Ca²⁺/Calmodulin-Dependent Protein Kinase II γ -Dependent Serine727 Phosphorylation Is Required for TMEM16A Ca²⁺-Activated Cl⁻ Channel Regulation in Cerebrovascular Cells

Cai-Xia Lin, PhD; Xiao-Fei Lv, PhD; Feng Yuan, PhD; Xiang-Yu Li, PhD;
Ming-Ming Ma, PhD; Can-Zhao Liu, PhD; Jia-Guo Zhou, PhD;
Guan-Lei Wang, PhD; Yong-Yuan Guan, PhD

Background: TMEM16A is a critical component of Ca²⁺-activated chloride channels (CaCCs) and mediates basilar arterial smooth muscle cell (BASM) proliferation in hypertensive cerebrovascular remodeling. CaMKII is a negative regulator of CaCC, and four CaMKII isoforms (α , β , γ and δ) are expressed in vasculature; however, it is unknown which and how CaMKII isoforms affect TMEM16A-associated CaCC and BASM proliferation.

Methods and Results: Patch clamp and small interfering RNA (siRNA) knockdown of different CaMKII isoforms revealed that only CaMKII γ inhibited native Ca²⁺-activated chloride currents (I_{Cl,Ca}) in BASMs. The TMEM16A overexpression evoked TMEM16A Cl⁻ current and inhibited angiotensin II (Ang II)-induced proliferation in BASMs. The co-immunoprecipitation and pull-down assay indicated an interaction between CaMKII γ and TMEM16A protein. TMEM16A Cl⁻ current was modulated by CaMKII γ phosphorylation at serine residues in TMEM16A. Serine525 and Serine727 in TMEM16A were mutated to alanine, and only mutation at Ser727 (S727A) reversed the CaMKII γ inhibition of the TMEM16A Cl⁻ current. Phosphomimetic mutation S727D markedly decreased TMEM16A Cl⁻ current and reversed TMEM16A-mediated suppression of BASM proliferation, mimicking the inhibitory effects of CaMKII γ on TMEM16A. A significant increase in CaMKII γ isoform content was observed in parallel to the decrease of TMEM16A and I_{Cl,Ca} in basilar artery proliferative remodeling in Ang II-infused mice.

Conclusions: Serine 727 phosphorylation in TMEM16A by CaMKII γ provides a new mechanism for regulating TMEM16A CaCC activity and Ang II-induced BASM proliferation.

Key Words: CaMKII γ ; Cerebrovascular remodeling; Chloride channels; Serine phosphorylation; TMEM16A

TMEM16A, also known as ANO1, has been recently identified as a critical molecular component of Ca²⁺-activated chloride channels (CaCCs) in a number of tissues and cell types.^{1–3} The heterologously expressed TMEM16A evokes the Ca²⁺-sensitive, voltage-dependent currents with properties similar to those of native Ca²⁺-activated Cl⁻ current (I_{Cl,Ca}).^{1–3} TMEM16A CaCC (or current) has been associated with a wide variety of physiological and pathological functions, such as epithelial fluid secretion, neuronal and cardiac excitability, vascular tone and remodeling, tumor growth and cancer progression.^{4,5} Recent evidence from our lab and others supports that in artery smooth muscle cells (SMCs), overexpression of

TMEM16A induced a Ca²⁺-activated, outward-rectifying Cl⁻ current, while TMEM16A antibody or siRNA knockdown of TMEM16A inhibited I_{Cl,Ca}, the TMEM16A Cl⁻ current and augmented angiotensin II (Ang II)-induced proliferation in basilar smooth muscle cells (BASMs) and human aortic SMCs.^{6–8} The most recent study showed the downregulation of TMEM16A expression in Ang II-induced vascular smooth muscle cell (VSMC) remodeling.⁸ The findings suggest that TMEM16A protein and the TMEM16A Cl⁻ current may play a role in regulating Ang II-induced vascular remodeling characterized by arterial SMC proliferation. However, the modulating mechanism of TMEM16A Cl⁻ current is still poorly understood.

Received June 1, 2017; revised manuscript received August 17, 2017; accepted September 29, 2017; released online October 28, 2017
Time for primary review: 49 days

Cardiac and Cerebral Vascular Research Center, Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

The first three authors contributed equally to this work (C.-X.L., X.-F.L., F.Y.).

Mailing address: Yong-Yuan Guan, PhD, or Guan-Lei Wang, PhD, Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2 Rd, Guangzhou 510089, China. E-mail: guanyy@mail.sysu.edu.cn; wangglei@mail.sysu.edu.cn

ISSN-1346-9843 All rights are reserved to the Japanese Circulation Society. For permissions, please e-mail: cj@j-circ.or.jp

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine kinase, is an important mechanism for regulating ion channel activity by Ca²⁺-dependent protein phosphorylation. CaMKII modulation of Cl⁻ channel activity has been demonstrated in different cell types and functionally linked to various fundamental cellular processes.⁹ In the cardiovascular system, CaMKII is generally accepted to be a negative regulator of CaCC in multiple vascular beds including basilar, pulmonary, coronary arteries and portal veins.^{7,10} Recently, we reported that the increase in blood pressure levels was associated with the increase in CaMKII activity and downregulation of CaCC.⁷ Moreover, KN-93, a specific CaMKII inhibitor significantly augmented TMEM16A CaCC activity in BASMC from hypertensive rats,⁷ suggesting the potential role of CaMKII in regulating cerebrovascular TMEM16A Cl⁻ channel activity.

CaMKII contains 4 highly homologous isoforms (α , β , γ and δ) isoforms that are differentially expressed in cells and tissues with the functional diversity.^{11,12} Recently, it has been shown that in double-knockout mice that had specific and complete deletion of both CaMKII γ and δ , cardiac myocytes are protected against cardiac dysfunction but that calcineurin-dependent cardiac hypertrophy develops, which is associated with profound hypophosphorylation of typical CaMKII phosphorylation targets. This double-knockout model also highlights the importance of CaMKII inhibition and CaMKII phosphorylation-dependent mechanisms under pathological conditions.¹³ However, it remains unclear whether and how these CaMKII isoforms affect the TMEM16A Cl⁻ channel and TMEM16A-associated arterial SMC functions.

In this study, we first examined which CaMKII isoform was responsible for regulating TMEM16A Cl⁻ currents in BASMCs. We then used site-specific mutation, patch clamp and siRNA knockdown to verify the target serine residue that is involved in phosphorylation of TMEM16A Cl⁻ currents by CaMKII. These results showed that only CaMKII γ , not the α , β or δ isoforms, regulated TMEM16A CaCC activity, and phosphorylation of serine727 in TMEM16A protein (TMEM16A-Ser727) was required for CaMKII γ -regulated TMEM16A CaCC activity and BASMC proliferation.

Methods

Materials and methods concerning BASMC isolation and culture, siRNA transfection, site-specific mutation, patch clamp, immunoprecipitation and Western Blot analysis, pull-down assay, determination of proliferation by cell counting kit-8 (cck8) and cell cycle by flow cytometry are detailed in the Supplementary Material online.

Animal Model

Ang II-infused hypertensive mice were prepared, as previously described.¹⁴ All animal experimental procedures complied with institutional guidelines for the health and care of experimental animals and conformed to the 'Guide for the Care and Use of Laboratory Animals' of the National Institute of Health in China, and were approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University.

Site-Specific Mutation

GPS2.1 software was used for the prediction of TMEM16A

(GenBank accession no. NM_001242349) phosphorylation sites. The site-directed mutation of TMEM16A was carried out with a Quick-change® 35 Lightning Site-Direct Mutagenesis Kit (Agilent Technologies, Santa Clara, USA), as previously described.¹⁵

Current Recording

Membrane Ca²⁺-activated Cl⁻ current was recorded by an Axopatch 200B Amplifier (Axon Instrument, Foster City, CA, USA), as previously described.⁷ The current was elicited with voltage steps from -100 to +100 mV in 20 mV increments for 250 ms, with an interval of 5 s from a holding potential of -50 mV, and digitalized at 5 kHz by a Digidata1550 (Axon Instrument).

TMEM16A Protein Phosphorylation Detection

The TMEM16A protein phosphorylation was analyzed by using the Immunoprecipitation method. BASMCs were transfected with CaMKII γ for 48 h, and then lysed in non-denaturing lysis buffer (mmol/L: 50 Tris, 150 NaCl, 1 EDTA, 1% Triton X-100) with 1% cocktail protease inhibitor at 4°C. The supernatants with 500 μ g total protein were incubated with an anti-Phosphoserine antibody (5 μ L) or anti-Phosphothreonine antibody (5 μ L), respectively, overnight at 4°C; then incubated with 20 μ L Protein A/G beads (Santa Cruz, CA, USA) for 4 h. Phosphorylated target proteins were detected by Western blotting using TMEM16A antibody; the expression intensity was quantified by Image J software.

Immunohistochemistry and Morphological Assessment

Mice were anaesthetized with pentobarbital sodium (60 mg/kg, i.p.) and were perfused with Krebs buffer containing heparin (100 U/kg) and nitroglycerol (0.3 μ g/kg), followed by 4% freshly depolymerized paraformaldehyde for 20 min. The brain was carefully removed, and sections (8 μ m) were prepared as previously described.¹⁶

Vascular Immunofluorescence Experiment

Frozen sections were dried for 30 min at 37°C, and then washed with phosphate buffer saline (PBS) twice. Then sections were carefully added 100 μ L of 0.1% Triton $\times$ 100 for 5 min at room temperature. After washing with PBS 3 times, sections were incubated with 5% bovine serum albumin (BSA) for 30 min at 37°C. Then sections were incubated with fibronectin antibody (1:200, Santa Cruz) or anti-BrdU antibody (1:500, sigma) overnight at 4°C. After washing with PBS 3 times, sections were incubated with mouse anti-rabbit IgG-Cy3 or rabbit anti-mouse IgG-FITC (1:200, Santa Cruz) for 1 h at 37°C. The cell nucleus were counterstained with 20 mmol/L Hoechst 33342 (Thermo Fisher Scientific) for 5 min in room temperature. All sections were observed by fluorescence microscopy (OLYMPUS, magnification $\times$ 400) and analyzed by Image-Pro Plus 6.0 software.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. A Student's 2-tailed unpaired t-test was used to determine significant differences between 2 groups. One-way or two-way ANOVA, followed by Bonferroni multiple comparison tests, were used to compare differences among 2 more groups. In all tests, P<0.05 was taken as being statistically significant.

Figure 1. CaMKII isoforms expressed in basilar arterial smooth muscle cells (BASMCs) and CaMKII γ regulating Ca^{2+} -activated chloride channels (CaCC). **(A)** The expressions of CaMKII α , β , γ and δ . **(B)** The small-interfering RNA (siRNA)-mediated silencing of CaMKII α , β , γ and δ at 40 nmol/L for 48 h in BASMCs ($n=5$, $*P<0.01$ vs. Control). **(C)** Representative traces of Ca^{2+} -activated Cl^- current ($I_{Cl,Ca}$) evoked by 500 nmol/L $[Ca^{2+}]_i$ in BASMCs (Control) and BASMCs transfected with negative siRNA, CaMKII isoforms siRNAs, pEGFP-C1 (vector) and pEGFP-CaMKII γ cDNA. Only CaMKII γ knockdown could increase the $I_{Cl,Ca}$ significantly ($n=6$, $P<0.05$). In parallel, CaMKII γ overexpression in BASMCs significantly decreased $I_{Cl,Ca}$. **(D)** Mean I-V curves for $I_{Cl,Ca}$ from experiments shown in **(C)**.

Results

CaMKII γ Regulates CaCC in BASMCs

We found that all CaMKII isoforms (α , β , γ and δ) were expressed in both BASMCs and A10 SMCs (Figure 1A).

The protein expression of 4 CaMKII isoforms could be reduced significantly by tested corresponding siRNAs in Western blots relative to the control siRNA (Figure 1B). We have previously confirmed that characteristics of $I_{Cl,Ca}$ in BASMCs are similar to those recorded in other SMCs.⁶⁻⁸

This outward-rectifying current is activated upon a rise in $[Ca^{2+}]_i$ and modulated by voltage. The reversal potential measurements have shown that the current is mainly carried by Cl^- and its reversal potential (-0.6 ± 2.4 mV) is near the equilibrium potential for Cl^- (0 mV) in our current experimental conditions. This Cl^- current can be significantly inhibited by a non-specific ICl_{Ca} blocker, niflumic acid (NFA) (100 μ mol/L). Therefore, the same protocol was applied to evoke the ICl_{Ca} in BASMCs by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to 500 nmol/L (Wang et al⁷), and the current densities of ICl_{Ca} in BASMCs were 4.98 ± 0.42 pA/pF at 100 mV (Figure 1C; n=6). Among all CaMKII isoforms, we found that only CaMKII γ siRNA significantly enhanced ICl_{Ca} from 4.98 ± 0.42 pA/pF to 14.11 ± 0.88 pA/pF at 100 mV (n=6, $P < 0.01$), whereas silencing CaMKII α , β and δ had no significantly effects on current densities of ICl_{Ca} ; that is, 4.97 ± 0.53 , 5.15 ± 0.93 and 5.10 ± 0.42 pA/pF at 100 mV in the presence of CaMKII α , β or δ siRNA respectively; the corresponding current-voltage relationships (I-V) is shown in Figure 1D. To further

examine the regulatory effects of CaMKII γ on ICl_{Ca} , CaMKII γ -cDNA was transfected and found to inhibit ICl_{Ca} in BASMCs (Figure 1C). These results indicated that CaMKII γ , not α , β or δ , may regulate ICl_{Ca} .

We then examined whether CaMKII γ would affect the protein expression of TMEM16A. As shown in Figure S1, neither knockdown nor overexpression of CaMKII γ could change TMEM16A protein expression. Taken together, the Western blot and patch clamp data indicated that CaMKII γ might regulate native ICl_{Ca} through affecting TMEM16A Cl^- channel activation, but not through its influence on TMEM16A protein expression.

CaMKII γ Enhanced Serine Phosphorylation of TMEM16A

The immunofluorescent images, co-immunoprecipitation and pull-down assay shows the co-localization of TMEM16A and CaMKII γ in BASMCs, and a direct interaction between both proteins (Figure S2). Therefore, CaMKII γ may modulate ICl_{Ca} through serine or threonine phosphorylation of TMEM16A. We transfected pEGFP-CaMKII γ plasmid into BASMCs for 48 h to induce CaMKII γ overexpression; and co-immunoprecipitation was then performed using anti-TMEM16A and anti-phosphoserine or phosphothreonine antibodies. Western blot analysis of the immune complex revealed the presence of CaMKII γ -dependent threonine and serine phosphorylation of TMEM16A protein. When CaMKII γ is overexpressed, serine phosphorylation of TMEM16A protein was remarkably enhanced (Figure 2B); but it is not the case for threonine phosphorylation of TMEM16A protein (Figure 2A). This result suggests that it may be serine, but not threonine phosphorylation of TMEM16A protein that works in the regulation of TMEM16A CaCC Cl^- current.

Ser727 Phosphorylation Is Required for CaMKII γ -Regulating TMEM16A Cl^- Currents

Two serine residues (S525, S727) in TMEM16A were predicted as CaMKII γ phosphorylation sites by GPS2.1 software. The positions of these serine residues are shown in schematic membrane topology of TMEM16A (Figure 3A). The site-directed mutagenesis was performed through mutating serine residue (S) to alanine (A), which eliminates the TMEM16A phosphorylation site. We then transfected these mutants into BASMCs and recorded whole-cell patch clamp Cl^- currents under the same conditions as described in Figure 1. The mean I-V curves of the TMEM16A Cl^- current are shown in Figure 3C. To exclude the interference from endogenous Cl^- currents, identical experiments were performed on BASMCs transfected with vector plasmids alone, which served as a parallel control. Only small endogenous voltage-dependent outward Cl^- currents were recorded from vector-transfected cells, and there was no difference in current amplitudes and I-V curves of control and vector-transfected cells. As shown in Figure 3B, expression of wild-type TMEM16A cDNA (WT-TMEM16A) yielded a large outward-rectifying Cl^- current in BASMCs. In WT-TMEM16A, S525A-TMEM16A and S727A-TMEM16A transfected cells, current densities of the TMEM16A Cl^- current were 12.22 ± 0.94 , 11.81 ± 1.68 and 14.09 ± 0.79 pA/pF at +100 mV (n=6, $P > 0.05$) respectively. Then, we examined the effects of mimicking serine 525 and 727 phosphorylation on TMEM16A Cl^- currents by substituting S525 and S727 with aspartate (S525D and S727D), a negatively charged

Figure 3. Serine727 (S727) is the phosphorylation site that CaMKII γ regulates TMEM16A CaCC. **(A)** Structure of TMEM16A showing CaMKII γ phosphorylates S525 and S727. **(B, C)** Representative traces and summary mean I-V curves for I_{Cl, Ca} evoked by 500 nmol/L [Ca²⁺]_i in BASMCs transfected with wild-type TMEM16A (WT-TMEM16A), S525A-TMEM16A mutants (S525A/S525D), S727A-TMEM16A mutants (S727A/S727D) and WT-TMEM16A + CaMKII γ , S525A + CaMKII γ , S727A + CaMKII γ , and S727D + CaMKII γ . Both S525 and S727 phosphomimetic mutations (S525D and S727D) inhibited TMEM16A I_{Cl, Ca}, while non-phosphorylated mutations (S525A and S727A) had no influence on TMEM16A I_{Cl, Ca}. The data from WT-TMEM16A + CaMKII γ , S525A + CaMKII γ , S727D + CaMKII γ , and S727A + CaMKII γ group indicated that S727, but not S525, is the CaMKII γ phosphorylation site regulating TMEM16A I_{Cl, Ca}. Abbreviations as in Figure 1.

residue. Both S525D- and S727D-transfected cells caused a significant reduction in TMEM16A Cl⁻ currents, and the current densities were reduced from 12.22±0.94 pA/pF (WT-TMEM16A) to 3.77±0.41 (S525D) and 4.25±0.47 pA/pF (S727D) at +100 mV (n=6, P<0.01 vs. WT-TMEM16A, **Figure 3B, C**). These results indicate that phosphorylations of Ser 525 and Ser 727 may be important for regulating the TMEM16A Cl⁻ channel.

In order to determine the specific serine site targeted by CaMKII γ for regulating TMEM16A Cl⁻ currents, WT-TMEM16A, S525A-TMEM16A and S727A-TMEM16A were co-transfected with pEGFP-CaMKII γ plasmid into BASMCs (**Figure 3B**). The mean I-V curves are summarized in **Figure 3C**. Overexpression of CaMKII γ abolished the large TMEM16A Cl⁻ currents. At +100 mV, the mean current densities were reduced from 12.22±0.94 (WT-TMEM16A) to 4.64±0.35 (WT-TMEM16A + CaMKII γ), indicating that CaMKII γ overexpression inhibited TMEM16A Cl⁻ currents. Further, compared with the

WT-TMEM16A + CaMKII γ group, the co-transfection of CaMKII γ with the S727A-TMEM16A mutant could reverse the CaMKII γ inhibition of TMEM16A Cl⁻ current, while the co-transfection with S525A-TMEM16A mutant had no influence. In parallel, the co-transfection of CaMKII γ with the S727D-TMEM16A phosphorylatable mutant showed no further influence on reduced Cl⁻ currents. These data indicate that S727A mutation prohibits CaMKII γ -induced S727 phosphorylation on TMEM16A and leaves TMEM16A Cl⁻ currents unchanged. Additionally, pDsred-tagged WT-TMEM16A and serine 727 mutants (S727A and S727D) displayed similar subcellular distribution, as shown by confocal images (**Figure S3**), which excluded the possibility that observed effects of TMEM16A mutants in response to CaMKII γ may due to the altered cellular distribution of TMEM16A protein. Together, these results suggest that S727 is the serine residue for CaMKII γ phosphorylation of the TMEM16A Cl⁻ channel.

Figure 4. Phosphorylation of TMEM16A S727 by CaMKII γ regulates angiotensin II (Ang II)-induced rat BASMC proliferation. **(A)** Summary data showing cell viability by CCK8 assay in rat BASMCs transiently expressing TMEM16A, TMEM16A S727A, TMEM16A S727D or co-expressing with CaMKII γ , after incubation with or without Ang II (0.5 μ mol/L) for 48h (n=6, *P<0.01 vs. Control group, #P<0.01 vs. Control group in the presence of Ang II; *P<0.01 vs. TMEM16A group in the presence of Ang II). **(B)** Summary data showing cell cycle transition detected by flow cytometry in rat BASMCs transiently expressing TMEM16A, TMEM16A S727A, TMEM16A S727D or co-expressing with CaMKII γ , after incubation with or without Ang II (0.5 μ mol/L) for 48h (n=4, *P<0.01 vs. Control group; #P<0.05 vs. Ang II group; *P<0.05 vs. TMEM16A group in the presence of Ang II). Abbreviations as in Figures 1,3.

Increased CaMKII γ Expression and Ser727 Phosphorylation Reverses TMEM16A-Mediated Suppression of BASMC Proliferation

The S727A and S727D mutants were respectively co-transfected with TMEM16A and CaMKII γ into cultured BASMCs to test their effects on Ang II-induced BASMC proliferation. As shown in **Figure 4A**, incubation of BASMCs with Ang II (500 nmol/L) for 48h significantly increased cell viability to 152.7 $\pm$ 8.0% of the control group (n=6, P<0.01), which was markedly inhibited by overexpression of TMEM16A (121.5 $\pm$ 6.7%, n=6, P<0.01). These data are consistent with previous reports.^{7,8} The inhibition of TMEM16A on cellular proliferation rates could be reversed to 145.3 $\pm$ 7.1% or 143.5 $\pm$ 6.1% in BASMCs co-transfected with WT-TMEM16A and CaMKII γ or transfected with phospho-mimic mutation S727D alone (n=6, P<0.01). In contrast, TMEM16A inhibition of cellular proliferation could not be altered by the co-transfection of the WT-TMEM16A+S727A mutant, because the cell viability was 118.3 $\pm$ 6.7% (n=6, P>0.05 vs. WT-TMEM16A). In addition, the non-phosphorylated S727A mutation yielded similar inhibitory effects on Ang II-induced BASMC proliferation as that of TMEM16A overexpression, whereas phospho-mimic mutation S727D had no effects on Ang II-induced cell proliferation. These data demonstrated that

the phosphorylation of TMEM16A-Ser727 and CaMKII γ -dependent regulation of TMEM16A is important for Ang II-induced BASMC proliferation. In comparison with the inhibitory effects of S727A mutant alone on Ang II-induced BASMC proliferation, the co-transfection of the CaMKII γ and S727A mutant could not cause any further change on cellular ability, indicating that Ser727 of TMEM16A is the target site for CaMKII γ -mediated phosphorylation involved in Ang II-induced BASMC proliferation.

A 500 nmol/L amount of Ang II accelerated the cell cycle transition from the G₀/G₁ phase to the S phase. Ang II also reduced the percentage of cells in the G₀/G₁ phase and elevated the percentage of cells in the S phase. The overexpression of TMEM16A inhibited Ang II-induced cell cycle transition, while co-transfection of TMEM16A and CaMKII γ reversed the inhibitory effects of TMEM16A on Ang II-induced cell cycle transition. In contrast, the transfection of S727A+CaMKII γ or S727A alone in BASMCs exhibited similar inhibitory effects on Ang II-induced cell cycle transition, whereas phospho-mimic mutation S727D had no effects on the cell cycle progression. In the absence of Ang II, the Ser727 mutant did not influence cell cycle progression from the G₀/G₁ to the S phase. Our data indicate that overexpression of CaMKII γ could reverse the

G₀/G₁ arrest by TMEM16A in Ang II-stimulated BASMCs, and the S727A mutation abolished effects of CaMKII γ on TMEM16A-arresting G₁/S transition.

Increased CaMKII γ Expression and Ser727 Phosphorylation on Cell Cycle Regulators in BASMC Proliferation

Ang II-induced cyclin E1/D1 expressions were significantly reduced in WT-TMEM16A and S727A mutant transfected cells. The co-transfection of WT-TMEM16A and CaMKII γ reversed the inhibitory effect of WT-TMEM16A on Ang II-induced cyclin E1/D1 expressions. Co-transfection of CaMKII γ and S727A mutant into cells could not further alter the effect of the S727A mutant on the protein expression of cell cycle regulators. In addition, the S727D mutant had the same influence as that of WT-TMEM16A and CaMKII γ co-transfection, which is consistent with the loss of TMEM16A function on cell cycle regulators and a G₀/G₁ arrest (**Figure 5A**). These data suggest that CaMKII γ inhibits TMEM16A CaCC activity through phosphorylation of Ser727 in TMEM16A, followed by the increase in cyclin E1/D1 expressions, accelerating cell cycle transition from G₀/G₁ phase to S phase and finally, enhancing cell proliferation. As shown in **Figure 5B**, Ang II and all of the serine mutants had no influence on p21, p27 and CDK2 protein expressions.

Enhanced CaMKII γ Protein Expression and Decreased I_{Cl,Ca} in Ang II-Induced Basilar Artery Proliferative Remodeling

Ang II (1.5 mg · kg⁻¹ · day⁻¹, 14-day release) infusions with osmotic minipumps into rats or mice have been used as the in vivo model of VSMC proliferation.¹⁷ As indicated by α -actin staining of smooth muscle layer (**Figure 6A**), Ang II infusion caused a significant increase in media thickness and the media cross-sectional area (CSA), and a decrease in internal lumen diameter, resulting in increased media-to-lumen ratio in the basilar arteries from WT mice relative to controls at 14 days. Medial CSA was significantly enhanced in Ang II-infused mice, a typical feature of hypertrophic remodeling. There was a marked increase in bromodeoxyuridine (BrdU) staining in the basilar artery from Ang II-infused mice, indicating that BASMC proliferation occurred during the development of cerebrovascular remodeling induced by Ang II. The immunostaining of fibronectin was markedly increased in the medial wall of the basilar artery after Ang II infusion, indicating an increase in extracellular matrix in cerebral vessels in Ang II-infused mice (**Figure 6A**).

Expression of CaMKII γ was markedly increased and TMEM16A was decreased in basilar arteries after Ang II infusion (**Figure 6B**). Patch clamp recordings indicated that there was a decrease in the amplitude and current densities of I_{Cl,Ca} induced by 500 nmol/L [Ca²⁺]_i in BASMCs freshly isolated from Ang II-infused mice (**Figure 6C**).

Discussion

We presently observed that CaMKII γ was a negative regulator of native I_{Cl,Ca} and TMEM16A CaCC in BASMCs under the basic state. We also provided evidence indicating that Ser727 in TMEM16A is critical for CaMKII γ phosphorylation of TMEM16A Cl⁻ currents and Ang II-induced BASMC proliferation; (1) Four CaMKII isoforms (α , β , γ and δ) are simultaneously expressed in BASMCs; only CaMKII γ was found to modulate the native I_{Cl,Ca} in BASMCs by using gene knockdown and

overexpression approaches; (2) by using co-immunoprecipitation and a pull-down assay, we showed a direct interaction between CaMKII γ and TMEM16A proteins in BASMCs. CaMKII γ caused serine, but not threonine phosphorylation of TMEM16A protein; (3) the TMEM16A overexpression evoked a large outward Cl⁻ current that is compatible with the native I_{Cl,Ca}. This heterologously expressed TMEM16A Cl⁻ current was almost abolished by the phosphomimetic mutation (S727D or S525D), but not influenced by the non-phosphorylatable mutation (S727A); (4) the co-transfection of CaMKII γ markedly decreased the expressed TMEM16A Cl⁻ current, which was reversed by S727A but not by S525A. Moreover, the TMEM16A overexpression inhibited Ang II-induced BASMC proliferation, which was mimicked by S727A, and reversed by CaMKII γ or S727D. Importantly, we found that CaMKII γ protein expression was upregulated in basilar arterial proliferative remodeling in Ang II-induced hypertensive mice, coinciding with the downregulation of TMEM16A and I_{Cl,Ca}.

CaMKII has been identified to negatively regulate CaCC and TMEM16A Cl⁻ currents in aortic, mesenteric, pulmonary and basilar arteries.^{6,10,18} Here, we further demonstrated that it is the CaMKII γ isoform that negatively regulated TMEM16A-mediated CaCC activity in BASMCs via phosphorylation of S727. We also identified a direct interaction between TMEM16A and CaMKII γ proteins in BASMCs, suggesting a direct influence of CaMKII γ on TMEM16A channel activities. These conclusions were consistent with the findings from the study by Jung et al that identified two highly conserved glutamic acid residues (E702 and E705) as calmodulin-binding motifs of ANO1/TMEM16A.¹⁹ S727 is close to this calmodulin binding site (**Figure 3**). The TMEM16A channel has also been found to be regulated by Ca²⁺ binding to the TMEM16A protein itself directly.²⁰ Our proliferation assays showed that S727 in TMEM16A, phosphorylated by CaMKII γ , reversed the inhibitory effect of TMEM16A on Ang-induced BASMC proliferation. As shown in **Figure S4**, CaMKII γ could be slightly phosphorylated under the basic state, which is consistent with a previous report showing the autophosphorylation of CaMKII;²¹ the incubation of BASMCs overexpressing CaMKII γ with 0.5 μ mol/L Ang II for 1 min induced a dramatic enhancement of CaMKII γ phosphorylation. The non-phosphorylated S727A mutation could abolish the effect of TMEM16A in Cl⁻ current (**Figure 3**) and the co-transfection of non-phosphorylated S727A mutation and CaMKII γ could abolish the effects of phospho-mimic mutation S727D alone on Ang II-induced BASMC proliferation, thus yielding similar inhibitory effects as that of TMEM16A overexpression on proliferation (**Figure 4**). Therefore, we speculate that the regulatory effects of CaMKII γ on TMEM16A in BASMCs can solely be ascribed to Ang II-induced phosphorylation mechanism. Further, under the baseline condition, the phosphorylation-dependent mechanism via CaMKII (including CaMKII γ) may play a small role in basilar arterial functions (e.g., in the absence of Ang II stimulation, **Figure 4**), while the enhancement of CaMKII γ (in vitro and in vivo) and consequently phosphorylation-dependent regulation of TMEM16A may exert significant effects on BASMC proliferation under pathological conditions (e.g., when Ang II stimulates). Together, these data suggest that a CaMKII-dependent phosphorylation mechanism may have a more prominent role under pathological conditions.

Figure 5. Effects of TMEM16A serine727 (S727) phosphorylation by CaMKII γ on cell cycle regulatory proteins. **(A)** The inhibition of TMEM16A on angiotensin II (Ang II)-induced cyclin D1 and cyclin E1 expression was reversed by phosphorylation of S727 in TMEM16A by CaMKII γ (n=6, *P<0.01 vs. Control group, #P<0.01 vs. Control group in the presence of Ang II; &P<0.01 vs. TMEM16A group in the presence of Ang II). **(B)** TMEM16A, CaMKII γ phosphorylation of TMEM16A S727 did not affect the protein expression of CDK2, p21, and p27. All representative blots shown are from 6 different experiments.

Figure 6. CaMKII γ expression is enhanced whereas TMEM16A expression and $I_{Cl,Ca}$ is decreased in cerebrovascular remodeling in Ang II-infused mice. **(A)** Cerebrovascular remodeling was developed after Ang II-infusion for 2 weeks. **(i)** Representative images showed the morphological characteristics of basilar arteries, detected by anti- α actin staining, immunofluorescence localization of fibronectin and bromodeoxyuridine (BrdU) incorporation assay. **(ii–iv)** Bar graphs indicated that the cross-sectional area, the expressions of fibronectin and BrdU (expressed in arbitrary units) was increased in Ang II-infused mice ($n=5$ mice per group, $*P<0.01$ vs. Control group). **(B)** Ang II-infusion caused a decrease of TMEM16A protein expression and an increase of CaMKII γ protein expression. **(i)** Representative Western blot images of protein expression of TMEM16A and CaMKII γ . **(ii,iii)** Bar graphs of TMEM16A and CaMKII γ protein expression ($n=6$ mice per group, $*P<0.01$ vs. Control group). **(C)** Activity of CaCCs in BASMCs was decreased in mice infused with Ang II. Representative traces **(i)** and summary mean I–V curves **(ii)** for $I_{Cl,Ca}$ evoked by 500 nmol/L $[Ca^{2+}]_i$ in BASMCs freshly isolated from Control or Ang II-infused mice ($n=6$ mice per group). Abbreviations as in Figures 1,4.

Consistent with our findings, the double-knockout mice lacking 2 CaMKII isoforms (γ and δ) demonstrates profound hypophosphorylation of typical CaMKII phosphorylation targets, including phospholamban, ryanodine receptor 2, histone deacetylase 4 and calcineurin. The absence of both CaMKII isoforms leads to calcineurin-dependent cardiac hypertrophy but protection against heart failure.¹³

In general, the consequence of hypertension is involved in SMC hypertrophy, hyperplasia, migration, apoptosis, and contraction. The accumulating evidence has demonstrated that promotion of VSMC proliferation occurs in some certain forms of hypertension^{15,22–25} whereby persistent elevation of Ang II level gives rise to vascular remodeling, indicating that VSMC proliferation may play a pivotal role in the progression of hypertensive vascular remodeling.²⁶ Ang II infusions with osmotic minipumps into rats or mice have been used as the in vivo model of hypertension as well as a model for VSMC proliferation.¹⁷ The in vivo BrdU data from the basilar artery of this hypertension model demonstrates that abnormal SMC proliferation occurs in BASMCs as the consequence of hypertension (**Figure 6A**), accompanied by decreased expression of TMEM16A. Moreover, our present study results from in vitro BASMCs experiments are in agreement with those from in vivo Ang II-infused mice, suggesting that the enhancement of CaMKII γ activity may cause the downregulation of TMEM16A Cl[−] channel activity, which can accelerate the cell cycle progression into the S phase, promoting BASMC proliferation.

It is noteworthy in both double kidney double clip hypertensive rat (2k2c) and the Ang II-infused hypertensive model that CaMKII γ was enhanced with the development of hypertension-induced cerebrovascular remodeling. However, our results are inconsistent with several reports from studies involving peripheral vessels. It was found that in the rat carotid artery, CaMKII γ was negatively mediated, whereas CaMKII δ positively mediated balloon injury-induced vascular remodeling and cell proliferation.^{27,28} The lack of CaMKII γ expression accelerated balloon injury-induced cell proliferation and remodeling through inhibition of p53/p21 signal pathway expression.²⁷ The first reasonable explanation for these contradictory findings may be due to the diverse CaMKII isoforms involved in different vascular functions and the signaling pathway, which may also be tissue specific. For example, in BASMCs, Ang II induced cell proliferation through the increase of cyclin D1 and E1 expression without alteration of cell cycle negative regulators, p21 and p27.⁷ However, in peripheral blood vessels, Ang II evoked cell proliferation by both the increase of cyclin D1/E1 expression and inhibition of p21 and p27.²⁹ The present study has several limitations. The functional link of CaMKII γ and TMEM16A-mediated BASMC proliferation is still absent in in vivo models. It is unknown why TMEM16A expression decreases during the development of hypertension-induced basilar arterial remodeling. To determine the specific role of CaMKII γ in TMEM16A-mediated vascular function, the use of tissue-specific CaMKII γ knockout or TMEM16A-transgenic mice will be helpful. The second possibility is that the portfolio of CaMKII γ variants in different vessels results in the different functions and associated signaling pathways. At least 6 different variants of CaMKII γ have been identified in rat aortic smooth muscle cells.¹¹ Further investigation is needed to identify

which variants of CaMKII γ are responsible for BASMC proliferation and cerebrovascular remodeling during hypertension.

Both TMEM16A and TMEM16A CaCC have recently emerged as a therapeutic approach for regulating vascular functions. Our data identified for the first time that the CaMKII γ isoform negatively regulates TMEM16A CaCC through phosphorylation at Serine727 within TMEM16A. This phosphorylation mechanism via CaMKII γ is critically involved in Ang II-induced BASMC proliferation and thus may play an important role in hypertension-induced cerebrovascular remodeling. Moreover, the present study provides evidence supporting that the regulation of TMEM16A via CaMKII γ phosphorylation may participate in pathological vascular processes involving abnormal VSMC proliferation. These results may provide new insights into the understanding of the molecular mechanisms regulating TMEM16A CaCC and potential targets against stroke.

Author Contributions

Y.-Y.G. and G.-L.W. proposed this study idea, designed experiments and revised the paper. C.-X.L., X.-F.L. and F.Y. performed all the experiments, analyzed the data, and contributed to the design of some experiments. X.-Y.L., M.-M.M., C.-Z.L., and J.-G.Z. performed some experiments and analyzed the data.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Key grants No. 81230082, No. 81173055, No. 81302771, No. 81370897) and CHINA-CANADA Joint Health Research Program from National Natural Science Foundation of China-Canadian Institutes of Health Research (No. 81361128011).

References

1. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, et al. TMEM16a, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 2008; **322**: 590–594.
2. Schroeder BC, Cheng T, Jan YN, Jan LY. Expression cloning of TMEM16a as a calcium-activated chloride channel subunit. *Cell* 2008; **134**: 1019–1029.
3. Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, et al. TMEM16a confers receptor-activated calcium-dependent chloride conductance. *Nature* 2008; **455**: 1210–1215.
4. Duran C, Thompson CH, Xiao Q, CrissHartzell H. Chloride channels: Often enigmatic, rarely predictable. *Annu Rev Physiol* 2010; **72**: 95–121.
5. Pedemonte N, Galletta LJ. Structure and function of TMEM16 proteins (Anoctamins). *Physiol Rev* 2014; **94**: 419–459.
6. Thomas-Gatewood C, Neeb ZP, Bulley S, Adebisi A, Bannister JP, Leo MD, et al. TMEM16a channels generate Ca(2+)-activated Cl(−) currents in cerebral artery smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2011; **301**: H1819–H1827.
7. Wang M, Yang H, Zheng LY, Zhang Z, Tang YB, Wang GL, et al. Downregulation of TMEM16a calcium-activated chloride channel contributes to cerebrovascular remodeling during hypertension by promoting basilar smooth muscle cell proliferation. *Circulation* 2012; **125**: 697–707.
8. Zhang XH, Zheng B, Yang Z, He M, Yue LY, Zhang RN, et al. TMEM16A and myocardin form a positive feedback loop that is disrupted by KLF5 during Ang II-induced vascular remodeling. *Hypertension* 2015; **66**: 412–421.
9. Wiwchar M, Ayon R, Greenwood IA, Leblanc N. Phosphorylation alters the pharmacology of Ca(2+)-activated Cl channels in rabbit pulmonary arterial smooth muscle cells. *Br J Pharmacol* 2009; **158**: 1356–1365.
10. Greenwood IA, Ledoux J, Leblanc N. Differential regulation of Ca(2+)-activated Cl(−) currents in rabbit arterial and portal vein smooth muscle cells by Ca(2+)-calmodulin-dependent kinase. *J Physiol* 2001; **534**: 395–408.
11. Gangopadhyay SS, Barber AL, Gallant C, Grabarek Z, Smith JL, Morgan KG. Differential functional properties of calmodu-

- lin-dependent protein kinase IIgamma variants isolated from smooth muscle. *Biochem J* 2003; **372**: 347–357.
12. Tombes RM, Faison MO, Turbeville JM. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene* 2003; **322**: 17–31.
 13. Kreusser MM, Lehmann LH, Keranov S, Hoting MO, Oehl U, Kohlhaas M, et al. Cardiac CaM Kinase II genes δ and γ contribute to adverse remodeling but redundantly inhibit calcineurin-induced myocardial hypertrophy. *Circulation* 2014; **130**: 1262–1273.
 14. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest* 2000; **105**: 1605–1612.
 15. Wang XG, Tao J, Ma MM, Tang YB, Zhou JG, Guan YY. Tyrosine 284 phosphorylation is required for CIC-3 chloride channel activation in vascular smooth muscle cells. *Cardiovasc Res* 2013; **98**: 469–478.
 16. Liu YJ, Wang XG, Tang YB, Chen JH, Lv XF, Zhou JG, et al. Simvastatin ameliorates rat cerebrovascular remodeling during hypertension via inhibition of volume-regulated chloride channel. *Hypertension* 2010; **56**: 445–452.
 17. Wolf G, Wenzel UO. Angiotensin II and cell cycle regulation. *Hypertension* 2004; **43**: 693–698.
 18. Waldsee R, Ahnstedt H, Eftekhari S, Edvinsson L. Involvement of calcium-calmodulin-dependent protein kinase II in endothelin receptor expression in rat cerebral arteries. *Am J Physiol Heart Circ Physiol* 2010; **298**: H823–H832.
 19. Jung J, Nam JH, Park HW, Oh U, Yoon JH, Lee MG. Dynamic modulation of ANO1/TMEM16A HCO₃⁻ permeability by Ca²⁺/calmodulin. *Proc Natl Acad Sci USA* 2013; **110**: 360–365.
 20. Yu Y, Kuan AS, Chen TY. Calcium-calmodulin does not alter the anion permeability of the mouse TMEM16A calcium-activated chloride channel. *J Gen Physiol* 2014; **144**: 115–124.
 21. Magupalli VG, Mochida S, Yan J, Jiang X, Westenbroek RE, Nairn AC, et al. Ca²⁺-independent activation of Ca²⁺/calmodulin-dependent protein kinase II bound to the C-terminal domain of CaV2.1 calcium channels. *J Biol Chem* 2013; **288**: 4637–4648.
 22. Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. *Circ Res* 1986; **58**: 427–444.
 23. Sumida T, Naito AT, Nomura S, Nakagawa A, Higo T, Hashimoto A, et al. Complement C1q-induced activation of beta-catenin signalling causes hypertensive arterial remodelling. *Nat Commun* 2015; **6**: 6241.
 24. Qi YX, Yao QP, Huang K, Shi Q, Zhang P, Wang GL, et al. Nuclear envelope proteins modulate proliferation of vascular smooth muscle cells during cyclic stretch application. *Proc Natl Acad Sci USA* 2016; **113**: 5293–5298.
 25. Chen KH, Guo X, Ma D, Guo Y, Li Q, Yang D, et al. Dysregulation of HSG triggers vascular proliferative disorders. *Nat Cell Biol* 2004; **6**: 872–883.
 26. Zhang Z, Wang M, Fan XH, Chen JH, Guan YY, Tang YB. Upregulation of TRPM7 channels by angiotensin II triggers phenotypic switching of vascular smooth muscle cells of ascending aorta. *Circ Res* 2012; **111**: 1137–1146.
 27. Saddouk FZ, Sun LY, Liu YF, Jiang M, Singer DV, Backs J, et al. Ca²⁺/calmodulin-dependent protein kinase II- γ (CaMKII γ) negatively regulates vascular smooth muscle cell proliferation and vascular remodeling. *FASEB J* 2016; **30**: 1051–1064.
 28. House SJ, Singer HA. CaMKII-delta isoform regulation of neointima formation after vascular injury. *Arterioscler Thromb Vasc Biol* 2008; **28**: 441–447.
 29. Hou Y, Okada K, Okamoto C, Ueshima S, Matsuo O. Alpha2-antiplasmin is a critical regulator of angiotensin II-mediated vascular remodeling. *Arterioscler Thromb Vasc Biol* 2008; **28**: 1257–1262.

Supplementary Files

Supplementary File 1

Supplementary Methods

Supplementary Results

Figure S1. Neither CaMKII γ knockdown nor overexpression had any influence on the protein expression of TMEM16A in basilar arterial smooth muscle cells (BASMCs).

Figure S2. CaMKII γ interacted with TMEM16A in basilar arterial smooth muscle cells (BASMCs).

Figure S3. TMEM16A-S727 phosphorylation by CaMKII γ had no influence on the subcellular distribution of TMEM16A.

Figure S4. Phosphorylation of overexpressed CaMKII γ was increased under Angiotensin II (Ang II) treatment.

Please find supplementary file(s);
<http://dx.doi.org/10.1253/circj.CJ-17-0585>