depolarization activates voltage-gated Ca^{2+} channels (8) and further increase intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), resulting in signal amplification, leading to VSMC contraction and contractile response of blood vessels. Transmembrane protein 16A (TMEM16A) was recently identified to be responsible for the CaCCs in basilar artery SMCs (BASMCs)

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and it is involved in the regulation of BASMCs proliferation during hypertension (9). Previous research regarding cerebral vasculature has revealed that pressure-induced membrane stretch may activate TMEM16A channels and thus contribute to the myogenic tone of basilar arteries (10). In addition to blood pressure, constriction of resistance arteries is also regulated by vasoconstrictors, such as angiotensin II (Ang II), thromboxane A₂ and 5-hydroxytryptamine; however, whether TMEM16A channels are also involved in vasoconstrictor-induced cerebral vasoconstriction remains unclear.

The renin-angiotensin system (RAS) is key in cardiovascular and renal physiology, and its overactivation is implicated in the induction and progression of hypertension (11). Circulating Ang II, the major contributing factor of the RAS, was significantly upregulated during hypertension (12), establishing it as an important and high-risk pathologic factor. Ang II acts directly on vascular smooth muscle as a vasoconstrictor in an either calcium-dependent or -independent manner; in the latter case, Ang II activates the RhoA/Rho-associated protein kinase (ROCK) signaling pathway and increases VSMC Ca²⁺ sensitization via Rho guanine nucleotide exchange factors (13), inducing phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1) and myosin light chains (MLCs), which finally causes SMC contraction. In a previous study, it was found that basilar artery constriction in response to Ang II was mediated by ROCK, while the vasoconstriction in response to KCl was not significantly influenced by this signaling pathway (14).

In the present study, the role of TMEM16A in Ang II-induced basilar artery vasoconstriction was investigated using its specific inhibitor, T16A-inhA01 (9,15). The basilar arteries were obtained from hypertensive rats at various time-points following 2-kidney, 2-clip (2k2c) surgery, and the influence of ROCK inhibitor, Y-27632 was also detected. Notably, the direct effect of Ang II on TMEM16A-mediated CaCC channels was determined in cultured BASMCs, and it was further revealed that the TMEM16A protein expression level affected Ang II-induced phosphorylation of MYPT1 and MLC via the RhoA/ROCK signaling pathway.

Materials and methods

Animal models and blood pressure measurement. The present study was approved by the ethics committee of Liuzhou People's Hospital (Liuzhou, China). All animal experimental procedures were performed according to the policies of the Second Medical University Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (16). Unless otherwise stated, all materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hypertensive rat models (2k2c) were established, as described previously (17). Briefly, 120 healthy male Sprague-Dawley rats (weight, 80–120 g; Laboratory Animal Centre, Guangxi Medical University, Nanning, China) were anaesthetized by sodium pentobarbital (40 mg/kg, i.p.), and following midline laparotomy, ring-shaped silver clips (internal diameter, 0.3 mm) were placed around the two renal arteries. All animals were maintained in a pathogen-free room with controlled temperature at ~25°C, under a 12-h light/dark cycle. Blood pressure was measured in conscious rats by tail-cuff

plethysmography (Powerlab 4/30; ADInstruments Pty Ltd., Bella Vista, NSW, Australia).

Basilar artery lumen diameter measurement. Following induction of deep anesthesia (pentobarbital; 200 mg/kg i.p.), the brain was removed from each of the rats and placed in Krebs buffer containing: 137 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl₂, 1.1 mM MgCl₂, 0.4 mM NaH₂PO₄, 5.6 mM glucose, 11.9 mM NaHCO₃, 105 U/l penicillin and 100 mg/l streptomycin, at 37°C. Basilar arteries were rapidly isolated and the connective tissue was carefully removed. As described previously (18), arteries were mounted onto glass micropipettes filled with Krebs buffer in an organ chamber, and allowed to equilibrate and reach a stable diameter for ≥30 min at a distending pressure of 60 mmHg. The stimuli and/or inhibitors were subsequently added. Ang II (10⁻¹¹–10⁻⁷ M) was added for 5 min for measurement and T16A-inhA01 (10 μM) and Y-27632 (10 μM) were added 5 min prior to Ang II stimulation. Vessel images were captured using an AxioImager Z1 microscope (Zeiss GmbH, Jena, Germany) and an S-100 video camera (Nikon Corporation, Tokyo, Japan), a VDA-10 electronic dimension analyzer (Living Systems Instrumentation, St. Albans, VT, USA) was then used to measure the lumen diameter.

Cell culture. Rat BASMCs were cultured from the rat basilar arteries, as previously described (19). Briefly, healthy male Sprague-Dawley rats (weight, 80–100 g) were anaesthetized with pentobarbital sodium (200 mg/kg i.p.). Basilar arteries were harvested immediately and immersed in iced Krebs solution. The vessels were isolated carefully and sliced into 0.2-mm rings in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) medium and the vessel segments were placed on the surface of the culture flask and incubated in DMEM/F12 medium supplemented with 20% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C, in an atmosphere of 5% CO₂ for 5–7 days. Cells from passage 3–6 were used in subsequent experiments.

Recording of CaCC currents in cultured BASMCs. The Ca²⁺-activated Cl⁻ current was recorded from cultured BASMCs using an Axopatch™ 200B Amplifier (Molecular Devices, LLC, Sunnyvale, CA, USA) according to a previously described, whole-cell patch clamp technique (20). The current was elicited using voltage steps from 60 to 100 mV in 20-mV increments for 250 msec from a holding potential of -40 mV and stored on a computer following digitalization at 5 kHz using a Digidata 1322A (Axon Instrument). The bath solution contained 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM NaCl, 140 mM NMDG-Cl, 10 mM HEPES and 10 mM glucose, which was adjusted to pH 7.4 using HCl. The Ca²⁺-buffered pipette solution contained 140 mM CsCl, 1 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, 4 mM Na₂-ATP, and 1.925 and 3.801 mM CaCl₂ to adjust the free Ca²⁺ concentration to 100 and 500 nM, respectively (calculated using the CaBuf program; www.jgp.org/cgi/content/full/jgp.201411339/DC1), which was adjusted to pH 7.2 using CsOH. The pipettes (Sutter Instrument Co, Novato, CA, USA) were pulled from borosilicate glass capillaries and had resistances of 3–5 MΩ after fire polishing. All experiments were performed at room temperature (22–25°C).

TMEM16A small interfering (si)RNA and adenovirus transfection. siRNA duplex (40 nM) against rat TMEM16A gene (sense, 5'-GGAGUUAUCAUCUAUAGAATT-3' and antisense, 5'-UUCUAUAGAUGAUAACUCCAA-3'; Qiagen GmbH, Hilden, Germany) was transiently transfected with Hiperfect Transfection Reagent (Qiagen GmbH), as previously described (19). A scrambled RNA (Qiagen GmbH) served as the negative control. Briefly, the siRNA strand and Hiperfect Transfection Reagent were diluted in serum- and antibiotic-free DMEM/F12 and maintained at room temperature for 10 min to form the transfection complexes. The complexes were added to BASMCs and mixed gently to ensure uniform distribution. Following incubation for 3 h at 37°C, transfection complexes were replaced with normal DMEM/F12 medium containing 10% FBS for 48 h. TMEM16A adenovirus (adv-TMEM16A) was constructed by Shengbo Biological Engineering Co., Ltd. (Shanghai, China). The adv-TMEM16A (multiplicity of infection, 200) was added into cultured BASMCs with serum- and antibiotic-free DMEM/F12 for 6 h, then cells were washed twice with phosphate-buffered saline and cultured in DMEM/F12 medium containing 10% FBS for another 48 h. adv-lacZ (Jikai Biological Engineering Co., Ltd., Shanghai, China) served as a negative control. Western blot analysis was used to examine the effect of TMEM16A siRNA (si-TMEM16A) and adv-TMEM16A.

Western blot analysis. Samples were gathered and protein was sequentially extracted according to the instructions of the Cell Lysis Buffer for Western and IP protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations in the samples were quantified by bicinchoninic acid protein assay using the BCA Protein assay kit (Beyotime Institute of Biotechnology). Protein (50 µg) was loaded into each lane and separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) by electrophoresis, and transferred at 200 mA for 1.5 h onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking in blocking buffer [PBS (pH 7.4) 5% skimmed milk powder and 0.05% Tween-20] for 1 h at room temperature, the membrane was incubated with primary and secondary antibodies. Rabbit polyclonal TMEM16A antibody was obtained from Novus Biologicals, LLC (Littleton, CO, USA; cat. no. NBPI-60076; dilution, 1:500). Rabbit polyclonal MLC (cat. no. 3672; dilution, 1:1,000), mouse monoclonal phosphorylated (p)-MLC (cat. no. 3675; dilution, 1:1,000), rabbit polyclonal MYPT1 (cat. no. 2634; dilution, 1:1,000) and rabbit polyclonal p-MYPT1 (cat. no. 5163; dilution, 1:1,000), were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal GAPDH as purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat. no. sc-365062; dilution, 1:2,000). Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. sc-2004; dilution, 1:1,000) and goat anti-mouse (cat. no. sc-2005; dilution, 1:1,000) immunoglobulin G antibodies were purchased from Santa Cruz Biotechnology, Inc. Detection was performed with the Immobilon Western Chemiluminescent HRP Substrate system (EMD Millipore).

RhoA activity assay. The change in RhoA activity results from the shift between the active GTP-bound state and the

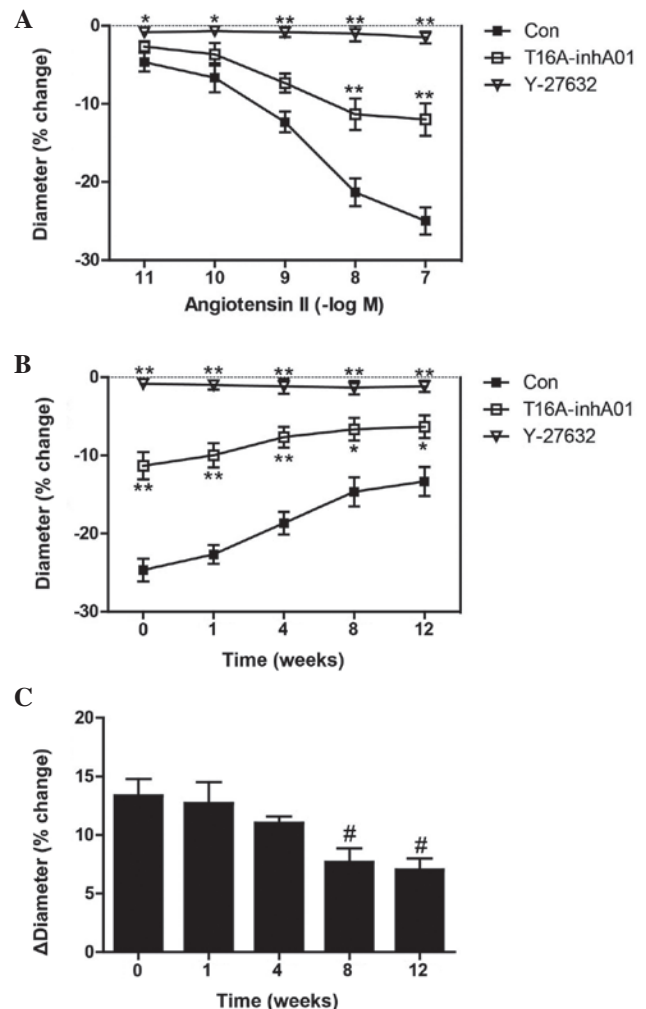


Figure 1. Inhibition of transmembrane protein 16A decreases rat basilar artery constriction in response to varying concentrations of Ang II. (A) Constriction of the basilar artery in response to Ang II in the absence and presence of T16A-inhA01 (10 µM) or Y-27632 (10 µM). (B) Constriction of the rat basilar arteries at week 0, and 1, 4, 8 and 12 weeks following 2-kidney, 2-clip surgery, in response to 100 nM Ang II, and in the absence and presence of T16A-inhA01 or Y-27632. (C) The bar graph indicates that T16A-inhA01 induced a decrease in the ΔDiameter in the presence of 100 nM Ang II. *P<0.05, **P<0.01 vs. Con; #P<0.05 vs. week 0 (n=6). ΔDiameter, basilar artery diameter; Ang II, angiotensin II; Con, control.

inactive guanosine diphosphate (GDP)-bound state. A RhoA Activation assay kit (Abcam, Cambridge, MA, USA) was used to detect the GTP-RhoA/RhoA ratio in the present study. The experiment was conducted according to the manufacturer's instructions. Briefly, BASMCs were washed twice with ice-cold PBS and lysed in the supplied lysis buffer (1 mM phenylmethanesulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin were added just prior to usage). Cell lysates were quantified as described above and total RhoA detection was performed using western blotting. For GTP-RhoA detection, the lysates were incubated with anti-active RhoA mouse monoclonal antibody and protein A/G agarose beads at 4°C in an end-over-end mixer (Shanghai Xiyuan Technology Development Co., Ltd., Shanghai, China) for 1 h. The beads were subsequently collected by centrifugation at 5,000 x g for 1 min at 4°C and washed three times with assay/lysis buffer, and were boiled for 5 min and centrifuged at 5,000 x g for

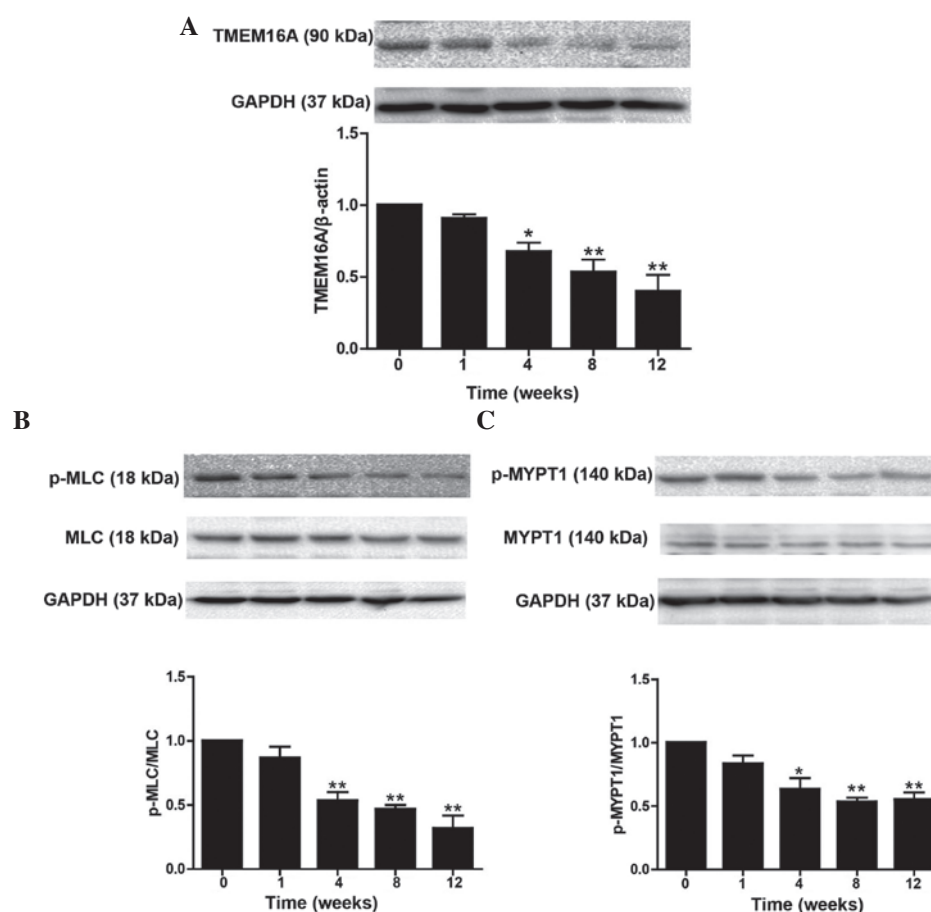


Figure 2. Decreased expression of TMEM16A, p-MLC/MLC and p-MYPT1/MYPT1 in rat basilar arteries during the development of hypertension. (A) Western blot analysis of TMEM16A in basilar arteries at various time-points after 2-kidney, 2-clip surgery. Western blot analysis of phosphorylation of (B) MLC and (C) MYPT1 in basilar arteries. GAPDH served as a loading control. * $P < 0.05$, ** $P < 0.01$ vs. week 0 ($n = 6$). TMEM16A, transmembrane protein 16A; MLC, myosin light chain; MYPT1, myosin phosphatase-targeting subunit 1; p, phosphorylated.

10 sec at $\sim 25^{\circ}\text{C}$. The pull-down supernatant was then subjected to western blot analysis. An anti-RhoA antibody served as the primary antibody and the secondary antibody was anti-rabbit (included in the RhoA activation assay kit).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data were expressed as mean \pm standard error of the mean. Student's *t*-test was used to test for differences between two groups, and one-way analysis of variance was used to test for differences among the treatment groups followed by the Bonferroni multiple comparison post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TMEM16A activity inhibition decreases Ang II-induced constriction in rat basilar arteries. Constriction of rat basilar artery in response to Ang II has previously been observed (14). The effect of a specific TMEM16A inhibitor, T16A-inhA01 on varying concentrations of Ang II induced basilar artery constriction (diameter change, %) was assessed in the present study. T16A-inhA01 (10 μM) partly reversed the Ang II-induced reduction in diameter of basilar arteries at 10 ($P < 0.01$) and 100 nM ($P < 0.01$) and

Rho kinase inhibitor, Y-27632 abolished the effect of Ang II (Fig. 1A) at 0.01, 0.1 ($P < 0.01$), 1, 10 and 100 nM ($P < 0.05$). Ang II (100 nM) was used in subsequent experiments. Similar results were observed in 2k2c hypertensive rats, and Ang II-induced constriction was gradually decreased in 2k2c hypertension rats (Fig. 1B). Furthermore, it was found that the T16A-inhA01-induced reduction in basilar artery constriction (Δ Diameter; % change) in the presence of 100 nM Ang II gradually decreased during the development of hypertension at 8 weeks ($P < 0.05$) and 12 weeks ($P < 0.05$; Fig. 1C), indicating less involvement of TMEM16A during progression of hypertension, which was consistent with the decreased level of TMEM16A protein expression in basilar arteries at 4 ($P < 0.05$), 8 ($P < 0.01$) and 12 weeks ($P < 0.01$; Fig. 2A). Phosphorylation of MLC and MYPT1 in basilar arteries from the hypertensive rats was also detected; western blot analysis demonstrated that p-MLC/MLC was significantly downregulated in a time-dependent manner at 4, 8 and 12 weeks (all $P < 0.01$) and p-MYPT1/MYPT1 was also significantly downregulated at 4 ($P < 0.05$), 8 ($P < 0.01$) and 12 weeks ($P < 0.01$) with the progression of hypertension (Fig. 2B and C), presenting further evidence of the reduced contractility of basilar arteries. These data strongly indicate that TMEM16A is involved in cerebral vasoconstriction during hypertension.

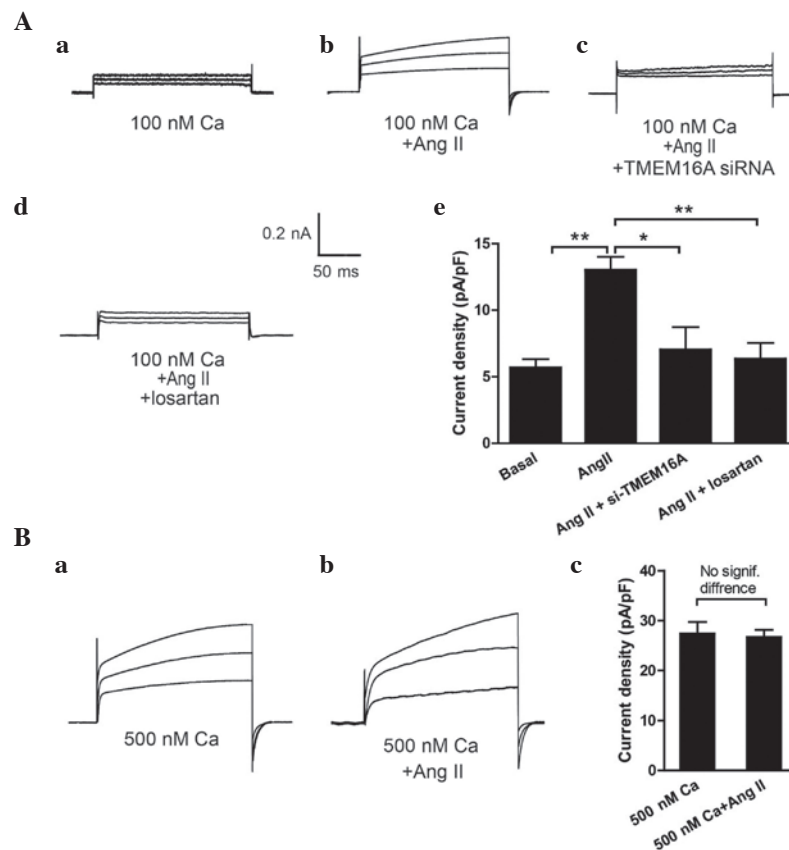


Figure 3. Ang II evoked a TMEM16A-mediated current using 100 nM $[Ca^{2+}]_i$ in BASMCs. (A) Representative traces of $I_{Cl,Ca}$ recorded in BASMCs. Cells were immersed in a bath solution and the current was recorded using a whole-cell patch clamp with (a) a basal intracellular calcium concentration (100 nM $[Ca^{2+}]_i$) and (b) immediately after 100 nM Ang II was perfused into the bath solution. Results from BASMCs pretreated with (c) TMEM16A siRNA and 100 nM $[Ca^{2+}]_i$, 100 nM Ang II-induced current for 48 h or (d) 10 μ M losartan for 15 min. (e) Bar graph of current density at 100 mV in the different groups. (B) $I_{Cl,Ca}$ was recorded in BASMCs with (a) 500 nM $[Ca^{2+}]_i$, and (b) the current wasn't enhanced by 100 nM Ang II. (c) Bar graph of current density at 100 mV in the two groups. * $P < 0.05$, ** $P < 0.01$ ($n = 8-11$). Ang II, angiotensin II; TMEM16A, transmembrane protein 16A; $[Ca^{2+}]_i$, intracellular Ca^{2+} ; BASMC, basilar artery smooth muscle cell; $I_{Cl,Ca}$, Ca^{2+} -dependent Cl^- channel.

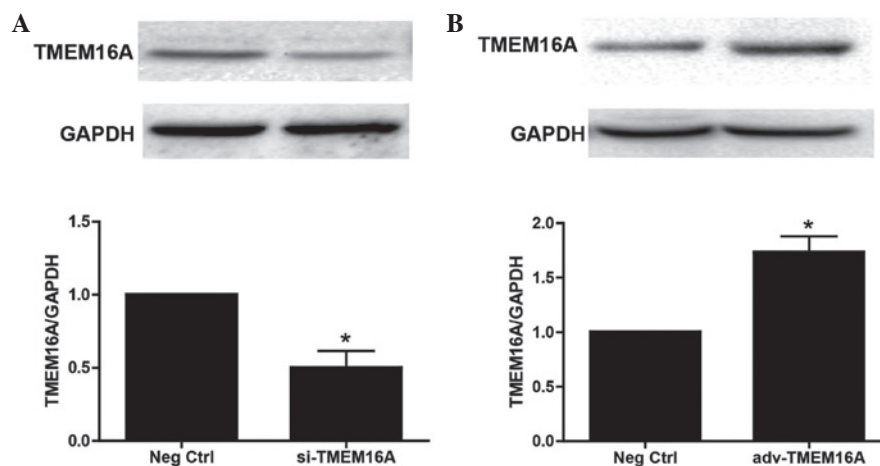


Figure 4. Effect of si-TMEM16A and adenovirus on TMEM16A expression in BASMCs. BASMCs were treated with (A) si-TMEM16A or (B) adv-TMEM16A for 3 and 6 h, respectively, and then cultured with 10% fetal bovine serum and Dulbecco's modified Eagle's medium/Ham's F-12 medium for another 48 h. TMEM16A protein expression was detected by western blot analysis and GAPDH served as a loading control. * $P < 0.05$ vs. Neg Ctrl. $n = 4$. TMEM16A, transmembrane protein 16A; BASMC, basilar artery smooth muscle cell; Neg Ctrl, negative control; si-TMEM16A, TMEM16A siRNA; adv-TMEM16A, TMEM16A adenovirus.

Ang II elicits a TMEM16A-mediated $CaCC$ current in BASMCs. In VSMCs, Ang II stimulates Ca^{2+} -dependent Cl^- channel ($I_{Cl,Ca}$) activity via increasing $[Ca^{2+}]_i$ (21,22). Ca^{2+} -induced $I_{Cl,Ca}$

($CaCCs$) in BASMCs was reported previously and TMEM16A was demonstrated to be a critical component of $CaCCs$ in basilar arteries (19). To investigate whether Ang II directly

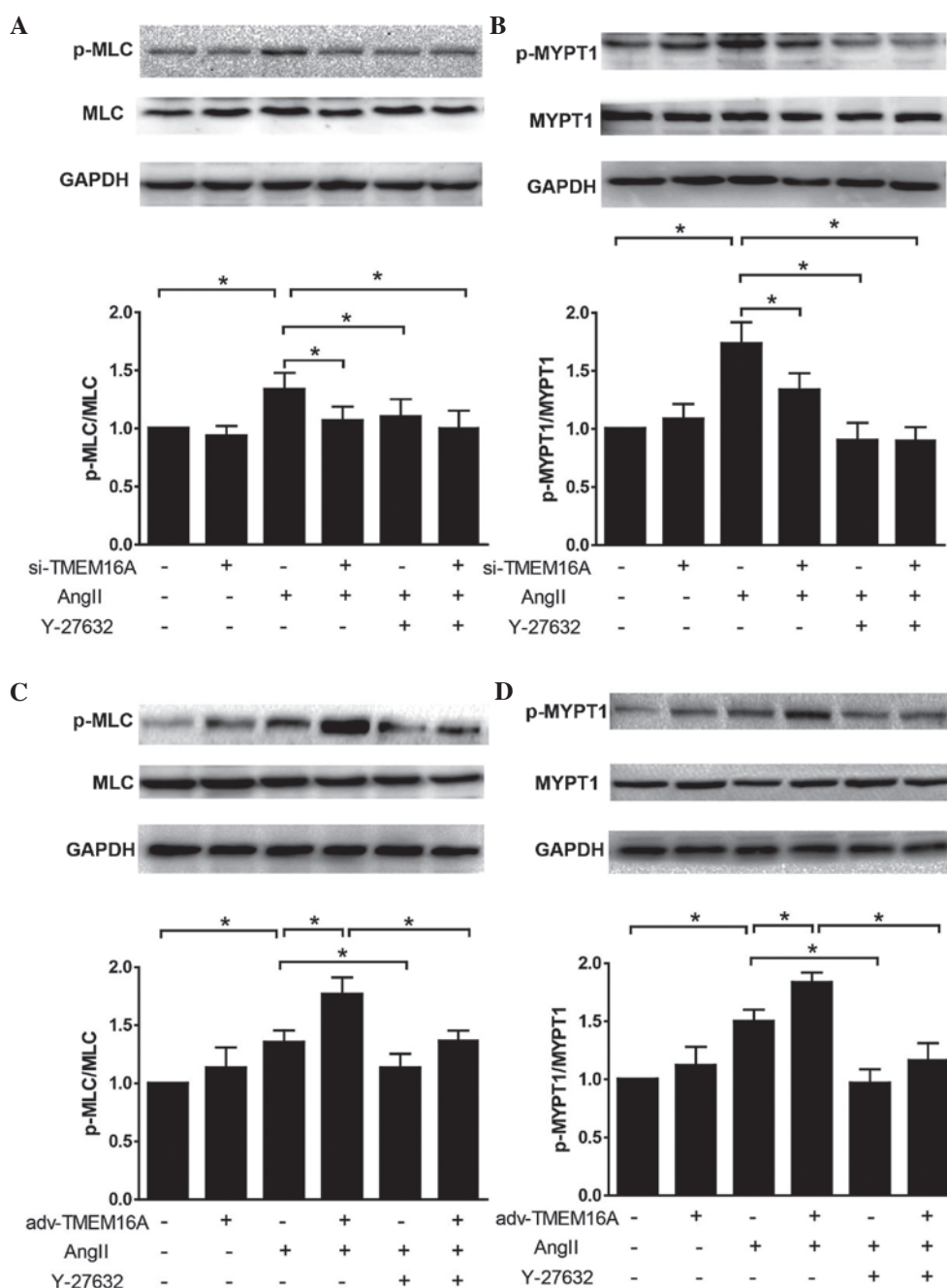


Figure 5. Regulation of Ang II-induced phosphorylation of MLC and MYPT1 by TMEM16A expression. BASMCs were treated with (A and B) si-TMEM16A or (C and D) adv-TMEM16A for 3 h or 6 h and cultured for another 48 h. Y-27632 (10 μ M) was added 10 min before the 5-min treatment of 100 nM Ang II. Cell lysates were collected and phosphorylation of (A and C) MLC and (B and D) MYPT1 were detected by western blot analysis. GAPDH served as a loading control. * $P < 0.05$; $n = 8$. Ang II, angiotensin II; MLC, myosin light chain; MYPT1, myosin phosphatase-targeting subunit 1; TMEM16A, transmembrane protein 16A; si, small interfering; si-TMEM16A, TMEM16A siRNA; BASMC, basilar artery smooth muscle cell; p, phosphorylated; adv-TMEM16A, TMEM16A adenovirus.

elicits TMEM16A-mediated CaCC channels, the effect of 100 nM Ang II on 100 nM $[Ca^{2+}]_i$ -induced $I_{Cl,Ca}$ (basal $I_{Cl,Ca}$) and 500 nM $[Ca^{2+}]_i$ -induced $I_{Cl,Ca}$ in BASMCs was analyzed. As shown in Fig. 3, Ang II enhanced basal $I_{Cl,Ca}$ to ~ 0.2 nA ($P < 0.01$; Fig. 3Aa and Ab) and this increase was abolished when cells were pretreated with TMEM16A siRNA ($P < 0.05$) and 10 μ M angiotensin type 1 (AT1) receptor blocker, losartan ($P < 0.01$; Fig. 3Ac and Ad). In addition, it was demonstrated that Ang II did not enhance the 500 nM $[Ca^{2+}]_i$ -induced $I_{Cl,Ca}$ further (Fig. 3Ba and Bb), indicating that this Ang II-elicited current was contained in TMEM16A-mediated CaCCs.

Effect of TMEM16A expression on phosphorylation of MLC and MYPT1 in BASMCs in response to Ang II and the influence of Y-27632. si-TMEM16A and adv-TMEM16A were evaluated in cultured BASMCs (Fig. 4) TMEM16A expression levels were significantly decreased by si-TMEM16A ($P < 0.01$) and increased by adv-TMEM16A. They were then used to test the effect of TMEM16A expression on cell contractility in response to Ang II. Western blot analysis demonstrated that downregulation of TMEM16A by si-TMEM16A decreased Ang II-induced phosphorylation of MLC and MYPT1 (Fig. 5A and B), while upregulation of TMEM16A by adv-TMEM16A

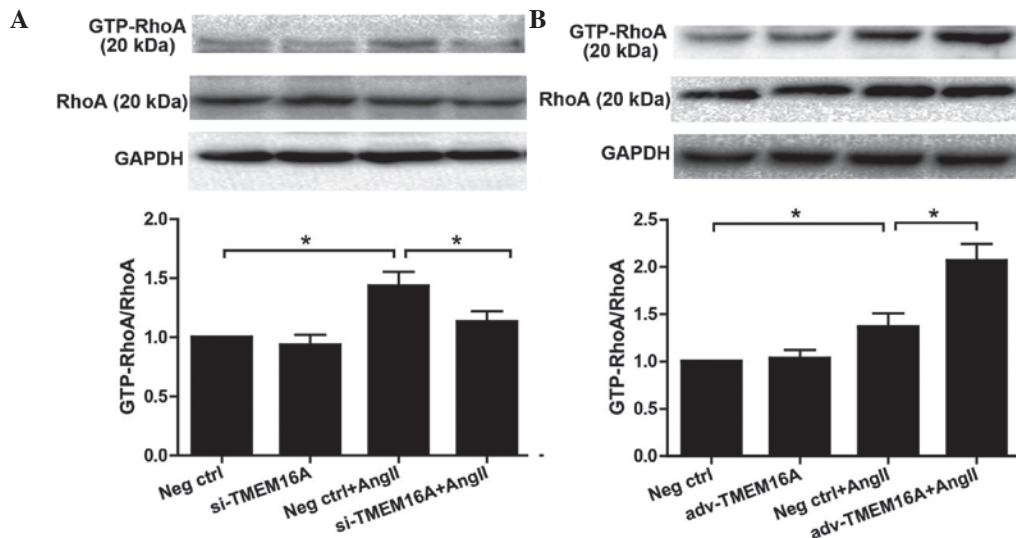


Figure 6. Expression of GTP-RhoA/RhoA in cultured BASMCs in response to 100 nM Ang II following TMEM16A downregulation or overexpression. BASMCs were treated with (A) si-TMEM16A or (B) adv-TMEM16A for 3 h or 6 h and then cultured with 10% fetal bovine serum and Dulbecco's modified Eagle's medium/Ham's F-12 medium for another 48 h. AngII (100 nM) was added for 5 min to induce cell contraction. Cell lysates were collected and GTP-RhoA and RhoA were detected by western blot analysis. GAPDH served as a loading control. * $P < 0.05$; $n = 8$. GTP, guanosine-5'-triphosphate; BASMC, basilar artery smooth muscle cell; TMEM16A, transmembrane protein 16A; si, small interfering; si-TMEM16A, TMEM16A siRNA; adv-TMEM16A, TMEM16A adenovirus; Neg Ctrl, negative control.

increased Ang II-induced phosphorylation of MLC ($P < 0.01$) and MYPT1 ($P < 0.01$; Fig. 5C and D). As shown in Fig. 1 and consistent with a previous study (14), Ang II-induced vasoconstriction is mediated to a great extent by ROCK. To investigate whether TMEM16A affects Ang II cell contractility via ROCK, a ROCK inhibitor, Y-27632 was applied to BASMCs treated with si-TMEM16A or adv-TMEM16A, and the data demonstrated that Y-27632 reversed the increase of contractility caused by overexpression of TMEM16A ($P < 0.01$; Fig. 5C and D), indicating that TMEM16A regulates Ang II-induced contraction of SMCs by affecting the RhoA/ROCK signaling pathway.

TMEM16A enhances Ang II-induced phosphorylation of MYPT1 and MLC in BASMCs by increasing RhoA activity. As shown in Fig. 1A and B, and Fig. 5C and D, TMEM16A regulated smooth muscle contraction in response to Ang II by affecting the RhoA/ROCK signaling pathway, therefore, the direct effect of TMEM16A on Ang II-induced RhoA activation was evaluated in BASMCs. BASMCs were pretreated with si-TMEM16A or adv-TMEM16A and then GTP-RhoA; the activated RhoA was detected following treatment of 100 nM Ang II for 5 min. Western blotting showed that downregulation of TMEM16A decreased the Ang II-induced GTP-RhoA increase ($P < 0.01$; Fig. 6A), while upregulation of TMEM16A further increased Ang II-induced augmentation of GTP-RhoA ($P < 0.01$; Fig. 6B).

Discussion

The results of the present study demonstrate that TMEM16A participates in basilar artery constriction via the RhoA/ROCK signaling pathway. The following were evidenced by the current study: i) Cerebral vascular contractility decreased in the 2k2c hypertensive rat model, which was indicated by the

decreased basilar artery constriction in response to Ang II, and by decreased phosphorylation of MLC and MYPT1; ii) the TMEM16A inhibitor, T16A-inhA01 partly inhibited Ang II-induced basilar artery constriction, and the inhibition ratio was decreasing as the hypertension progressed, in parallel with a decline in the level of TMEM16A protein expression; iii) Ang II elicited CaCC in BASMCs, which was blocked by si-TMEM16A and the AT1 receptor inhibitor, losartan. Notably, Ang II did not further enhance the 500 nM $[Ca^{2+}]_i$ -activated I_{CLCa} , which has been demonstrated as a TMEM16A-mediated CaCC in BASMCs, indicating that the Ang II-elicited current and the 500 nM $[Ca^{2+}]_i$ -induced current were mediated by the same channel, TMEM16A. The data from the present study provide the first direct evidence of the association between Ang II and activation of the TMEM16A channel in cerebral SMCs; and iv) TMEM16A overexpression increased Ang II-induced phosphorylation of MYPT1 and MLC by regulating RhoA activation in BASMCs. TMEM16A downregulation decreased Ang II-induced phosphorylation of MYPT1 and MLC by regulating RhoA activation in BASMCs.

In the circulatory system, vascular resistance is critical for regulating partial constriction. Small resistance arteries (such as mesenteric, renal and basilar arteries) constrict when subjected to KCl (depolarization), which increases intraluminal pressure and levels of vasoconstrictors, such as endothelin 1 and Ang II (23). The mechanisms of various stimuli (including endothelin-1, 5-hydroxytryptamine and thromboxane A2) have been investigated in past decades, and the present study focuses on cerebral vasoconstriction induced by Ang II (24-26). Ang II is the primary effector pleiotropic hormone of the RAS cascade, which mediates physiological control of electrolyte balance and blood pressure (27). Ang II exerts its effect via activation of two receptor subtypes, AT1 and AT2 (28,29); the AT1 receptor is noteworthy, as it is critical in regulating vessel constriction and blood pressure (30).

Earlier studies of cerebral arteries have demonstrated the vasoconstrictor effects of Ang II on vascular tone (31,32), and the effects are generally considered to result from the activation of AT1 receptors, which in turn stimulate phospholipase C (PLC) and phospholipase A2 (PLA2). PLC and PLA2 then generate inositol trisphosphate/ Ca^{2+} and diacylglycerol/protein kinase C, which activate multiple downstream signaling cascades that result in SMC contraction (33). Ang II has been reported to affect numerous ion channels, such as the large conductance Ca^{2+} -activated K^{+} channel, a key determinant of vascular tone (34), TRP cation channels (35) and voltage-dependent Ca^{2+} channels (36). In the present study, the influence of Ang II on CaCCs was elucidated in BASMCs. Previous studies hypothesized TMEM16A to be an important component of CaCCs (20,37,38) and its function was then widely investigated. It has been reported that TMEM16A is critical in Cl^{-} secretion of epithelial cells of the airways (20,39), SMC proliferation and the nervous system by controlling the excitability of various neurons. A strong association between CaCC activity and the concentration of free Ca^{2+} has been demonstrated in previous studies (40,41). Ang II is involved in the regulation of intracellular Ca^{2+} , therefore, the present study hypothesized whether Ang II influenced $\text{I}_{\text{Cl,Ca}}$ in BASMCs. The data indicates that Ang II directly evoked TMEM16A-mediated CaCCs and the inhibition of which suppressed constriction of the basilar arteries, indicating that TMEM16A may be involved in Ang II-induced cerebral vasoconstriction. In addition, the effect of TMEM16A inhibitor, T16A-inhA01 [a compound that inhibited CaCC currents in TMEM16A-transfected Fisher rat thyroid cells with a half maximal inhibitory concentration of $\sim 1 \mu\text{M}$ (42)], on the constriction of basilar arteries in response to Ang II was analyzed in the present study. The data showed that inhibition of TMEM16A reduced Ang II-induced basilar artery constriction, and the impact of the inhibitor was abrogated in the hypertensive rats. Ang II acts as a vasoconstrictor for acute stimulation and, in the long term, causes vascular remodeling. The increase of plasma Ang II levels in hypertensive rats is attributed to vascular remodeling (19) and TMEM16A expression levels decrease during SMC proliferation. However, this is a different process to vascular contraction, which is the focus of the current study. The decline of TMEM16A observed during hypertension in the present study illustrates our hypothesis of the role of TMEM16A in vasoconstriction. Subsequent findings in cultured BASMCs demonstrated that Ang II-induced phosphorylation of MYPT1 and MLC were upregulated by TMEM16A, which confirmed our hypothesis that TMEM16A is involved in cerebral vascular tone.

The small GTPase, Rho and ROCK regulate vascular smooth muscle contraction and blood pressure (43). Once Rho is activated by agonists of receptors coupled to cell membrane G proteins, such as Ang II and phenylephrine, it then activates ROCK. Activated ROCK phosphorylates MYPT1, a subunit of MLC phosphatase, which is then inhibited, leading to phosphorylation of MLC stimulating vascular smooth muscle contraction and cell migration (44). Ang II-induced vasoconstriction in basilar arteries was demonstrated to be predominantly mediated by ROCK using the ROCK inhibitor, Y-27632 (14). In a previous study, ROCK inhibitor, Thiazovivin reversed Ang II-induced MYPT1 and MLC

phosphorylation (45); therefore, whether the RhoA/ROCK signaling pathway mediated the effect of TMEM16A on Ang II-induced BASMC contraction was investigated in the current study. The results demonstrated that this was the case and, furthermore, it was found that TMEM16A expression regulated Ang II-induced activation of RhoA. These data are the first, to the best of our knowledge, to provide a mechanism by which TMEM16A contributes to Ang II-induced cerebral vascular constriction.

In conclusion, the current study reveals that TMEM16A, the recently identified component of CaCCs, is involved in Ang II-induced cerebral constriction. Decreased TMEM16A activity and expression reduces basilar artery contractility, and this effect of TMEM16A is mediated by affecting RhoA activation. These data also indicate that Ang II activates TMEM16A-mediated CaCCs in BASMCs. Therefore, TMEM16A has been demonstrated as a potent therapeutic target for the control of vascular function, hypertension and stroke. Future studies are required to determine how TMEM16A affects the RhoA/ROCK signaling pathway, providing a greater insight into the underlying mechanism.

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