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 (BASMC) 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 BASMC proliferation.

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

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

Key Words: CaMKIIγ; Cerebrovascular remodeling; Chloride channels; Serine phosphorylation; TMEM16A
Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine kinase, is an important mechanism for regulating ion channel activity by Ca\textsuperscript{2+}-dependent protein phosphorylation. CaMKII modulation of Cl\textsuperscript{-} channel activity has been demonstrated in different cell types and functionally linked to various fundamental cellular processes.\textsuperscript{3} 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.\textsuperscript{7,8} Recently, we reported that the increase in blood pressure levels was associated with the increase in CaMKII activity and downregulation of CaCC.\textsuperscript{7} Moreover, KN-93, a specific CaMKII inhibitor significantly augmented TMEM16A CaCC activity in BASMC from hypertensive rats,\textsuperscript{7} suggesting the potential role of CaMKII in regulating cerebrovascular TMEM16A Cl\textsuperscript{-} 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.\textsuperscript{9,10} Recently, it has been shown that in double-knockout mice that had specific and complete deletion of both CaMKII\textsubscript{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.\textsuperscript{11,12} However, it remains unclear whether and how these CaMKII isoforms affect the TMEM16A Cl\textsuperscript{-} channel and TMEM16A-associated arterial SMC functions.

In this study, we first examined which CaMKII isoform was responsible for regulating TMEM16A Cl\textsuperscript{-} 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\textsuperscript{-} currents by CaMKII. These results showed that only CaMKII\textsubscript{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\textsubscript{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 (cck-8) 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.\textsuperscript{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_001243249) phosphorylation sites. The site-directed mutation of TMEM16A was carried out with a Quick-change\textsuperscript{®} 35 Lightning Site-Direct Mutagenesis Kit (Agilent Technologies, Santa Clara, USA), as previously described.\textsuperscript{15}

**Current Recording**

Membrane Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current was recorded by an Axopatch 200B Amplifier (Axon Instrument, Foster City, CA, USA), as previously described.\textsuperscript{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 Digidata1500 (Axon Instrument).

**TMEM16A Protein Phosphorylation Detection**

The TMEM16A protein phosphorylation was analyzed by using the Immunoprecipitation method. BASMCs were transfected with CaMKII\textsubscript{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 μg total protein were incubated with an anti-Phosphoserine antibody (5μL) or anti-Phosphothreonine antibody (5μL), respectively, overnight at 4°C; then incubated with 20μL 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 anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and were perfused with Krebs buffer containing heparin (100 U/kg) and nitroglycerol (0.3 μg/kg), followed by 4% freshly depolymerized paraformaldehyde for 20 min. The brain was carefully removed, and sections (8μm) were prepared as previously described.\textsuperscript{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 μL of 0.1% TritonX100 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×400) and analyzed by Image-Pro Plus 6.0 software.

**Statistical Analysis**

All data are expressed as mean±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.
CaMKIIγ and Serine727 Phosphorylation in TMEM16A

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).

Results

CaMKIIγ 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).
This outward-rectifying current is activated upon a rise in \([\text{Ca}^{2+}]\) 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 enhanced \(I_{\text{Cl,Ca}}\) from 4.98 ± 0.42 pA/pF at 100 mV (\(n=6, P<0.01\)) respectively. Then, we examined the effects of mimicking serine 525 and 727 phosphorylation on current densities of \(I_{\text{Cl,Ca}}\); 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 CaMKIIγ on \(I_{\text{Cl,Ca}}\), CaMKIIγ-cDNA was transfected and found to inhibit \(I_{\text{Cl,Ca}}\) in BASMCs (Figure 1C). These results indicated that CaMKIIγ, not α, β or δ, may regulate \(I_{\text{Cl,Ca}}\).

We then examined whether CaMKIIγ would affect the protein expression of TMEM16A. As shown in Figure S1, neither knockdown nor overexpression of CaMKIIγ could change TMEM16A protein expression. Taken together, the Western blot and patch clamp data indicated that CaMKIIγ might regulate native \(I_{\text{Cl,Ca}}\) through affecting TMEM16A Cl− channel activation, but not through its influence on TMEM16A protein expression.
CaMKIIγ and Serine727 Phosphorylation in TMEM16A

WT-TMEM16A+CaMKIIγ group, the co-transfection of CaMKIIγ with the S727A-TMEM16A mutant could reverse the CaMKIIγ inhibition of TMEM16A Cl– current, while the co-transfection with S525A-TMEM16A mutant had no influence. In parallel, the co-transfection of CaMKIIγ with the 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 CaMKIIγ for regulating TMEM16A Cl– currents, WT-TMEM16A, S525A-TMEM16A and S727A-TMEM16A were co-transfected with pEGFP-CaMKIIγ plasmid into BASMCs (Figure 3B). The mean I–V curves are summarized in Figure 3C. Overexpression of CaMKIIγ 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+CaMKIIγ), indicating that CaMKIIγ overexpression inhibited TMEM16A Cl– currents. Further, compared with the WT-TMEM16A+CaMKIIγ group, the co-transfection of CaMKIIγ with the S727A-TMEM16A mutant could reverse the CaMKIIγ inhibition of TMEM16A Cl– current, while the co-transfection with S525A-TMEM16A mutant had no influence. In parallel, the co-transfection of CaMKIIγ with the S727D-TMEM16A phosphorylatable mutant showed no further influence on reduced Cl– currents. These data indicate that S727A mutation prohibits CaMKIIγ-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 CaMKIIγ may due to the altered cellular distribution of TMEM16A protein. Together, these results suggest that S727 is the serine residue for CaMKIIγ phosphorylation of the TMEM16A Cl– channel.
Increased CaMKIIγ Expression and Ser727 Phosphorylation Reverses TMEM16A-Mediated Suppression of BASMC Proliferation

The S727A and S727D mutants were respectively co-transfected with TMEM16A and CaMKIIγ 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 48 h significantly increased cell viability to 152.7±8.0% of the control group (n=6, P<0.01), which was markedly inhibited by overexpression of TMEM16A (121.5±6.7%, n=6, P<0.01). These data are consistent with previous reports. The inhibition of TMEM16A on cellular proliferation rates could be reversed to 145.3±7.1% or 143.5±6.1% in BASMCs co-transfected with WT-TMEM16A and CaMKIIγ 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±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 CaMKIIγ-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 CaMKIIγ and S727A mutant could not cause any further change on cellular ability, indicating that Ser727 of TMEM16A is the target site for CaMKIIγ-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 CaMKIIγ reversed the inhibitory effects of TMEM16A on Ang II-induced cell cycle transition. In contrast, the transfection of S727A+CaMKIIγ 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 S727 mutant did not influence cell cycle progression from the G0/G1 to the S phase. Our data indicate that overexpression of CaMKIIγ could reverse the

Figure 4. Phosphorylation of TMEM16A S727 by CaMKIIγ 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 CaMKIIγ, after incubation with or without Ang II (0.5 μmol/L) for 48 h (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 CaMKIIγ, after incubation with or without Ang II (0.5 μmol/L) for 48 h (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.
G0/G1 arrest by TMEM16A in Ang II-stimulated BASMCs, and the S727A mutation abolished effects of CaMKIIγ on TMEM16A-arresting G0/G1 transition.

Increased CaMKIIγ 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 CaMKIIγ reversed the inhibitory effect of WT-TMEM16A on Ang II-induced cyclin E1/D1 expressions. Co-transfection of CaMKIIγ 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 CaMKIIγ 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 CaMKIIγ 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 CaMKIIγ Protein Expression and Decreased I{subscript}C{subscript}Ca in Ang II-Induced Basilar Artery Proliferative Remodeling

Ang II (1.5 mg kg{superscript−1} day{superscript−1}, 14-day release) infusions with osmotic minipumps into rats or mice have been used as the in vivo model of VSMC proliferation. 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 CaMKIIγ 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{subscript}C{subscript}Ca induced by 500 nmol/L Ca{superscript2+} in BASMCs freshly isolated from Ang II-infused mice (Figure 6C).

Discussion

We presently observed that CaMKIIγ was a negative regulator of native I{subscript}C{subscript}Ca and TMEM16A CACC in BASMCs under the basic state. We also provided evidence indicating that Ser727 in TMEM16A is critical for CaMKIIγ phosphorylation of TMEM16A Cl{superscript−} currents and Ang II-induced BASMC proliferation: (1) Four CaMKII isoforms (α, β, γ and δ) are simultaneously expressed in BASMCs; only CaMKIIγ was found to modulate the native I{subscript}C{subscript}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 CaMKIIγ and TMEM16A proteins in BASMCs. CaMKIIγ caused serine, but not threonine phosphorylation of TMEM16A protein; (3) the TMEM16A overexpression evoked a large outward Cl{superscript−} current that is compatible with the native I{subscript}C{subscript}Ca. This heterologously expressed TMEM16A Cl{superscript−} 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 CaMKIIγ markedly decreased the expressed TMEM16A Cl{superscript−} 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 CaMKIIγ or S727D. Importantly, we found that CaMKIIγ protein expression was upregulated in basilar arterial proliferative remodeling in Ang II-induced hypertensive mice, coinciding with the downregulation of TMEM16A and I{subscript}C{subscript}Ca.

CaMKIIγ has been identified to negatively regulate CaCC and TMEM16A Cl{superscript−} currents in aortic, mesenteric, pulmonary and basilar arteries. Here, we further demonstrated that it is the CaMKIIγ isoform that negatively regulated TMEM16A-mediated CaCC activity in BASMCs via phosphorylation of S727. We also identified a direct interaction between TMEM16A and CaMKIIγ proteins in BASMCs, suggesting a direct influence of CaMKIIγ 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. As S727 is close to this calmodulin binding site (Figure 3), the TMEM16A channel has also been found to be regulated by Ca{superscript2+} binding to the TMEM16A protein itself directly. Our proliferation assays showed that S727 in TMEM16A, phosphorylated by CaMKIIγ, reversed the inhibitory effect of TMEM16A on Ang-induced BASMC proliferation. As shown in Figure S4, CaMKIIγ could be slightly phosphorylated under the basic state, which is consistent with a previous report showing the autophosphorylation of CaMKII; the incubation of BASMCs overexpressing CaMKIIγ with 0.5 μmol/L Ang II for 1 min induced a dramatic enhancement of CaMKIIγ phosphorylation. The non-phosphorylated S727A mutation could abolish the effect of TMEM16A in Cl{superscript−} current (Figure 3) and the co-transfection of non-phosphorylated S727A mutation and CaMKIIγ 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 CaMKIIγ on TMEM16A in BASMCs may potentially play a small role in basilar arterial functions (e.g., in the absence of Ang II stimulation, Figure 4), while the enhancement of CaMKIIγ (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 CaMKIIγ 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 CaMKIIγ (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, CaMKIIγ 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. CaMKIIγ 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 CaMKIIγ protein expression. (i) Representative Western blot images of protein expression of TMEM16A and CaMKIIγ. (ii,iii) Bar graphs of TMEM16A and CaMKIIγ 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+}] 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.\textsuperscript{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\textsuperscript{15,12-25} 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.\textsuperscript{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.\textsuperscript{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 CaMKIIγ 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 CaMKIIγ was enhanced with the development of hypertension-induced cerebrovascular remodeling. However, our results are inconsistent with several reports from from studies involving peripheral vessels. It was found that in the rat carotid artery, CaMKIIγ was negatively mediated, whereas CaMKIIδ positively mediated balloon injury-induced vascular remodeling and cell proliferation.\textsuperscript{27,28} The lack of CaMKIIγ expression accelerated balloon injury-induced cell proliferation and remodeling through inhibition of p53/p21 signal pathway expression.\textsuperscript{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.\textsuperscript{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.\textsuperscript{29} The present study has several limitations. The functional link of CaMKIIγ 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 CaMKIIγ in TMEM16A-mediated vascular function, the use of tissue-specific CaMKIIγ knockout or TMEM16A-transgenic mice will be helpful. The second possibility is that the portfolio of CaMKIIγ variants in different vessels results in the different functions and associated signaling pathways. At least 6 different variants of CaMKIIγ have been identified in rat aortic smooth muscle cells.\textsuperscript{11} Further investigation is needed to identify which variants of CaMKIIγ 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 CaMKIIγ isoform negatively regulates TMEM16A CaCC through phosphorylation at Serine727 within TMEM16A. This phosphorylation mechanism via CaMKIIγ 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 CaMKIIγ 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.

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**Supplementary Files**

**Supplementary File 1**

**Supplementary Methods**

**Supplementary Results**

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

**Figure S2.** CaMKIIγ interacted with TMEM16A in basilar arterial smooth muscle cells (BASMCs).

**Figure S3.** TMEM16A-S727 phosphorylation by CaMKIIγ negatively regulated vascular smooth muscle cell proliferation and vascular remodeling. *FASEB J* 2016; 30: 1051 – 1064.

**Figure S4.** Phosphorylation of overexpressed CaMKIIγ was increased under Angiotensin II (Ang II) treatment. Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-17-0585