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Journal Title: British Journal of Pharmacology
Volume: Volume 171, Number 15
Publisher: Wiley: 12 months | 2014-07-17, Pages 3680-3692
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1111/bph.12730
Permanent URL: https://pid.emory.edu/ark:/25593/s2fjg

Final published version: http://dx.doi.org/10.1111/bph.12730

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Hypoxia augments the calcium-activated chloride current carried by anoctamin-1 in cardiac vascular endothelial cells of neonatal mice

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BACKGROUND AND PURPOSE
The molecular identity of calcium-activated chloride channels (CaCCs) in vascular endothelial cells remains unknown. This study sought to identify whether anoctamin-1 (Ano1, also known as TMEM16A) functions as a CaCC and whether hypoxia alters the biophysical properties of Ano1 in mouse cardiac vascular endothelial cells (CVECs).

EXPERIMENTAL APPROACH
Western blot, quantitative real-time PCR, confocal imaging analysis and patch-clamp analysis combined with pharmacological approaches were used to determine whether Ano1 was expressed and functioned as CaCC in CVECs.

KEY RESULTS
Ano1 was expressed in CVECs. The biophysical properties of the current generated in the CVECs, including the Ca²⁺ and voltage dependence, outward rectification, anion selectivity and the pharmacological profile, are similar to those described for CaCCs. The density of Iₐ(Ca) detected in CVECs was significantly inhibited by T16Ainh-A01, an Ano1 inhibitor, and a pore-targeting, specific anti-Ano1 antibody, and was markedly decreased in Ano1 gene knockdown CVECs. The density of Iₐ(Ca) was significantly potentiated in CVECs exposed to hypoxia, and this hypoxia-induced increase in the density of Iₐ(Ca) was inhibited by T16Ainh-A01 or anti-Ano1 antibody. Hypoxia also increased the current density of Iₐ(Ca) in Ano1 gene knockdown CVECs.

CONCLUSIONS AND IMPLICATIONS
Ano1 formed CaCC in CVECs of neonatal mice. Hypoxia enhances Ano1-mediated Iₐ(Ca) density via increasing its expression, altering the ratio of its splicing variants, sensitivity to membrane voltage and to Ca²⁺. Ano1 may play a role in the
pathophysiological processes during ischaemia in heart, and therefore, Ano1 might be a potential therapeutic target to prevent ischaemic damage.

**Abbreviations**

Ano1, anoctamin-1; CaCC, calcium-activated chloride channel; \( I_{\text{Cl(Ca)}} \), calcium-activated chloride current; CVEC, cardiac vascular endothelial cell

**Introduction**

Calcium-activated chloride channels (CaCCs) play an important role in epithelial secretion of electrolytes and water, sensory transduction, regulation of neuronal and cardiac excitability, and regulation of vascular tone (Hartzell et al., 2005; channel nomenclature follows Alexander et al., 2013). A member of a Ca\(^{2+}\)-sensitive Cl\(^-\) channel family (CLCA1), volume-regulated chloride channel (CLC-3), bestrophins and tweety were proposed as the CaCC candidate proteins; however, the biophysical features of the currents mediated by these channels are different from those of native classical CaCCs (Hartzell et al., 2009). Heterologous expression of the proteins anoctamin-1 (Ano1; TMEM16A) and Ano2 (TMEM16B) in a variety of cell types produced calcium-activated chloride currents \([I_{\text{Cl(Ca)}}]\), with biophysical characteristics similar to those of native classical CaCCs. Therefore, Ano1 and Ano2 have been proposed as the novel candidate proteins for CaCCs (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Pifferi et al., 2009). Subsequent studies suggested that the gene product of Ano1 was the protein involved in native CaCC in gland acinar cells (Yang et al., 2008; Huang et al., 2009; Romanenko et al., 2010), epithelial cells (Ousingsawat et al., 2009; Rock et al., 2009; Dutta et al., 2011), interstitial cells of Cajal (Hwang et al., 2009) and smooth muscle cells (SMCs) (Davis et al., 2010; Manoury et al., 2010).

CaCCs are expressed in various endothelial cell types of different species, including calf pulmonary artery endothelial cells (Nilius et al., 1997), mouse aorta endothelial cells (Suh et al., 1999) and human umbilical vein endothelial cells (Zhong et al., 2000). CaCCs have been implicated in the control of the membrane potential, which helps to maintain the driving force for Ca\(^{2+}\). In vascular endothelial cells, the resting membrane potential (approximately –40 mV) is more negative than the equilibrium potential for Cl\(^-\) (approximately –28 mV) (Ono et al., 1998). As a consequence of agonist-induced increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{c}\)), CaCCs are activated and Cl\(^-\) leaves the cell, which results in a shift of the membrane potential of endothelial cells towards the Cl\(^-\) equilibrium potential, and possibly inducing a negative feedback during agonist stimulation. Therefore, CaCCs are functionally important in the control of the membrane potential, the driving force for Ca\(^{2+}\) influx and regulation of Ca\(^{2+}\) signalling in endothelial cells (Hosoki and Iijima, 1994). CaCCs may also be involved in control of volume, cell shape and cell proliferation, as well as intracellular pH regulation (Nilius et al., 1997; Hartzell et al., 2005). The CaCCs are involved in many important physiological processes in vascular endothelial cells but their molecular identity and roles in ischaemic conditions remain unknown.

In the present study, we investigated whether Ano1 functions as a CaCC and whether hypoxia alters the biophysical properties of Ano1 in cardiac vascular endothelial cells (CVECs) isolated from neonatal mice. The novel findings of the functional properties of Ano1 in the normal and ischaemic CVECs may provide new therapeutic target for the treatment of ischaemia-induced damage in the vascular system.

**Methods**

**Isolation and culture of CVECs and Ano1 gene silencing in CVECs**

All animal care and experimental procedures were approved by the Harbin Medical University Animal Supervision Committee. All studies involving animals are reported in accordance with the ARrrive guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010). Over 200 animals were used in the experiments described here.

Neonatal BALB/c mice were purchased from Experimental Animal Center of Harbin Medical University. CVECs were isolated from the left ventricles of hearts from neonatal mice, as previously described (Zhou et al., 2010), with slight modifications. Briefly, each pup was placed on a 37°C temperature-controlled pad for general anesthesia from spontaneous respirations of 3.0% isoflurane in oxygen. After anesthesia, the hearts were quickly excised and washed, then minced in ice-cold serum-free DMEM. The atrial, cardiac valves and right ventricle were stripped under a microscope. Then the isolated left ventricles were immersed in 70% ethanol for 10 s to devitalize epicardial mesothelial and endocardial endothelial cells. The left ventricular tissue was finely minced and transferred into a tube containing 5 mL of trypsin (0.25%) for twice for 5 min at 37°C. As a consequence, the functional properties of Ano1 in cardiac vascular endothelial cells (CVECs) may provide new therapeutic target for the treatment of ischaemia-induced damage in the vascular system.

CVECs were cultured in 6-well plates. After cells became 80% confluent, Ano1-specific small hairpin RNAs (shRNAs) were
transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The sequences of shRNA constructs are listed in Supplement Table 1. The shRNAs were cloned into the pGPU6/GFP/Neo-shRNA vector by inserting constructs into the 3’-UTR of the GFP gene (GenePharma, Shanghai, China). Evaluation of gene silencing efficiency assays (qRT-PCR and western blot assays) were performed 48 hrs after transfection. In addition, the green fluorescent protein (GFP) was used as an additional indicator for success of transfection.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from CVECs using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with the RT system protocol in a 20 μl reaction mixture. Total RNA (1 μg) was used in the reaction, and a random primer was used for the initiation of cDNA synthesis, the reaction mixture was incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min, respectively. Then qRT-PCR was performed using the ABI Prism 7500 sequence detection system with SYBR Green PCR reagents (Applied Biosystems, USA). The primers used are listed in Supplement Table 1. After circle reaction, the threshold cycle (Ct) was determined and normalized by the intensity of β-actin level in each sample.

For determining whether hypoxia leads to an altered expression ratio of Ano1 splicing variants in CVECs, we used the primers listed in Supplement Table 2, in which exons 6b, 13 and 15 were excluded. The values for the mRNA products of these Ano1 splicing variants, obtained either from normoxic or hypoxic condition, were then normalized to the values of Ano1 mRNA product obtained using the primers shown in Supplement Table 3 under normoxic condition.

**Western blotting**

CVECs were homogenized in RIPA lysis buffer containing 10 μL mL⁻¹ protease inhibitor cocktail, and centrifuged at 13,500g for 15 min at 4°C to precipitate cell debris. Equal concentration of denatured proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked by 5% nonfat dry milk for 1 hr, followed by incubating with rabbit polyclonal anti-Ano1 antibody (Abcam ab53213, 1:500 dilution) for overnight. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz-2793, CA, 1:4000 dilution) for 1 hr, labeled proteins were visualized with enhanced chemiluminescence (ECL) (Invitrogen WP20005, Carlsbad, CA, USA) and quantified by scanning densitometry (Bio-Rad). The intensities of interesting band were normalized by the intensity of β-actin (Santa Cruzsc-47778, 1:1000 dilution) bands.

**Immunostaining**

Neonatal mouse heart was fixed with 4% paraformaldehyde for 2 hrs followed by 18% sucrose 16 hrs, then preserved in optimum cutting temperature compound (−80°C). Longitudinal sections of the left ventricular wall were cut with a cryostat microtome (CM 3500 cryostat, Israel) at a thickness of 4 mm and permeabilized with 0.2% Triton X-100 (PBST) for 10 min and blocked with 5% bovine serum albumin (BSA) PBST for 1 hr. Rabbit polyclonal anti-Ano1 antibody (1:1000 dilution) (Yu et al., 2010) and sheep polyclonal antibody to von Willebrand factor (vWF) were added in 1% BSA/PBST for overnight at 4°C. The sections were washed in PBST and incubated with Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Invitrogen A21206, 1:1000 dilution) or TRITC conjugated donkey anti-sheep (Abcam ab6897, 1:1000 dilution) for 1 hr.

The endothelial cells of passage one were washed with ice-cold PBS. After fixation with 4% paraformaldehyde at room temperature (22–24°C), cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 5% BSA/PBST for 30 min. Rabbit polyclonal anti-Ano1 antibody (1:1000 dilution) (Yu et al., 2010), endothelial cells markers CD31 (Abcam ab7388, 1:1000 dilution) was added in 1% BSA/PBS overnight at 4°C. Subsequently, the cells were washed in PBS containing 0.1% Tween and incubated with Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Invitrogen A21206, 1:1000 dilution) or Alexa Fluor 594 conjugated donkey anti-rat IgG (Invitrogen A21209, 1:1000 dilution) for 1 hr. All slides were imaged using a confocal microscope (Olympus, Fluoview1000, Japan). Identical acquisition settings were used for all images.

**Cell proliferation assays**

3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was employed to evaluate cell proliferation rate. The CVECs were plated into 96-well plates at a density of 50,000 cells per well in 200 μl culture medium. After 8 hrs recovery, cells were treated with 30 μM T16Ainh-A01 for 48 hrs. For hypoxic group CVECs were cultured under normal condition for 32 hrs and followed by culturing in the hypoxic incubator for 16 hrs, in the absence or in the presence of 30 μM T16Ainh-A01. Then 20 μl PBS-buffered MTT (5 mg mL⁻¹) solution was added to each well and the cells were incubated at 37°C for 4 hrs. Subsequently, the supernatant was then discarded form each well and 100 μL DMSO was added into each well and mixed thoroughly for 10 min to ensure all crystals were dissolved. The absorbance of samples was measured by a microplate reader at wavelength of 570 nm (Tecan, Mannedorf, Switzerland). The quantity of formazan product would be proportional to the number of living cells in culture.

**Exposure of CVECs to hypoxia**

For hypoxic treatment, ~80% confluent CVECs were placed in a hypoxic incubator (Thermo Scientific Series WJ 8000, Waltham, MA, USA) and were kept at 37°C for 16 h with a constant stream of water-saturated 93% N₂, 5% CO₂ and 2% O₂. There was no apoptosis that occurred under this condition, as detected by TUNEL assays (data not shown).

**Patch-clamp recording**

The whole-cell patch-clamp technique was used to record I_{Ca,IC} in voltage-clamp mode using an Axopatch 200B amplifier, and data were filtered at 1 kHz and sampled at 5 kHz (Axon Instruments, Foster City, CA, USA). Borosilicate glass electrodes were pulled with a four-stage puller (Sutter, Novato, CA, USA) and had a resistance of 1–2 MΩ when filled
with pipette solution. $I_{\text{Ca(Ca)}}$ was recorded using a holding potential of 0 mV followed by voltage steps from −100 to +100 mV in 20 mV increment for a duration of 1000 ms, and then repolarized to −100 mV for 300 ms. Membrane current densities were obtained by dividing the measured current amplitude by the cell capacitance. The current density−voltage relationships ($I−V$) were plotted using the calculated current density from the beginning (instantaneous) or the end (steady state) of 1000 ms, as a function of voltages. The activation time constant ($\tau_{\text{act}}$) and the inactivation time constant ($\tau_{\text{inact}}$) were, respectively, obtained by fitting 400 ms of whole-cell current following the end of the capacitance transient and by fitting 200 ms of whole-cell current following the repolarization currents measured after capacitance transient to a single exponential function. The macroscopic chord conductance ($G$) was calculated by an equation: $G = I/V_m$, where $I$ represents the amplitude of steady-state current measured at the end of 1000 ms of each voltage, $V_m$ indicates the membrane potential and $V_{\text{rev}}$ is the reversal potential (since $V_{\text{rev}}$ is −0 mV for all records, we set $V_{\text{rev}}$ to 0 mV for all calculations). The $G$ obtained from each tested voltage was then normalized to the $G$ calculated from +100 mV ($G_{\text{max}}$, the maximal chord conductance). These data points were plotted as a function of voltages and were fitted by the Boltzmann function: $G = G_{\text{max}}/[1 + \exp(-(V_m - V_{1/2})/k)]$, where $V_{1/2}$ represents the voltage at which 50% of activation occurs and $k$ is the slope factor. Prior to analysis, the whole-cell recording traces were further filtered to 100 Hz (Clampfit 10.2; Molecular Devices, Sunnyvale, CA, USA).

For anion selectivity experiments, the data were corrected for junction potentials at the ground bridge (3 M KCl in 3% agar), which ranged from 2 to 4 mV as determined with a free-flowing KCl electrode. $V_{\text{rev}}$ for chloride and for each test anion were used to calculate relative permeability [anion $X$ to Cl$^-$ permeability ($P_{X}/P_{\text{Cl}}$)] values according to the Goldman–Hodgkin–Katz equation (Hille, 1992).

The extracellular solution contained (in mM): 150 NaCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 10 sucrose, 10 HEPES (pH 7.4 with NaOH); the pipette solution contained (in mM): 130 CsCl, 10 EGTA, 1 MgCl$_2$, 10 HEPES, 1 ATP and different concentrations of CaCl$_2$ to obtain the desired free Ca$^{2+}$ concentration (calculated according to the method posted at http://www.stanford.edu/~cspotton/); 1 mM for 18 nM, 6 mM for 290 nM, 7 mM for 453 nM, 8 mM for 777 nM, 8.5 mM for 1.1 μM, and 10 mM for 36.5 μM (pH = 7.3 with CsOH), as determined with the Ca-EGTA calculator.

### Data analyses

Data are reported as mean values ± SEM for n observations. All data gathered in Excel were plotted using Origin 8.5 software (OriginLab, Northampton, MA, USA). Significance was determined using Student’s $t$-tests for paired or unpaired data. $P < 0.05$ was considered statistically significant.

### Materials

Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The specific Ano1 inhibitor, T64Ainh-A01, was purchased from EMD Millipore Biosciences (Billerica, MA, USA).

### Results

#### A Ca$^{2+}$- and voltage-dependent macroscopic current was detected in CVECs

A group of macroscopic currents was recorded from mouse CVECs in the presence of a variety concentrations of free [Ca$^{2+}$], (Fig. 1A–F). The current recorded, in the presence of 18 nM free [Ca$^{2+}$], exhibited no outward rectification and time-dependent relaxation (Fig. 1A and G). The amplitude of the outward currents was amplified gradually and the outward rectification and time-dependent relaxation became more profound, as free [Ca$^{2+}$], was increased from 290 nM to 1.1 μM (Fig. 1B–E and G). However, when free [Ca$^{2+}$], reached 36.5 μM, the inward and outward currents were nearly equal in amplitude, and time-dependent relaxation was almost lost (Fig. 1F and G). The macroscopic currents were deactivated by switching membrane potential to −100 mV. The average instantaneous tail current density measured at −100 mV after pre-pulses to different membrane voltage was plotted as a function of free [Ca$^{2+}$], and the data points were fitted to the Hill equation (Fig. 1H). The data show that EC$_{50}$ of free [Ca$^{2+}$], decreased by about fourfold [2.08 ± 1.04 μM at 0 mV ($n = 7–11$) vs. 0.53 ± 0.06 μM at +100 mV ($n = 7–11$)]. These results suggest that the gating of the macroscopic currents recorded from CVECs is Ca$^{2+}$- and voltage-dependent.

#### A chloride channel mediates the voltage- and Ca$^{2+}$-dependent currents in CVECs

For the rest of the experiments, 777 nM free [Ca$^{2+}$], was used. We assessed anion selectivity experiments to determine whether the voltage- and Ca$^{2+}$-dependent macroscopic current is mediated by a chloride channel. The magnitude of outward currents was significantly reduced by replacing extracellular Cl$^-$ with gluconate, and the $V_{\text{rev}}$ shifted from near zero (−1.37 ± 0.79 mV) to depolarizing potential (52.5 ± 2.31 mV) ($n = 5$) (Fig. 2A–C). Substitution of extracellular Cl$^-$ with NO$_3^-$ resulted in a dramatic increase in the amplitude of outward current, and the $V_{\text{rev}}$ shifted from near zero (−0.92 ± 0.68 mV) to hyperpolarizing potentials (−23.11 ± 1.04 mV) ($n = 8$) (Fig. 2D–F). The relative permeability ratios for $P_{\text{Na}}/P_{\text{Cl}}$ and $P_{\text{NO3}}/P_{\text{Cl}}$ were 0.13 ± 0.01 and 2.30 ± 0.10. Moreover, the macroscopic current was reversibly blocked by 100 μM nifluamic acid (NFA), a non-specific chloride channel blocker ($n = 6$) (Fig. 2G–J). These data together suggest that the gating of the macroscopic currents recorded from CVECs is mediated by a chloride channel.

#### Ano1 presents in CVECs isolated from neonatal mouse

The biophysical features and pharmacological profile of the $I_{\text{Ca(Ca)}}$ detected in CVECs are similar to those of CaCCs. We reasoned that $I_{\text{Ca(Ca)}}$ might be mediated by Ano1. As seen in Fig. 3A, Ano1 mRNA, but not Ano2 mRNA, was detected in CVECs (Fig. 3A). Western blot analysis demonstrated that CVECs expressed Ano1 (Fig. 3B). We further stained the neonatal mouse heart with anti-Ano1 antibody and anti-vWF antibody, a marker for endothelial cells, and sliced the left ventricular walls in longitudinal sections. Ano1 (green) was nicely present at endothelium and co-localized with endothelial...
lial marker (red) (Fig. 3C; the white arrow heads indicate blood vessels). Confocal imaging analyses also revealed that a co-localization of Ano1 (green) and CD31 (red) occurred at the plasma membrane of CVECs (Fig. 3D) because the highest merged fluorescence intensity (peaks) appeared in the vicinity of the plasma membrane (Fig. 3E). These results led us to hypothesize that the $I_{\text{Cl(Ca)}}$ is carried by Ano1 in CVECs.

The $I_{\text{Cl(Ca)}}$ in CVECs is mediated by Ano1

T16Ainh-A01, a newly identified specific Ano1 inhibitor (Forrest et al., 2012; Davis et al., 2013), and a specific pore-targeting anti-Ano1 antibody (Thomas-Gatewood et al., 2011) were respectively applied to the bath solution to test the hypothesis that the $I_{\text{Cl(Ca)}}$ recorded from CVECs is mediated by Ano1. Figure 4A–D showed that the $I_{\text{Cl(Ca)}}$ was significantly and reversibly inhibited by 30 μM T16Ainh-A01 (~60%). The specific pore-targeting Ano1 antibody also dramatically inhibited $I_{\text{Ca(Cl)}}$ (~57%) (Fig. 4E–G); however, the boiled-specific pore-targeting, anti-ANO1 antibody had no effect on the amplitude of the $I_{\text{Ca(Cl)}}$ (Fig. 4H–J). Furthermore, the $I_{\text{Ca(Cl)}}$ was almost completely blocked in Ano1-specific shRNA transfected CVECs; in contrast, the $I_{\text{Ca(Cl)}}$ was not altered in CVECs expressing scramble shRNA (Fig. 4K–N) (refer to Supporting Information Fig. S1 for gene silencing efficiency; since shRNAs were linked with GFP, the cells showing green were used for patch-clamp analyses). These data strongly suggest that the $I_{\text{Ca(Cl)}}$ recorded from CVECs is mediated by Ano1.

**Hypoxia potentiates Ano1-mediated $I_{\text{Cl(Ca)}}$ in CVECs**

As shown in Fig. 5A, B, F and G, the steady-state current ($I_s$) and the instantaneous current ($I_m$) were significantly potentiated by hypoxia, although with different extent (approximately twofold for $I_s$ and approximately fourfold for $I_m$ at depolarizing potentials). Hypoxia-induced increased in the amplitude of $I_{\text{Ca(Cl)}}$ was significantly inhibited by the specific pore-targeting Ano1 antibody (Fig. 5C, F and G) and by T16Ainh-A01 (data not shown). Moreover, hypoxia also significantly enhanced the amplitude of $I_{\text{Ca(Cl)}}$ in Ano1 knockdown CVECs (Fig. 5D and E; $I–V$ data not shown), suggesting that the residual Ano1 was up-regulated by hypoxia. These data further confirm that the $I_{\text{Ca(Cl)}}$ recorded in CVECs is mediated by Ano1. The data shown in Fig. 5H and I demonstrate that the mRNA and protein expression levels of Ano1
were slightly, but significantly increased by exposure of CVECs to hypoxia. However, the increased Ano1 protein expression levels (~20%) were not in agreement with hypoxia-induced increase in current density (approximately twofold for $I_{ss}$ and approximately fourfold for $I_{inst}$), suggesting that there might be other mechanisms by which hypoxia induces an increase in the current density of Ano1. Moreover, the proliferation rate of CVECs was increased ~20% by hypoxia. This hypoxia-induced increase in proliferation rate was not affected by T16Ainh-A01 in either control cells or in the cells exposed to hypoxia (Fig. 5J).

**Hypoxia amplifies the current density of Ano1 via enhancing its sensitivity to Ca$^{2+}$ and to membrane voltage**

Figure 6A shows that in hypoxic CVECs, the ratios of $I_{\text{mol}}/I_{ss}$ (obtained from the records shown in Fig. 5A and B) were increased by hypoxia at the depolarizing potentials. Furthermore, the average $\tau_{\text{act}}$ values were significantly smaller in the cells exposed to hypoxia than in control cells (Fig. 6B). These data suggest that hypoxia alters the activation kinetics of Ano1 in CVECs during sustained depolarization, which is sensitive to Ca$^{2+}$ (Kuruma and Hartzell, 2000; Xiao et al., 2011). The $\tau_{\text{max}}$ values were increased in CVECs exposed to the hypoxia, suggesting that hypoxia retarded the deactivation of Ano1 channels (Fig. 6E). Furthermore, EC$_{50}$ of [Ca$^{2+}$], for activating Ano1-mediated $I_{Ca(Cl)}$ was 534 ± 63.2 nM for control ($n = 7$–11 for different data points) and 385 ± 35.4 nM for hypoxia ($n = 6$–8 for different data points) ($P<0.05$), with Hill coefficients of 2.20 for control and 4.33 for hypoxia (Fig. 6F). The data described earlier suggest that hypoxia potentiates the sensitivity of Ano1-mediated $I_{Ca(Cl)}$ to Ca$^{2+}$, for example, activation of Ano1-mediated $I_{Ca(Cl)}$ requires much lower [Ca$^{2+}$] under hypoxic condition. Figure 6G shows that the half maximum activation voltage ($V_{1/2}$) was shifted from 103 ± 10 mV for control ($n = 11$) to 8.21 ± 2.30 mV for hypoxia ($n = 8$) ($P<0.01$), with the corresponding slope factor ($k$) values of 42.77 ± 2.10 and 53.01 ± 2.61, respectively.
suggesting that hypoxia enhances sensitivity of Ano1-mediated $I_{\text{Ca}(\text{Cl})}$ to membrane voltage, for example, activation of Ano1-mediated $I_{\text{Ca}(\text{Cl})}$ requires significantly less membrane depolarization.

**Hypoxia induces an altered expression ratio of the Ano1 variants**

The human Ano1 splicing variants (deletion of exons 6b, 13 and 15) are known to generate the currents with distinct biophysical properties to wild-type Ano1 (Ferrera et al., 2009; Mazzone et al., 2011). Furthermore, there is a variety of Ano1 splicing variants in mouse heart and these splicing events are highly conserved between human and mouse (O’Driscoll et al., 2011). Therefore, we examined whether hypoxia may cause the changes in the ratio of Ano1 splicing variants, which, in turn, alter the biophysical properties of Ano1-mediated $I_{\text{Ca}(\text{Cl})}$. Our qRT-PCR data showed that under hypoxic condition, the mRNA expression levels of Ano1 splicing variants lacking of exons 6b and 13 (Fig. 7A and B), but not lacking exon 15 (Fig. 7C), were significantly higher than those of control (the primers used are listed in Supporting Information Table S2). These results suggest that changes in the ratio of Ano1 splicing variants may involve in the altered biophysical properties of Ano1-mediated $I_{\text{Ca}(\text{Cl})}$ under hypoxic condition.

**Discussion**

The major findings of the present study are as follows: (i) demonstrating that mouse CVECs express Ano1; (ii) the $I_{\text{Ca}(\text{Cl})}$ recorded from CVECs is mediated by Ano1 and the biophysical properties of the Ano1-encoded $I_{\text{Ca}(\text{Cl})}$ are compatible with those of CaCCs; (iii) hypoxia up-regulates Ano1-mediated $I_{\text{Ca}(\text{Cl})}$ via enhancing its expression levels and an altered expression ratio of Ano1 splicing variants, thereby potentiating its sensitivity to $\text{Ca}^{2+}$ and the membrane voltage.

Although the $I_{\text{Ca}(\text{Cl})}$ in endothelial cells was discovered more than 20 years ago, its molecular identity remains uncertain (Hartzell et al., 2005). Therefore, we aimed to identify the gene responsible for CaCCs in mouse CVECs. We detected a NFA-sensitive macroscopic current with profound outward rectification, time-dependent relaxation and an anionic selectivity sequence of $\text{NO}_3^- > \text{Cl}^- > \text{Glu}^-$. The gating of this macroscopic current was dependent upon $\text{Ca}^{2+}$ and upon membrane voltage. These data suggest that the macroscopic current detected in CVECs is an $I_{\text{Cl}(\text{Ca})}$. The biophysical features of the $I_{\text{Cl}(\text{Ca})}$ in CVECs are consistent with what has been reported for CaCC (Qu et al., 2003). The results obtained from qRT-PCR, Western blot and immunofluorescence imaging analyses suggest that Ano1 is present in CVECs. The $I_{\text{Cl}(\text{Ca})}$ recorded from CVECs was reversibly blocked by T16Ainh-A01, a specific Ano1 inhibitor (Forrest et al., 2012; Davis et al., 2014).
2013), and was significantly inhibited by a specific pore-targeting anti-Ano1 antibody (Thomas-Gatewood et al., 2011) and was greatly diminished in Ano1 knockdown CVECs. Furthermore, hypoxia-induced augmentation of the \( I_{\text{Cl(Ca)}} \) was completely inhibited by the specific pore-targeting anti-Ano1 antibody. These results strongly suggest that the \( I_{\text{Cl(Ca)}} \) recorded from mouse CVECs is mediated by Ano1.

Recent reports suggest that translation of mouse Ano1 yields a molecular weight of \( \sim 110 \text{kDa} \) (Davis et al., 2010). The exclusion of exon 18 in the mouse transcript could result in a truncated Ano1 protein of \( \sim 60-75 \text{kDa} \) (O’Driscoll et al., 2011). We have to notice that the molecular weight of Ano1 in mouse CVECs, detected by Western blots, is \( \sim 150 \text{kDa} \), which is similar to those described in distal colonic epithelial cells and neck squamous cell carcinoma (He et al., 2011; Duvvuri et al., 2012). Human Ano1 has various protein isoforms generated by alternative splicing and has been labelled the four identified alternative segments as a, b, c and d (Ferrera et al., 2009). The TMEM16 (abc)-mediated channel was strongly inactivated by 1.25 \( \mu \text{M} \text{Ca}^{2+} \) (Ferrera et al., 2011). In comparison, we were able to generate Ano1 current at 36.5 \( \mu \text{M} \text{a}2^{+} \), albeit with no outward rectification and time-dependent relaxation. Our result is very compatible with those obtained from HEK293 cells overexpressing Ano1 (Xiao et al., 2011). Our data show that mouse Ano1 has a much lower \( EC_{50} \) for \( \text{Ca}^{2+} \) (\( \sim 0.53 \mu \text{M} \) at +100 mV) compared with those human varieties of Ano1 (\( \sim 3.32 \mu \text{M} \) for (abc) and \( \sim 0.85 \mu \text{M} \) for (ac)) (Ferrera et al., 2009). It is no surprise because alternative splicing events are thought to affect \( \text{Ca}^{2+} \) sensitivity, voltage dependence and the kinetics of activation and deactivation of the Ano1 channel (Ferrera et al., 2009; 2011; Xiao et al., 2011).

**Figure 4**

(A–C) Representative macroscopic currents were respectively generated in the same cell under control condition, in the presence of 30 \( \mu \text{M} \) T16Ainh-A01 followed by washing out. (D) Steady-state current densities obtained from indicated experimental conditions were plotted as a function of the membrane voltages. The macroscopic currents were significantly and reversibly inhibited by T16Ainh-A01 (\( n = 5 \)). (E and F) Representative whole-cell currents were recorded from the same cell under control condition and 10 min after application of specific pore-targeting anti-Ano1 antibody. (G) \( I-V \) relationships show that the macroscopic currents were significantly inhibited by anti-Ano1 antibody (\( n = 6 \)). (H and I) Representative macroscopic currents were respectively obtained from the same cell, in the absence or in the presence of boiled anti-Ano1 antibody. (J) \( I-V \) relationships show that the boiled anti-Ano1 antibody did not affect the macroscopic current density (\( n = 6 \)). (K–M) Representative macroscopic currents were respectively generated from control cells (\( n = 8 \)), the cells transfected with scramble shRNA (\( n = 6 \)) and the cells transfected with shRNA against Ano1 (\( n = 6 \)). (N) \( I-V \) relationships show that the macroscopic currents were greatly diminished in Ano1 knockdown CVECs.
Hypoxia is associated with angina pectoris, myocardial infarction, heart failure and peripheral artery disease. Severe hypoxia induces cell proliferation and angiogenesis (Li et al., 2007). Vascular endothelial cell proliferation plays an essential role in angiogenesis and revascularization of the myocardium following myocardial infarction (Isner and Losordo, 1999). Sun and co-workers suggested that an increase in expression levels of Ano1 in SMCs, isolated from chronic-hypoxic pulmonary hypertension rat, is responsible for enhanced current density of Ano1 (Sun et al., 2012). A similar result was observed in monocrotaline-induced pulmonary hypertension rat model (Forrest et al., 2012). In comparison, our results demonstrate that hypoxia enhances the density of Ano1-mediated \( I_{\text{Cl(Ca)}} \) not only through increasing expression of Ano1 but also through potentiating its sensitivity to \( \text{Ca}^{2+}\) and to membrane voltage. Recent studies suggest that a variety of Ano1 splicing variants are present in mouse heart (O’Driscoll et al., 2011). Therefore, we speculated that the hypoxia-induced increase in Ano1 expression levels and altered biophysical features of Ano1 in CVECs might be associated with the changes in the ratio of Ano1 splicing variants. We found that the relative mRNA expression levels of exons 6b and 13 excluded Ano1 variants were significantly up-regulated by hypoxia. Due to overexpression of exon 6b, omitted human Ano1 in HEK293 cells led to a significantly increased sensitivity to \( \text{Ca}^{2+}\) (Ferrera et al., 2009); we also speculate that hypoxia-induced increase in \( \text{Ca}^{2+}\) sensitivity of Ano1-mediated \( I_{\text{Cl(Ca)}} \) could be attributable to elevated expression levels of exon 6b excluded Ano1 variant in CVECs. Furthermore, hypoxia-induced increase in voltage sensitivity of Ano1-mediated \( I_{\text{Cl(Ca)}} \) might be caused by the increased fraction of exon 13 excluded Ano1 variant, since a reduction of the voltage dependence of activation was found in HEK293 cells expressing exon 13 deleted human Ano1 variant (Ferrera et al., 2009). Overexpression of exon 15 deleted human Ano1 variant in HEK293 resulted in a significant faster activation
and deactivation kinetics (Mazzone et al., 2011). However, we did not detect if hypoxia caused changes in the relative ratio of exon 15 excluded Ano1 variant in CVECs. Finally, this hypoxia-induced increase in Ano1 current density might be, at least in part, due to Ca$^{2+}$ overload under this condition (Abdallah et al., 2007).

Ano1 may be involved in hypertension-induced cerebrovascular remodelling, based upon the facts that knockdown of Ano1 facilitates and overexpression of Ano1 inhibits angiotensin II-induced cell cycle transition and cell proliferation in rat basilar SMCs (Wang et al., 2012). Other studies suggest that the function of Ano1 is an important factor for contraction of SMCs both in membrane potential-dependent and membrane potential-independent ways (Dam et al., 2013). Several studies have suggested that Ano1 expression levels are associated with cell proliferation including interstitial cells of Cajal in the small intestine (Stanich et al., 2011), head and neck squamous cell carcinoma cells, the T24 bladder cancer cells (Duvvuri et al., 2012) and the gastrointestinal stromal tumour cells (Simon et al., 2013). Our data suggest that hypoxia also induces an increase in the proliferation rate of CVECs; however, it appears that this hypoxia-induced proliferation is not due to enhanced Ano1 activity because T16Ainh-A01 (up to 30 μM for 48 h) did not affect the cell growth.

In summary, we suggest that Ano1 forms CaCC in CVECs and that hypoxia up-regulates Ano1-mediated $I_{\text{Ca,C}}$ via enhancing its expression levels and an altered expression ratio of Ano1 splicing variants, thereby potentiating its sensitivity to Ca$^{2+}$ and the membrane voltage.
Acknowledgements

This study was supported by Key Project of Chinese National Program for Fundamental Research and Development (973 Program 2014CB542401, 2012CB517803 to Z. Z.), National Natural Science Foundation of China (30871007, 81270340 and 81320108002 to Z. Z.), Doctoral Tutor Foundation of Ministry of Education (20122307110008 to Z. Z.), Overseas Talent Foundation of Department of Education, Heilongjiang Province (1154HZ11 to Z. Z.) and the Natural Science Foundation of Heilongjiang Province (ZD200807-01, ZD200807-02 to Z. Z. and QC2010097 to D. Z.). This study was also supported by American Heart Association Western States Affiliate Grant-in-Aid (11GRNT7610161 to D. D.) and the National Institute of Health Grant (HL106256 to D. D.).

Author contributions

Z-RZ, DZ and DDD conceived and designed the experiments; M-MW, JL, B-LS, Y-FG, Y-CL, CY, Q-SW, T-XM and KM collected, analysed and interpreted the data; Z-RZ, DZ and M-MW drafted the manuscript; HCH and DDD revised the manuscript; and all authors approved the final version of the manuscript.

Conflict of interest

None declared.

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Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
http://dx.doi.org/10.1111/bph.12730

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Supporting information
Figure S1 (A) Immunofluorescence images demonstrate the transfection efficiency. Images were taken by fluorescence microscope. The green colour represents GFP. Scale bars represent 40 µm. (B) qRT-PCR demonstrating the expression levels of Ano1 mRNA under indicated conditions (n = 6). (C) Representative immunoblots demonstrating the expression levels of Ano1 protein under different experimental conditions. (D) Summarized bar graph represents the abundance of Ano1 protein under different experimental conditions as indicated (n = 6). **Indicates P < 0.01 compared to either control or scramble shRNA transfected group.

Table S1 Specific shRNAs sequences for Ano1.
Table S2 Primers used for qRT-PCR of Ano1 exon variants.
Table S3 Oligo nucleotide primers for qRT-PCR.
Appendix S1 Methods and results.