Downregulation of TMEM16A Calcium-Activated Chloride Channel Contributes to Cerebrovascular Remodeling During Hypertension by Promoting Basilar Smooth Muscle Cell Proliferation

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Background—The Ca$^{2+}$-activated chloride channel (CaCC) plays an important role in a variety of physiological functions. In vascular smooth muscle cells, CaCC is involved in the regulation of agonist-stimulated contraction and myogenic tone. The physiological functions of CaCC in blood vessels are not fully revealed because of the lack of specific channel blockers and the uncertainty concerning its molecular identity.

Methods and Results—Whole-cell patch-clamp studies showed that knockdown of TMEM16A but not bestrophin-3 attenuated CaCC currents in rat basilar smooth muscle cells. The activity of CaCC in basilar smooth muscle cells isolated from 2-kidney, 2-clip renovascular hypertensive rats was decreased, and CaCC activity was negatively correlated with blood pressure (n=25; P<0.0001) and medial cross-sectional area (n=24; P<0.0001) in basilar artery during hypertension. Both upregulation of CaMKII activity and downregulation of TMEM16A expression contributed to the reduction of CaCC in the hypertensive basilar artery. Western blot results demonstrated that angiotensin II repressed TMEM16A expression in basilar smooth muscle cells (n=6; P<0.01). Knockdown of TMEM16A facilitated and overexpression of TMEM16A inhibited angiotensin II–induced cell cycle transition and cell proliferation determined by flow cytometry and BrdU incorporation (n=6 in each group; P<0.05). TMEM16A affected cell cycle progression mainly through regulating the expression of cyclin D1 and cyclin E.

Conclusions—TMEM16A CaCC is a negative regulator of cell proliferation. Downregulation of CaCC may play an important role in hypertension-induced cerebrovascular remodeling, suggesting that modification of the activity of CaCC may be a novel therapeutic strategy for hypertension-associated cardiovascular diseases such as stroke. (Circulation. 2012;125:697-707.)

Key Words: ANO 1 protein ■ cerebrovascular disorders ■ chloride channels ■ hypertension ■ remodeling

The Ca$^{2+}$-activated chloride channel (CaCC) is ubiquitously expressed in almost all eukaryotic cell types. CaCC has been reported to be involved in the regulation of a variety of physiological activities, including epithelial fluid and electrolyte secretion, neuronal and cardiac excitability, vascular contractility, and oocyte fertilization.$^{1-4}$ Although CaCC has been studied extensively for almost 3 decades, the functions of this channel are not fully understood. The major obstacle is the unknown molecular identity of the channel. Several candidates, including CLCA, CIC-3, and Tweety, have been proposed for CaCC, but these hypotheses were not supported by subsequent studies.$^{2-6}$ In 2002, bestrophins were proposed to be a CaCC in that heterogeneous expression of bestrophins could induce a Ca$^{2+}$-sensitive Cl$^{-}$ current that shared some similarities with native Ca$^{2+}$-activated chloride current (I$_{Cl,Ca}$)$^{1,3,4,6-8}$ However, the conclusion that bestrophins are responsible for the CaCC remains controversial because inconsistent or even conflicting data has been reported.$^{9,10}$

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The TMEM16A/anoctamin (Ano) proteins are a family of transmembrane proteins that consists of 10 members (TMEM16A–H, TMEM16J, and TMEM16K). Most recently, these proteins were proposed to be the candidates for CaCC based on the observations that heterogeneous expression of TMEM16A/Ano1 or TMEM16B/Ano2 produced a Cl$^{-}$ cur-
rent with the same characteristics as the native $I_{\text{CaCC}}$, including Ca$^{2+}$ and voltage dependence, outward rectification, and ion selectivity.\textsuperscript{11-13} Accumulating evidence has suggested that TMEM16A is critical for the normal development of trachea,\textsuperscript{14} transepithelial Cl$^-$/secretion,\textsuperscript{15} and bradykinin-induced inflammatory pain\textsuperscript{16} and is associated with numerous types of cancers.\textsuperscript{17-19} In the cardiovascular system, the expression of TMEM16A was initially identified in mouse portal vein by Sones et al.\textsuperscript{20} Later, Davis et al\textsuperscript{21} and Manoury et al\textsuperscript{22} reported that TMEM16A is expressed in vascular smooth muscle cells of thoracic aorta, carotid artery, and pulmonary artery and was suggested to be responsible for the CaCC in these cells. However, the functions of TMEM16A in the vessels remain elusive.

Importantly, the activity of Cl$^-$ channels has been found to be cell cycle dependent in ascidian embryos and B lymphocytes,\textsuperscript{23,24} indicating that Cl$^-$ channels may be critical for cell cycle transition and cell proliferation. Previous studies from our laboratory and others have demonstrated that the volume-regulated chloride channel plays an important role in the regulation of cell cycle progression and cell proliferation in a variety of cell types.\textsuperscript{25-30} Moreover, our recent results revealed that the activity of volume-regulated chloride channel was increased in hypertensive rat basilar smooth muscle cells (BASMCs) and that the upregulation of volume-regulated chloride channel contributed to the hypertension-induced cerebrovascular remodeling by promoting BASMC proliferation.\textsuperscript{31,32} Although a recent study in Ehrlich-Lettre ascites cells suggested that CaCC may be also a critical regulator of cell proliferation,\textsuperscript{33} whether CaCC is involved in cerebrovascular remodeling during hypertension is still a mystery.

In the present study, we examined the contributions of TMEM16A and bestrophin-3 to the CaCC in BASMCs and investigated whether the TMEM16A CaCC is involved in the regulation of cell proliferation and hypertension-induced cerebrovascular remodeling and their underlying mechanisms.

\section*{Methods}

An expanded Materials and Methods section is provided in the online-only Data Supplement.

\subsection*{Animal Model and Blood Pressure Measurement}

All animal experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in China. Hypertensive rats (2-kidney, 2-clip [2k2c]) were operated on, and blood pressure was measured in conscious rats by tail-cuff plethysmography as described previously.\textsuperscript{26,31} Cell number was determined with a hemocytometer. BrdU incorporation was measured at 450 and 540 nm on an Elisa microplate reader (BIO-TEK Synergy HT).

\subsection*{Western Blot Analysis}

Western blotting was performed as described previously.\textsuperscript{26,30,31} Briefly, proteins from each group were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Millipore Corp, Bedford, MA). After incubation with primary and appropriate secondary antibodies, the target bands were visualized by enhanced chemiluminescence. \(eta\)-Actin was used as the loading control. The anti–bestrophin-3 antibody (Fabgenix Inc) recognizes the sequence near the C-terminal end of rat bestrophin-3 protein (sequence: EHTEE-PKGTTPPRPTWF; amino acids 655–672). The anti-TMEM16A antibody (from Abcam, UK), recognizes the N-terminal region of TMEM16A protein.

\subsection*{Flow Cytometry}

Cell cycle was evaluated by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL) to characterize the population fraction in each phase as described previously.\textsuperscript{26}

\subsection*{CaMKII Activity Assay}

Cerebral arteries were isolated and frozen in liquid nitrogen. After the tissue was homogenized and centrifuged, the CaMKII activity of the tissue was homogenized and centrifuged, the CaMKII activity was measured in conscious rats by tail-cuff plethysmography as described previously.\textsuperscript{26,31} Cell number was determined with a hemocytometer. BrdU incorporation was measured at 450 and 540 nm on an Elisa microplate reader (BIO-TEK Synergy HT).

\subsection*{Reverse-Transcriptase Polymerase Chain Reaction}

The expression of bestrophins (bestrophin-1, bestrophin-2 and bestrophin-3), members of TMEM16 family (TMEM16A–H, J, and K), and TMEM16A splice variants (a, b, c, and d) was assessed by reverse-transcriptase polymerase chain reaction (RT-PCR) with a OneStep RT-PCR Kit (Qiagen). Synthesis of specific primers (Tables II–IV in the online-only Data Supplement) was performed by Shangon Biotech (Shanghai, China).

\subsection*{Statistical Analysis}

All data are expressed as mean±SEM. For Western blot, RT-PCR, and cell proliferation assays, \( n \) represents the number of independent experiments on different batches of cells or different rats. For patch-clamp studies, \( n \) represents the number of recorded cells from at least 4 different batches of cells or 5 different rats. The correlation analyses were determined by the Pearson correlation test. Statistical analysis was determined by an unpaired 2-tailed Student t test or 1-way ANOVA followed by the Bonferroni multiple comparison post hoc test with a 95% confidence interval. Differences in current
density-voltage relationships were analyzed by 2-way ANOVA. Values of $P < 0.05$ were considered significant.

**Results**

**Ca$^{2+}$-Activated Cl$^{-}$ Current in BASMCs**

In the freshly isolated rat BASMCs, an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) evoked a [Ca$^{2+}$]$_i$-dependent outward-rectifying current. The half-effective concentration of Ca$^{2+}$ (EC$_{50}$) to activate this current is $286.1 \pm 13.5$ nmol/L ($n=6–9$; Figure 1A). The reversal potential ($1.3 \pm 3.6$ mV) of this current was near the equilibrium potential for Cl$^{-}$ (0 mV) in our experimental conditions. A reduction of the extracellular Cl$^{-}$ concentration (from 133 to 44 mmol/L) shifted the reversal potential of the current from 1.3±3.6 to 20.8±2.3 mV ($n=8$; $P<0.01$).

**TMEM16A But Not Bestrophin-3 Is Responsible for CaCC in Rat BASMCs**

In rat BASMCs, bestrophin-3 is the most abundantly expressed isoform compared with bestrophin-1 and bestrophin-2 (Figure IIA in the online-only Data Supplement). To determine the relationship between bestrophins and CaCC in BASMCs, we examined the effect of knockdown of bestrophin-3 on the activity of CaCC in BASMCs. Although antibestrophin-3 siRNA transfection (20 nmol/L) for 48 hours significantly decreased bestrophin-3 expression by $90.3 \pm 3.8$% (Figure IIB in the online-only Data Supplement), the current densities of I$_{Cl, Ca}$ in BASMCs did not change (Figure IIC in the online-only Data Supplement). The data excluded the possibility that bestrophin-3 contributes to CaCC in BASMCs.

Recently, 2 members of the TMEM16 family, TMEM16A and TMEM16B, have been proposed to be the candidates for CaCC.11–13 In BASMCs, RT-PCR analysis showed that the
transcripts for TMEM16A, TMEM16C, TMEM16E through TMEM16H, TMEM16J, and TMEM16K are present; however, the transcripts for TMEM16B and TMEM16D were not detected (Figure 2A).

Because TMEM16B expression was not detected in BASMCs, we next investigated whether TMEM16A is responsible for CaCC in BASMCs. Consistent with previous studies, we found that phosphorylated enhanced green fluorescent protein (pEGFP)-TMEM16A plasmid but not pEGFP-N1 vector transfection increased TMEM16A expression in BASMCs (n=6; *P<0.05). The primer specific for b amplified 3 bands in our results, indicating that the TMEM16A transcripts containing or lacking this exon are both expressed in BASMCs. As for exon d, only the short spliced band was amplified in BASMCs, indicating that the TMEM16A transcripts lack this exon (Figure 2A).

Alteration of $I_{\text{Cl,Ca}}$ in BASMCs From Hypertensive Rats

The 2k2c hypertensive rat model was established as we described previously. At 4, 8, and 12 weeks postoperatively, the mean values of blood pressure in the 2k2c group were significantly higher than those in sham-operated group (n=30 in each group; P<0.05, P<0.01 versus corresponding sham group; Figure IV in the online-only Data Supplement).

In BASMCs isolated from 2k2c hypertensive rats, ATP- or thapsigargin-evoked $I_{\text{Cl,Ca}}$ was gradually decreased from 1 week after 2k2c operation (Table V in the online-only Data Supplement). To exclude the possibility that the abnormal signaling pathway activated by ATP or thapsigargin contributed to the decrease of $I_{\text{Cl,Ca}}$ during the development of hypertension, we further compared the current amplitude of $I_{\text{Cl,Ca}}$ in rat BASMCs between the
hypertension and sham groups when [Ca^{2+}] was clamped at 500 nmol/L. Our results showed that both the current amplitude and current densities of I_{Cl, Ca} were remarkably reduced in hypertensive rat BASMCs from 1 week after surgery and that the I_{Cl, Ca} was negatively correlated with blood pressure (Figure 3A and 3C). At 12 weeks, the cell size of BASMCs from hypertensive rats also increased (n=6–10; P<0.05 versus corresponding sham group; Figure 2B), suggesting that the increment of cell volume is one of the contributors to the decline of the current density of I_{Cl, Ca} after 12 weeks. Moreover, we also observed the I_{Cl, Ca} in BASMCs from 4-week hypertensive rats and the corresponding sham rats under different [Ca^{2+}]. The calculated EC_{50} of [Ca^{2+}] for I_{Cl, Ca} activation in hypertension group was 282.6±12.2 nmol/L (n=6), which was not different from that in the sham group (277.9±12.8 nmol/L; n=5; P>0.05; Figure V in the online-only Data Supplement), indicating that the Ca^{2+} sensitivity of CaCC did not change.

I_{Cl, Ca} Is Negatively Correlated With Hypertension-Induced Cerebrovascular Remodeling

In agreement with our previous studies,\textsuperscript{31,32} cerebral vessels exhibited inward remodeling in 2k2c hypertensive rats. In basilar arteries, the wall and lumen diameter decreased and the wall-to-lumen area ratio and mean values of medial cross-sectional area increased in the hypertension group from 4 weeks postoperatively compared with those in the corresponding sham groups (Figure 4A–4D). More interestingly, we found that I_{Cl, Ca} was negatively correlated with the medial cross-sectional area of basilar artery during hypertension (Figure 4E), suggesting that the decrease of I_{Cl, Ca} may be involved in hypertension-induced cerebrovascular remodeling.

**Downregulation of TMEM16A in the Basilar Artery From Hypertensive Rats**

Because TMEM16A is a critical component of the CaCC in BASMCs, we next investigated whether the reduced expression of TMEM16A attributed to the decrease of I_{Cl, Ca} during hypertension. Western blot results showed that TMEM16A expression in basilar artery did not change in the sham group during the time course (0–12 weeks) we studied. However, its expression was gradually decreased in 2k2c hypertensive rats from 4 weeks after operation. At 4, 8, and 12 weeks after surgery, TMEM16 expression was reduced by 17%, 43%, and 74%, respectively (n=5; P<0.05, P<0.01 versus sham; Figure 5). Our results indicate that the decreased expression of TMEM16A underlies, at least in part, the downregulation of I_{Cl, Ca} in BASMCs during hypertension.

**Regulation of I_{Cl, Ca} by CaMkII in BASMCs From 2k2c Hypertensive Rats**

It is noteworthy, however, that the decrease of I_{Cl, Ca} preceded the downregulation of TMEM16A expression, indicating that a reduction of TMEM16A expression is not the only mechanism attenuating the activity of CaCC during hypertension. Therefore, we next examined the effect of CaMkII on I_{Cl, Ca} because previous studies have demonstrated that CaMkII is a negative regulator of CaCC in rabbit pulmonary and coronary arterial smooth muscle cells.\textsuperscript{34} In freshly isolated rat BASMCs, KN-93, a specific
CaMKII inhibitor, significantly augmented I_{Cl, Ca}. At 100 mV, KN-93 (10 \mu mol/L) increased I_{Cl, Ca} by nearly 32.3% from 26.6 ± 1.3 to 35.2 ± 1.4 pA/pF (n = 6; P < 0.01; Figure 6A). A similar effect of KN-93 on TMEM16A-mediated Cl\(^{-}\) current was also observed in TMEM16A-overexpressing BASMCs (Figure 6B), suggesting the involvement of CaMKII in regulating the activity of TMEM16A channel. Moreover, we found that KN-93 induced a more dramatic increase in I_{Cl, Ca} in the hypertensive groups than in the corresponding sham groups (Figure 6C). Furthermore, the activity of CaMKII in basilar artery began to increase from 1 week after surgery and its activity was elevated gradually along with the development of hypertension (Figure 6D and 6E). These results suggest that the elevated CaMKII activity also contributes to the downregulation of I_{Cl, Ca} during hypertension.

Effect of TMEM16A on Angiotensin II–Induced BASMC Proliferation

In cultured BASMCs, angiotensin II (AngII; 0.5 \mu mol/L) treated for 48 hours significantly decreased TMEM16A expression (n = 6; P < 0.01 versus control; Figure 7A). This prompted us to further examine whether the downregulation of TMEM16A exerts a functional role in the regulation of AngII-induced cell proliferation because vascular smooth muscle cell proliferation plays an important role in hypertension-induced cerebrovascular remodeling. Our results demonstrated that knockdown of TMEM16A with anti-TMEM16A siRNA (40 nmol/L) promoted AngII-induced BASMC proliferation (n = 6; P < 0.05 versus control; P < 0.05 versus AngII-only group; Figure 7B and 7F and Figure VIA in the online-only Data Supplement). In contrast, overexpression of TMEM16A inhibited AngII-induced...
BASMC proliferation \( n=6; P<0.05 \) versus control; \( P<0.05 \) versus AngII-only group; Figure 7C and Figure VIB in the online-only Data Supplement). The data suggests that down-regulation of TMEM16A is involved in AngII-induced cell proliferation.

Effect of TMEM16A on Cell Cycle in AngII-Stimulated BASMCs

As shown in Figure 7, AngII \((0.5\mu \text{mol/L})\) promoted the cell cycle transition from the G0/G1 phase to the S phase in BASMCs. AngII decreased the percentage of cells in the G0/G1 phase and increased the percentage of cells in the S phase. Knockdown of TMEM16A increased the effect of AngII on cell cycle transition, which decreased the percentage of cells in the G0/G1 phase from 72.3\(\pm\)4.7\% to 59.4\(\pm\)3.1\% and enhanced the percentage of cells in the S phase from 23.3\(\pm\)1.5\% to 36.1\(\pm\)3.3\% \( n=6; P<0.05 \) versus control; \( P<0.05 \) versus AngII-only group; Figure 7D). In contrast, overexpression of TMEM16A inhibited AngII-induced cell cycle progression with a decrease in the cell population in the S phase from 25.7\(\pm\)1.7\% to 14.2\(\pm\)1.3\% and an increase in the cell population in the G0/G1 phase from 72.9\(\pm\)1.2\% to 83.3\(\pm\)2.3\% \( n=6; P<0.05 \) versus control; \( P<0.05 \) versus AngII-only group; Figure 7E). Our data indicates that downregulation of TMEM16A facilitates cell entrance from the G0/G1 phase to the S phase in BASMCs and thus promotes cell proliferation.

Effect of TMEM16A on Cell Cycle Regulators

Cell cycle transition is a tightly controlled event that is positively regulated by cyclins and cyclin-dependent kinases and negatively regulated by cyclin-dependent kinase inhibitors. To explore the molecular mechanism by which TMEM16A affects G1/S transition, we analyzed the proteins regulating cell cycle progression in these cell phases, including cyclin-dependent kinase-2, cyclin D1, cyclin E, p21CIP, and p27KIP. We found that knockdown of TMEM16A increased and overexpression of TMEM16A decreased AngII-induced expression of cyclin D1 and cyclin E \( n=6; P<0.05 \) versus control group; \( P<0.05 \) versus AngII-treated group; Figure 8A and 8B). However, knockdown or overexpression of TMEM16A had no effect on cyclin-dependent kinase-2, p21CIP, and p27KIP expression in BASMCs (Figure 8C and 8D).

Discussion

Currently, the physiological functions of CaCC are not fully understood because of the lack of specific channel blockers and the uncertainty about its molecular identity. In 2002, Sun et al.\(^6\) proposed that bestrophins may be the molecular candidates for CaCC based on the observation that hBest1 overexpression in human embryonic kidney 293 cells induced a\(Ca^{2+}\)-sensitive Cl\^-- current. This hypothesis was further supported by several subsequent studies.\(^7,8,39,40\) However, skepticism remains because \(Ca^{2+}\)-sensitive Cl\^-- current still exists in mBest1 or hBest2 knockout mice.\(^9,10\) In vascular smooth muscle cells, 2 types of \(I_{\text{Cl, Ca}}\), “classic” \(I_{\text{Cl, Ca}}\) and cGMP-dependent \(I_{\text{Cl, Ca}}\), have been found. Interestingly, a recent work demonstrated that bestrophin-3 is responsible for cGMP-dependent \(I_{\text{Cl, Ca}}\) but not classic \(I_{\text{Cl, Ca}}\) in vascular smooth muscle cells.\(^41\) In the present study, we found that...
bestrophin-3 is the most abundantly expressed isoform in rat basilar artery. However, knockdown of bestrophin 3 with siRNA had no effect on I_{Cl,Ca}, indicating that bestrophin-3 contributes little to the CaCC in BASMCs.

Several recent lines of evidence support that TMEM16A (Ano1) and TMEM16B (Ano2), 2 members of anoctamin family, encode CaCC in various cell types.11,13,15,16,22,42,43 Most recently, the expression profile of TMEM16 family members and their contributions to the CaCC in vascular smooth muscle cells have been examined in several types of blood vessels. For example, Sones et al20 detected the expression of TMEM16A in murine portal vein. Davis et al21 observed the expression of TMEM16A and TMEM16B in murine conduit artery. Manoury et al22 identified the expression of TMEM16A-B, TMEM16D-F, and TMEM16K in cultured pulmonary arterial smooth muscle cells. Most recently, a study published while we were preparing our revision reported that only the transcript for TMEM16A is expressed in cerebral arterial smooth muscle cells.38 Here, in basilar artery, we found that TMEM16A, TMEM16C, TMEM16E, TMEM16F, and TMEM16K are expressed at high levels, whereas TMEM16B and TMEM16D are not detected. The differences in the expression profile of the TMEM16 family in our study compared with previous studies may be due to the different species and the size and types of vessels studied. Because TMEM16B was not detected in BASMCs, we next examined the relationship between TMEM16A and CaCC in BASMCs. We found that heterogeneous expression of TMEM16A could induce a Ca^{2+}-sensitive Cl^{-} current with properties similar to the native I_{Cl,Ca} in BASMCs. Moreover, we found that TMEM16A is endogenously expressed in rat BASMCs. Knockdown of TMEM16A with siRNA remarkably attenuated I_{Cl,Ca} in BASMCs. The data suggests that TMEM16A is responsible for the CaCC in BASMCs.

Cerebrovascular remodeling has been thought to be an important determinant of the increased risk of stroke that accompanies chronic hypertension.44,45 Alteration of several kinds of ion channels, including the Ca^{2+} channel, K^{+} channel, and volume-regulated Cl^{-} channel, has been suggested to play a critical role in hypertension-induced cerebrovascular remodeling.46–48 The CaCC has been reported to be

Figure 7. TMEM16A inhibited angiotensin II (AngII)–induced basilar smooth muscle cell (BASMC) proliferation and cell cycle transition. A, AngII decreased TMEM16A expression in BASMCs. BASMCs were treated with 0.5 μmol/L AngII for 48 hours, and then the cell lysates were collected for Western blot (n = 6; *P < 0.05). B, Downregulation of TMEM16A promoted AngII-induced BASMC proliferation. Growth-arrested BASMCs were pretreated with 40 nmol/L anti-TMEM16A siRNA; 48 hours later, 0.5 μmol/L AngII was added to the culture medium for another 48 hours, and the cells were then collected for BrdU assay (n = 6; *P < 0.05 vs control; #P < 0.05 vs AngII-treated group). C, Overexpression of TMEM16A inhibited AngII-induced BASMC proliferation. Growth-arrested BASMCs were transiently transfected with phosphorylated enhanced green fluorescent protein (pEGFP)-TMEM16A for 48 hours; after that, 0.5 μmol/L AngII was added to the culture medium for another 48 hours, and the cells were then collected for BrdU assay (n = 6; *P < 0.05 vs control; #P < 0.05 vs AngII-treated group). D, Downregulation of TMEM16A accelerated AngII-induced G1/S transition in BASMCs. Growth-arrested BASMCs were pretreated with 40 nmol/L anti-TMEM16A siRNA; 48 hours later, 0.5 μmol/L AngII was added to the culture medium for another 48 hours; then, the cells were collected for flow cytometric analysis (n = 6; *P < 0.05 vs control; #P < 0.05 vs AngII-treated group). E, Overexpression of TMEM16A inhibited AngII-induced G1/S transition in BASMCs. Growth-arrested BASMCs were transiently transfected with pEGFP-TMEM16A for 48 hours; after that, 0.5 μmol/L AngII was added to the culture medium for another 48 hours, and then the cells were collected for flow cytometric analysis (n = 6; *P < 0.05 vs control; #P < 0.05 vs AngII-treated group). Neg indicates negative siRNA; Re, Hyperfect transfection reagent.
involved in the regulation of cerebrovascular contraction\textsuperscript{2–4}; however, the possible functions of this channel in cerebrovascular remodeling are not fully appreciated. Our results demonstrated that in BASMCs isolated from 2k2c hypertensive rats, both I\textsubscript{Cl, Ca} and TMEM16A expression were reduced. Moreover, the activity of I\textsubscript{Cl, Ca} negatively correlated with blood pressure and the medial cross-sectional area of basilar artery during hypertension development. These findings indicate that downregulation of CaCC may be involved in cerebrovascular remodeling during the development of hypertension.

It is noteworthy that the downregulation of TMEM16A expression cannot fully explain the decreased activity of CaCC in 2k2c hypertensive rats because I\textsubscript{Cl, Ca} began to drop at 1 week after operation but TMEM16A reduction was observed until 4 weeks after surgery. To further determine the mechanisms underlying the downregulation of CaCC during hypertension, we investigated the effect of CaMKII on I\textsubscript{Cl, Ca} in BASMCs because CaMKII has been proposed to be a negative regulator of CaCC in rabbit coronary myocytes.\textsuperscript{34} Consistent with this study, we found that the native I\textsubscript{Cl, Ca} and TMEM16A-mediated currents were negatively regulated by CaMKII. Addition of KN-93, a CaMKII inhibitor, potentiated the native I\textsubscript{Cl, Ca} in BSMCs and TMEM16A-mediated Cl\textsuperscript{−} currents. Moreover, we found that the percentage increases of I\textsubscript{Cl, Ca} by KN-93 were substantially elevated and accompanied by blood pressure increases, suggesting that CaMKII activity may be upregulated in hypertension. Indeed, the activity of CaMKII in basilar artery was augmented from 1 week after operation. Our findings were in agreement with previous work demonstrating that CaMKII activity was upregulated in AngII-induced vascular hyperplasia and hypertension.\textsuperscript{34,49} Together, our data demonstrates that the activity of CaCC in BASMCs was reduced in hypertension. Both the increased activity of CaMKII and decreased expression of TMEM16A contributed to the hypertension-induced downregulation of CaCC.

Abnormal vascular smooth muscle cell proliferation is a critical contributor to hypertension-induced vascular remodeling.\textsuperscript{31,44} Recent growing evidence has supported that Cl\textsuperscript{−} channels play an important role in regulating cell proliferation and cell cycle transition in a variety of cell types.\textsuperscript{23,24,26–30} In Ehrlich-Lettre ascites cells, a previous study demonstrated that the activity of CaCC was cell cycle dependent and that I\textsubscript{Cl, Ca} was decreased when the cell entered the S phase from the quiescent G\textsubscript{0} phase,\textsuperscript{33} suggesting that CaCC may be a critical cell cycle regulator and may play an important role in cell proliferation. In the present study, we found that AngII treatment decreased TMEM16A expression in rat BASMCs. Knockdown of TMEM16A facilitated the AngII-induced cell cycle transition from the G0/G1 phase to the S phase and promoted cell proliferation. In contrast,
overexpression of TMEM16A arrested the cell cycle at the G0/G1 phase and inhibited AngII-induced cell proliferation. Moreover, our results revealed that knockdown of TMEM16A further increased and overexpression of TMEM16A decreased AngII-induced upregulation of cyclin D1 and cyclin E in BASMCs. These observations indicated that TMEM16A-mediated CaCC is an effective cell proliferation inhibitor by preventing cell cycle transition from the G0/G1 phase to the S phase via inhibition of cyclin D1 and cyclin E expression. Downregulation of TMEM16A-mediated CaCC is involved in AngII-induced BASMC proliferation. Our results seems to be contradictory to previous studies which reported that TMEM16A was upregulated in several cancers, including oral cancer and gastrointestinal stromal tumors, before it was identified as CaCC.17–19 Of note, however, is that the exact functions of TMEM16A upregulation in cancer cells remain enigmatic.

Conclusions

Our present study provides evidence that TMEM16A but not bestrophin-3 is responsible for the CaCC in BASMCs. TMEM16A-mediated CaCC is a negative regulator of cell proliferation by arresting the cell cycle at the G0/G1 phase through a reduction of cyclin D1 and cyclin E expression. During hypertension, the activity of CaCC in BASMCs is decreased as a result of upregulation of CaMKII activity and downregulation of TMEM16A expression. These results suggest that a reduction of CaCC in hypertension may play an important role in the regulation of vascular smooth muscle cell proliferation and thus contribute to hypertension-induced cerebrovascular remodeling, indicating that modulation of the activity of CaCC may be a novel therapeutic approach for hypertension-associated cardiovascular diseases such as stroke.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

During hypertension, cerebral arterioles undergo remodeling of the vascular walls, which contributes to the increased risk for stroke. Accumulating evidence suggests that chloride channels play an important role in regulation of cell cycle transition and cell proliferation and that the upregulation of volume-regulated chloride channel is involved in hypertension-induced cerebrovascular remodeling. Recently, TMEM16A was proposed to be the molecular candidate of the calcium-activated chloride channel (CaCC). TMEM16A has been found to be abundantly expressed and to mediate the calcium-activated chloride current in several types of vascular smooth muscle cells. However, the molecular identity of CaCC and its functions in cerebrovascular smooth muscle cells remain enigmatic. In present study, we demonstrate that TMEM16A is responsible for the CaCC in rat basilar smooth muscle cells. The activity of CaCC in basilar smooth muscle cells is remarkably reduced in hypertensive rats. Upregulation of CaMKII activity and downregulation of TMEM16A expression contribute to the attenuation of CaCC in hypertension. In addition, TMEM16A negatively regulates cell proliferation and cell cycle transition from the G0/G1 phase to the S phase through modifying cyclin D1 and cyclin E expression. These results provide evidence that the reduction of CaCC activity is an important contributor to hypertension-induced vascular smooth muscle cell proliferation and cerebrovascular remodeling, indicating that restoration of the TMEM16A CaCC activity could exert beneficial effects on hypertension-associated cardiovascular diseases such as stroke.
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Downregulation of TMEM16A calcium-activated chloride channel contributes to cerebrovascular remodeling during hypertension through promoting basilar smooth muscle cell proliferation

Wang et al. TMEM16A CaCC and cerebrovascular remodeling

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Supplemental Methods

Animals were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China.

Animal models

2-kidney, 2 clip (2k2c) renovascular stroke-prone hypertensive rats were operated as previously described\(^1\)\(^-\)\(^3\). Briefly, healthy male Sprague-Dawley rats were anaesthetized by injection of 10% chloral hydrate (3 mg/kg, i.p.), and ring-shaped silver clips with the internal diameter of 0.3 mm were placed around both right and left renal arteries. The sham-operated rats underwent the same surgical procedure except for the placement of silver clips and served as control. Stroke-prone spontaneous hypertensive rats and WKY rats were kindly provided by Dr. DF Su (The Second Military Medical University, Shanghai, China). Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (powerlab 4/30, ADInstruments, Australia). All rats were maintained in pathogen-free facilities with a 12-hour light/dark cycle.

Immunohistochemistry

Rats were anesthetized with 10% chloralhydrat and were perfused intracardiacly with 0.1 mol/L phosphate buffer containing heparin (100 U/kg)
and nitroglycerol (0.3 μg/kg), followed by 4°C fixative solution containing 4% freshly depolymerized paraformaldehyde in 0.1 mol/L phosphate buffer for 15 minutes, the pressure was controlled ≈100mmHg. The rat brain was carefully removed and sections (8 µm) were prepared from freshly frozen rat basilar arteries as previously described\(^1\)-\(^2\). Randomly selected 3 sections in each group were pretreated with the solution of 3% hydrogen peroxide and methanol at the ratio of 1:50 for 30min at room temperature, and then blocked with 5% bovine serum albumin in PBS for 30min. Then the sections were exposed to α-actin monoclonal antibody (Sigma; dilution 1:400) at 4°C overnight and then were treated with goat FITC-conjugated secondary antibody (Cell Signaling Technology; dilution 1:400) at room temperature for 30min. The quantification of α-SM-actin staining was analyzed using confocal system (OLYMPUS, FV500-IX 81, magnification 400 ) and Image-Pro Plus 5.0. For each tested group at each time point, 10 rat brains were taken for the experiment, respectively.

**Fresh isolation of rat basilar artery smooth muscle cells**

Fresh isolation of rat basilar artery smooth muscle cells (BASMCs) was carried out as we previously described\(^2\). Briefly, hypertensive rats and their corresponding controls were anaesthetized with chloral hydrate and decapitated. The basilar arteries was harvested immediately and placed in a solution containing (in mmol/L): 130 NaCl, 5 KCl, 0.8 CaCl\(_2\), 1.3 MgCl\(_2\), 10 HEPES and 5 glucose, pH 7.4. After fat and connective tissue was cleaned,
the basilar arteries were cut into 0.2 mm rings and were incubated in low Ca\textsuperscript{2+} solution (in mmol/L: 0.2 CaCl\textsubscript{2}, 130 NaCl, 5 KCl, 1.3 MgCl\textsubscript{2}, 10 HEPES, 5 glucose, pH 7.4) containing collagenase (type II, 0.5g/l), elastase (type II-A, 0.5g/l), hyaluronidase (type IV-S, 0.5 g/l) and deoxyribonuclease I (0.1g/l) for 1 hour at room temperature. After that, the rings were washed in fresh low Ca\textsuperscript{2+} solution containing trypsin inhibitor (0.5g/l) and deoxyribonuclease I (0.1g/l) and were then triturated gently. Isolated cells were plated on glass coverslips and stored at 4 °C in the above buffer solution containing 0.8 mmol/L CaCl\textsubscript{2} and fatty acid-free bovine serum albumin (2g/l). All the cells were used for electrophysiological studies within 10 hours.

Cell culture

Rat basilar smooth muscle cells (BASMCs) were isolated and cultured from rat basilar arteries using explant method as previously described\textsuperscript{1,2,4}. Briefly, male Sprague-Dawley rats (150~180g) were anaesthetized with ether and decapitated. The basilar arteries were harvested immediately and immersed in Kreb’s solution containing (mmol/L): NaCl 137, KCl 5.4, CaCl\textsubscript{2} 2.0, MgCl\textsubscript{2} 1.1, NaH\textsubscript{2}PO\textsubscript{4} 0.4, Glucose 5.6, NaHCO\textsubscript{3} 11.9, 10\textsuperscript{5} U/L penicillin and 100 mg/L streptomycin. After fat and connective tissue was cleaned, the basilar arteries were cut into small pieces about 0.5 mm long and the vessel segments were placed on the surface of the culture dish. The dish was then incubated in Dulbecco’s Modified Essential Medium (DMEM)/F-12 supplemented with 20% fetal calf serum (FCS) at 37°C, 5% CO\textsubscript{2}. 3 days later, the floating tissues were
discarded and fresh DMEM/F-12 containing 20% FCS was added. The cells will migrate from the attached vascular specimens about 7-10 days. After reaching 70% confluence, cells were then subcultured with 0.25% trypsin with 0.02% EDTA. Passage 8 to 12 of BASMCs at 70% to 90% confluence were growth-arrested by incubation in DMEM/F12 with 0.5% FCS for 24 hours before experiments. BASMCs were identified by positive immunocytochemical staining with a monoclonal antibody against smooth muscle α-actin and by the characteristic “hill and valley” growth pattern. Passage 8 to 12 of cultured BASMCs were used for cell proliferation assays.

**siRNA transfection**

The siRNA duplexex against rat TMEM16A gene and bestrophin-3 gene (Qiagen, Table S1) was transiently transfected with Hiperfect Transfection Reagent (Qiagen) according to the instructions as previously described. A scrambled RNA (Qiagen) was used as negative control. siRNA strand and Hiperfect Transfection Reagent were diluted in serum and antibiotics free DMEM/F12. The final concentration of Bestrophin-3 and TMEM16A siRNA were 20 nmol/L and 40 nmol/L respectively. The mixture was kept at room temperature for 10 min to form the transfection complexes. The complexes were then added to BASMCs and were swirled gently to ensure uniform distribution. After incubation for 6h at 37°C, transfection complexes were replaced with normal DMEM/F12 containing 10% Fetal calf serum (Gibco, USA). 48 hour later, western blot was used to examine the effect of TMEM16A
siRNA and negative siRNA on TMEM16A protein expression. The intensities of bands of TMEM16A and β-actin were quantified by digital densitometry using Quantity One software (Bio-Rad). TMEM16A band intensities normalized to β-actin from TMEM16A siRNA and negative siRNA transfected groups were compared on the same membranes.

**Plasmid transfection**

pEGFP-TMEM16A plasmid (kind gift from Dr. Jan LY, University of California, San Fransisco, USA) was transfected with lipofectamine2000 reagent (Invitrogen) as previously described1. Briefly, BASMCs were plated on 24-well plate at a density of 1-1.5×10⁵/ml. 24 hours later, pEGFP-TMEM16A or pEGFP-N1 plasmids were transfected into the cells with LipofectAMINE2000 reagent (Invitrogen, Life Technologies, Inc.) in OPTI-MEM® reduced serum medium (GIBICO) according to the manufacturer’s instructions. 6h later, cells were rinsed with PBS and switched to 10% or 0.5% serum-containing medium for patch clamp or cell proliferation assays.

**Cell proliferation assay**

Cell proliferation was assessed by cell counts and 5-bromo-2'-deoxyuridine (BrdU) incorporation as we reported previously1, 4. For the cell counts, BASMCs were trypsinized and plated into 6-well culture plates at a density of 5×10⁵ cells/well in DMEM/F-12 supplemented with 10% FCS. After 24 hours, cells were rendered quiescent in 0.5% FCS for 24 hours before addition of fresh growth medium containing the appropriate supplements. Cell number
was determined in triplicate using a hemocytometer.

The DNA synthesis was assessed by BrdU incorporation. After starvation for 24 hours, cells were treated with the same supplements used in the cell count experiment before adding 10 mmol/L BrdU to the medium. 18 hours later, cells were fixed and treated with anti-BrdU primary antibody for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated goat anti-IgG for 30 min, 100 mmol/L 3,3′,5,5′-tetramethylbenzidine was then added as the substrate for horseradish peroxidase. The incorporation was measured at 450 and 540nm on an Elisa microplate reader (BIO-TEK synergy HT, American).

**Ca^{2+}-activated Cl⁻ current in BASMCs**

Ca^{2+}-activated Cl⁻ current was recorded at room temperature using whole-cell patch clamp technique with an Axopatch 200B patch clamp amplifier (Axon instrument, Foster City, CA, USA) as previously described⁵, ⁶, ⁸-¹¹. Patch pipettes were pulled from borosilicate glass with p-97 puller (Sutter Instrument Co., USA). The resistance of the pipettes used in this study was 3-6 MΩ after filling with pipette solution. Currents were filtered at 2 kHz and sampled at 5 kHz. A 3 mol/L KCl-agar salt bridge between the bath solution and the Ag-AgCl reference electrode was employed to minimize the changes of liquid junctional potentials. During experiment, the cells were held at -50mV, and the test potentials were applied from -100mV to +100mV for 250ms in +20mV increments at an interval of 5s. Data acquisition and analysis were
performed with Clampex 8 (Axon instrument, USA).

The extracellular solution contained (mmol/L): NMDG·Cl 125, KCl 5, CaCl₂ 1.5, MgSO₄ 1, HEPES 10, Glucose 10, pH was adjusted to 7.4 with NMDG. The pipette solution contained (mmol/L): CsCl 130, Mg·ATP 1, MgCl₂ 1.2, HEPES 10, EGTA 2, and CaCl₂ 1.639, pH was adjusted to 7.3 with CsOH. The intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was 500 nmol/L.

In antibody dialysis experiments, anti-bestrophin-3 or anti-TMEM16A antibody was diluted and added to the pipette solution (final concentration ranged from 0.5ug/ml to 8ug/ml) respectively. In preabsorbed experiments, antibody and the corresponding antigen were mixed in a ratio of 1:10, then stored at 4 °C overnight. The mixture was added to pipette solution before experiments.

**Western blot analysis**

Total proteins were prepared from the cultured BASMCs or pooled basilar arteries from hypertensive rats and the corresponding controls (3 basilar arteries from each group were used as 1 sample), and western blotting was performed as previously described. Briefly, the protein content was quantified with Coomassie Brilliant Blue. Aliquots containing 30 µg of proteins from each group were subjected to SDS-PAGE and were then transferred to nitrocellulose membranes (Schleicher & Schuell). After incubation with the blocking solution at room temperature for 1 hour, the membranes were incubated with primary antibodies against TMEM16A (Abcam, UK), p21⁰⁰⁰⁰
(Santa Cruz, USA), cyclin D1, cyclin E, CDK2, p27Kip (Cell Signaling Technology, USA) and bestrophin-3 (FabGennix, USA), respectively, for 1 hour at room temperature, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology; dilution 1:1000) for 1 hour at room temperature. Final detection was carried out with LumiGLO chemiluminescent reagent (New England Biolabs) as described by the manufacturer. The densities of target bands was accurately determined by the computer-aided 1-D gel analysis system.

**Flow cytometry**

After appropriate treatment, BASMCs were collected by centrifugation at 200g for 5 min at 4°C. Pellets were rinsed twice with ice-cold phosphate-buffered-saline (PBS) and fixed in 70% ethanol for 24 h. Samples were then stained with staining buffer (PBS containing 50 μg/ml propidium iodide, 10 μg/ml RNase A, 0.1% sodium citrate and 0.1 Triton X-100) for 60 min at room temperature in the dark. DNA content was analysed by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL, USA) to determine the cell distribution in cell cycle.

**Measurement of CaMKII activity**

For CaMKII activity assay, cerebral arteries were frozen in liquid nitrogen. Then the tissue was homogenized at 4 °C by sonication in a buffer containing 20 mmol/L Tris-HCl, 2 mmol/L EGTA, 2 mmol/L EDTA, 20μg/ml soybean trypsin inhibitor, 10μg/ml aprotinin, 5μg/ml leupeptin, 2 mmol/L DTT, 25 mmol/L
benzamidin, 1 mmol/L PMSF (in 100% ethanol), pH 8.0. Since more than 95% CaMKII activity is residual to the supernatant fraction, the insoluble material was removed by centrifugation at 15,000 X g for 5 min and the supernatant fraction was taken for the total protein assay. 5–10µg of protein (5µl) was added to a preheated reaction mix composed of (final concentration): 2.5µl CaM KII Biotinylated Peptide Substrate, 5 mmol/L CaCl₂, 5 µmol/L calmodulin, 0.1mg/ml BSA, 250 mmol/L Tris-HCl (pH 7.5), 50 mmol/L MgCl₂, 2.5 mmol/L DTT, 3000Ci/mmol [γ-32P]ATP. The control mix is composed of (final concentration): 5 mmol/L EGTA (pH 7.2), 250 mmol/L Tris-HCl (pH 7.5), 50 mmol/L MgCl₂, 2.5mmol/L DTT and 3000Ci/mmol [γ-32P] ATP. The reaction was run at 30°C for 3 minutes and terminated by 7.5 mol/L guanidine hydrochloride. 10µl of the reaction mix was spotted onto a prenumbered square of the SAM Membrane and absorbed for 2–3 s before washing. The incorporation of γ-32P onto the peptide was measured by liquid scintillation counting (Wallac 1409, Turku, Finland), and enzyme activity was determined as described previously¹³.

**RT-PCR**

Total RNA was extracted from rat basilar arteries using Trizol reagent according to the manufacturer's instructions (Invitrogen). The expression of bestrophins (bestrophin-1, bestrophin-2 and bestrophin-3), members of TMEM16 family (TMEM16A-H, TMEM16 J and TMEM16K) and TMEM16A splice variants (a, b, c and d) were assessed by RT-PCR using Onestep
RT-PCR Kit (Qiagen). Specific primers (Table S2, S3 and S4) were synthesized by Shangon Biotech (Shanghai, China). PCR was performed in 50 µl reaction volume containing 1µl dNTPs (10 mmol/L), 1µl of each primer (50 pmol), 5 µl cDNA solution, 3 µl MgCl₂ (15 mmol/L), 5 µl PCR buffer (10×), water (33 µl) and 1 µl Taq DNA polymerase (Invitrogen) on a Thermal Cycler (Thermo Scientific). Denaturation was carried out at 94°C for 1min, annealing at 55°C for 1min and elongation at 72°C for 1min for 35 cycles, followed by 72°C for 10min. PCR products were detected in 1% agarose gel containing 1% ethidium bromide. The specificity of the reaction was confirmed by sequencing the products that had the expected sizes.
Supplemental Tables

Table S1  Sequences of the siRNAs against bestrophin-3 and TMEM16A.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Target</th>
<th>Gene Accession</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>Bestrophin-3</td>
<td>XM_001080943</td>
<td>5’- ACGUUACUCUGGUAGUGA A-3’</td>
</tr>
<tr>
<td>strand</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td></td>
<td>5’- UUCACUACCAGAGUAACGU-3’</td>
</tr>
<tr>
<td>strand</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>TMEM16A</td>
<td>NM_001107564</td>
<td>5’-GGAGUUAUCAUCUAGAATT-3’</td>
</tr>
<tr>
<td>strand</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td></td>
<td>5’- UUCUAUAGAGUAGUACUCCA-3’</td>
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### Table S2  Sequences of the primers for bestrophins.

<table>
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<tr>
<th>Experiment</th>
<th>Target (GeneID)</th>
<th>mRNA (accession number)</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>Bestrophin-1 (293735)</td>
<td>NM_001011940</td>
<td>5'-CCGTGGACTCTACAGAATGG-3'</td>
<td>5'-GTGTTGAAGACTGGGAAGAC-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>Bestrophin-2 (364973)</td>
<td>NM_001108895</td>
<td>5'-GCCACCGCTTTCTGCCTCA-3'</td>
<td>5'-AACCGCACTCAACGCCTCA-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>Bestrophin-3</td>
<td>XM_235161</td>
<td>5'-TCAGCTGTACCCACTA-3'</td>
<td>5'-TGTCGTTTCCCTATCCA-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
**Table S3**  Sequences of the primers for alternative splicing variants (a, b, c and d) of TMEM16A.

<table>
<thead>
<tr>
<th>Splicing variants</th>
<th>Primer pair (5'-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Fw: CACAAGAGAGCCTCGGGTAG&lt;br&gt;Rw: ATCTTCACAACCGACACC</td>
<td>267</td>
</tr>
<tr>
<td>b</td>
<td>Fw: CAAAACCCGGAGCAAAATAG&lt;br&gt;Rw: CAGGAGTTTCCCTGCTCTG</td>
<td>175 or 241</td>
</tr>
<tr>
<td>c</td>
<td>Fw: CTCTGGGCTGCCACCTTC&lt;br&gt;Rw: TGGCTTCATACTCTGCTCTGC</td>
<td>118 or 130</td>
</tr>
<tr>
<td>d</td>
<td>Fw: TCCCAGAGCAAGATATGAAG&lt;br&gt;Rw: AGATGGGAGGAGTTCATGC</td>
<td>230 or 305</td>
</tr>
</tbody>
</table>

Fw, forward primer; Rw, reverse primer; bp, base pair
**Table S4**  Sequences of the primers for members of TMEM16A family.

<table>
<thead>
<tr>
<th>gene</th>
<th>Primer pair (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| TMEM16A  | Fw: TTCGTCACCCTGTTCGT  
           Rw: GTGCCATTCTGGAAGTCG | 329                |
| TMEM16B  | Fw: CCAGGGGAAGCAGAGTTCTTG  
           Rw: TGTTGTGGCTCGAGAACAG | 297                |
| TMEM16C  | Fw: TCGGACTGCTACACTGGCCCT  
           Rw: CAGCGTGCCCAGCGCTCATA | 290                |
| TMEM16D  | Fw: TGGCTTTCAGGGCCAAGGACA  
           Rw: TGAGCCGGGTCCCCGGGTAGTC | 319                |
| TMEM16E  | Fw: ACGGCCAAAGTACACCACCCCT  
           Rw: ACTGGCCATCATGGAGCGGG | 293                |
| TMEM16F  | Fw: AGCCATCTGGGCGTGTTGA  
           Rw: GCCCGGGTGTTGTCGAAGG | 304                |
| TMEM16G  | Fw: TGGCGCCGCTTGTTCGCGCTA  
           Rw: GCAAGTGCGGTTGTGTGACG | 302                |
| TMEM16H  | Fw: AAGTTCGCCACCCACGGCCAC  
           Rw: TGATGCGCTGTCACGCCACG | 324                |
| TMEM16J  | Fw: GGCTGGCCTGTGGAAAGCTGG  
           Rw: GCCACAGGGGTCGAGTGGA | 327                |
| TMEM16K  | Fw: GGCTACCCGCAGGCCAATTG  
           Rw: CCCACACGAACAGGATGGGC | 280                |
| GAPDH    | Fw: CACCAGCATCACCCCCATTT  
           Rw: CCATCAAGGACCCCTTCATT | 180                |

Fw, forward primer; Rw, reverse primer; bp, base pair
Table S5  Alterations of $I_{\text{Cl, Ca}}$ activated by ATP and thapsigargin (TG) in BASMCs at 1-, 4-, 8- and 12-week after 2k2c operation. The current densities at +100 mV were shown  (Mean±SEM, n=7-12).

<table>
<thead>
<tr>
<th></th>
<th>Current density at +100mV (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1w</td>
</tr>
<tr>
<td>$I_{\text{Cl, ATP}}$</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>42.1±3.3</td>
</tr>
<tr>
<td>Htn</td>
<td>30.0±3.6*</td>
</tr>
<tr>
<td>$I_{\text{Cl, TG}}$</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>20.0±1.3</td>
</tr>
<tr>
<td>Htn</td>
<td>16.1±1.5*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs. corresponding sham group.
Supplemental Figures

Figure S1

Figure S1. ATP and thapsigargin (TG) activated $I_{\text{Cl,Ca}}$ in BASMCs. The currents were recorded in 1.5 mmol/L Ca$^{2+}$-containing extracellular solution at voltage steps from -100mV to +100mV for 250ms in +20mV increments at an interval of 5s. The holding potential was -50mV. Representative traces of ATP (upper panel) and TG (lower panel) induced $I_{\text{Cl,Ca}}$ from 6 different experiments were shown.
Figure S2

Figure S2. Effect of knockdown of bestrophin-3 on $I_{Cl, Ca}$ in BASMCs. A. RT-PCR showed that bestrophin-3 is expressed at high levels in BASMC. A weak expression of bestrophin-2 and bestrophin-1 was also observed in BASMCs. B. Anti-bestrophin-3 siRNA (20 nmol/L) transfection for 48 hours significantly decreased endogenous bestrophin-3 protein expression in BASMCs (n=6, *p<0.05 vs. control). C. Knockdown of bestrophin-3 with siRNA had no effect on $I_{Cl, Ca}$ in rat BASMCs. i), representative traces of $I_{Cl, Ca}$ in control, bestrophin-3 siRNA or negative siRNA transfected BASMCs., ii) I-V curves of $I_{Cl, Ca}$ from experiments as those shown in i) (n=6).
Figure S3. Effect of nifilumic acid (NFA) on TMEM16A-mediated current. 100 μmol/L NFA significantly inhibited Ca\(^{2+}\)-activated Cl\(^{-}\) current in TMEM16A overexpressed BASMCs (n=7).
Figure S4. Blood pressure of 2k2c hypertensive (Htn) and the corresponding sham (Sham) rats measured at 1-, 4-, 8- and 12-week after operation (mmHg). Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography. (n=30; *p<0.05, **p<0.01 vs. corresponding sham groups).
Figure S5. $I_{\text{Cl,Ca}}$ recorded in BASMCs isolated from sham and 2k2c hypertensive rats at 4 weeks after operation in different concentrations of $[\text{Ca}^{2+}]$. The calculated $EC_{50}$ for $I_{\text{Cl,Ca}}$ activation in the sham and hypertension group were $277.9\pm12.8$ nmol/L (n=5) and $282.6\pm11.2$ nmol/L (n=6), respectively.
Figure S6

**Figure S6.** TMEM16A inhibited angiotensin II (AngII)–induced BASMC proliferation. A. Downregulation of TMEM16A promoted Ang II–induced BASMC proliferation. Growth-arrested BASMCs were pretreated with 40 nmol/L anti-TMEM16A siRNA, 48 hours later, 0.5 µmol/L AngII was added into the culture medium for another 48 hours and then the cells were collected for cell counts (n=6; *p<0.05 vs. control; #p<0.05 vs. AngII-treated group). B. Overexpression of TMEM16A inhibited Ang II–induced BASMC proliferation. Growth-arrested BASMCs were transiently transfected with pEGFP-TMEM16A for 48 hours, after that, 0.5 µmol/L AngII was added into the culture medium for another 48 hours and then the cells were collected for cell counts (n=6; *p<0.05 vs. control; #p<0.05 vs. AngII-treated group).
Supplemental References


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