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High blood pressure is the leading risk factor for death worldwide. One of the hallmarks is a rise of peripheral vascular resistance, which largely depends on arteriole tone. Ca\(^{2+}\)-activated chloride currents (CaCCs) in vascular smooth muscle cells (VSMCs) are candidates for increasing vascular contractility. We analyzed the vascular tree and identified substantial CaCCs in VSMCs of the aorta and carotid arteries. CaCCs were small or absent in VSMCs of medium-sized vessels such as mesenteric arteries and larger retinal arterioles. In small vessels of the retina, brain, and skeletal muscle, where contractile intermediate cells or pericytes gradually replace VSMCs, CaCCs were particularly large. Targeted disruption of the calcium-activated chloride channel TMEM16A, also known as ANO1, in VSMCs, intermediate cells, and pericytes eliminated CaCCs in all vessels studied. Mice lacking vascular TMEM16A had lower systemic blood pressure and a decreased hypertensive response following vasoconstrictor treatment. There was no difference in contractility of medium-sized mesenteric arteries; however, responsiveness of the aorta and small retinal arterioles to the vasoconstriction-inducing drug U46619 was reduced. TMEM16A also was required for peripheral blood vessel contractility, as the response to U46619 was attenuated in isolated perfused hind limbs from mutant mice. Out data suggest that TMEM16A plays a general role in arteriolar and capillary blood flow and is a promising target for the treatment of hypertension.

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Disruption of vascular Ca\(^{2+}\)-activated chloride currents lowers blood pressure

Christoph Heinze,1 Anika Seniuk,2 Maxim V. Sokolov,3 Antje K. Hübner,1 Agnieszka E. Klementowicz,3 István A. Szi jártó,4,5 Johanna Schleifenbaum,4 Helga Vitzthum,2 Maik Gollasch,4 Heimo Ehmke,2 Björn C. Schroeder,3 and Christian A. Hübner1

1Institut für Humangenetik, Universitätsklinikum Jena, Friedrich-Schiller Universität Jena, Jena, Germany. 2Institut für Zelluläre und Integrative Physiologie, Universitätssklinikum Hamburg Eppendorf, Hamburg, Germany. 3Max-Delbrück Centrum für Molekulare Medizin (MDC) and NeuroCure, Berlin, Germany. 4Medizinische Klinik mit Schwerpunkt Nephrologie und Internistische Intensivmedizin, Charité – Universitätsmedizin Berlin, Experimental and Clinical Research Center (ECRC), Berlin, Germany. 5Interdisziplinäres Stoffwechsel-Centrum, Charité – Universitätsmedizin Berlin, Berlin, Germany.

High blood pressure is the leading risk factor for death worldwide. One of the hallmarks is a rise of peripheral vascular resistance, which largely depends on arteriole tone. Ca\(^{2+}\)-activated chloride currents (CaCCs) in vascular smooth muscle cells (VSMCs) are candidates for increasing vascular contractility. We analyzed the vascular tree and identified substantial CaCCs in VSMCs of the aorta and carotid arteries. CaCCs were small or absent in VSMCs of medium-sized vessels such as mesenteric arteries and larger retinal arterioles. In small vessels of the retina, brain, and skeletal muscle, where contractile intermediate cells or pericytes gradually replace VSMCs, CaCCs were particularly large. Targeted disruption of the calcium-activated chloride channel TMEM16A, also known as ANO1, in VSMCs, intermediate cells, and pericytes gradually replace VSMCs, CaCCs were particularly large. Targeted disruption of the calcium-activated chloride channel TMEM16A, also known as ANO1, in VSMCs, intermediate cells, and pericytes eliminated CaCCs in all vessels studied. Mice lacking vascular TMEM16A had lower systemic blood pressure and a decreased hypertensive response following vasoconstrictor treatment. There was no difference in contractility of medium-sized mesenteric arterioles; however, responsiveness of the aorta and small retinal arterioles to the vasoconstriction-inducing drug U46619 was reduced. TMEM16A was also required for peripheral blood vessel contractility, as the response to U46619 was attenuated in isolated perfused hind limbs from mutant mice. Out data suggest that TMEM16A plays a general role in arteriolar and capillary blood flow and is a promising target for the treatment of hypertension.

Introduction

With more than 25% of the adult population being affected, hypertension is an important public health challenge worldwide (1). It is a major risk factor for cardiovascular disease, myocardial infarction, stroke, and chronic renal failure. The pathogenesis of hypertension as well as the basic mechanisms of blood pressure control, however, are still insufficiently understood. A hallmark of hypertension is the increased tone of arterial blood vessels and therefore elevation of total peripheral vascular resistance (2).

Vascular tone depends on a complex interplay of vasodilator and vasoconstrictor stimuli, which are integrated by VSMCs and transformed into the activity of the contractile apparatus. Accumulation of Cl\(^{-}\) by the Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) co-transporter NKCC1 (3) and the Cl\(^{-}\)/HCO3\(^{-}\) exchanger AE2 (4) raises the Cl\(^{-}\) equilibrium potential above the resting membrane potential in VSMCs. Therefore, opening of Cl\(^{-}\) channels within the plasma membrane of VSMCs is predicted to cause Cl\(^{-}\) efflux and membrane depolarization (5). Ca\(^{2+}\)-activated chloride currents (CaCCs) have been described in VSMCs of various blood vessels (4, 6, 7). Because CaCC-dependent depolarization can activate voltage-gated Ca\(^{2+}\) channels and thus further increase intracellular Ca\(^{2+}\), CaCCs may enhance the contractile response of blood vessels (8).

Recently, it has been shown that TMEM16A, also known as ANO1, mediates CaCCs (9–11). TMEM16A belongs to a family of 10 homologous transmembrane proteins of approximately 900 amino acids. So far four members have been shown to be associated with hereditary disorders, including craniocervical dystonia (12), muscular dystrophy (13), the bleeding disorder Scott syndrome (14), and cerebellar ataxia (15). Despite their obvious physiological importance, a function in ion transport has not been established for most family members. Interestingly, TMEM16F and other family members have been associated with scramblase activity (16). TMEM16A expression has been reported for VSMCs of the thoracic aorta, the carotid artery, and some arteries in the brain, and it has been suggested that TMEM16A mediates CaCCs of these cells (17, 18). Knockdown of TMEM16A in cultured medium-sized cerebral arteries from rat resulted in a reduction of pressure-induced vasoconstriction (19). Moreover whole-cell patch-clamp studies showed that knockdown of TMEM16A attenuated CaCCs in rodent large cerebral artery VSMCs (20, 21). However, the role of CaCCs in VSMCs in the regulation of systemic arterial blood pressure could not be addressed so far, as Tmem16a knockout mice have a complex phenotype with early mortality, malformations of the trachea, and intestinal obstruction (22). Moreover, specific CaCC inhibitors are currently not available.

Here we show that disruption of TMEM16A under the control of the smooth myosin heavy chain promoter abolishes CaCCs in contractile cells of blood vessels and lowers arterial blood pressure. This hypotensive effect of disruption of TMEM16A is most likely mediated via small-diameter arterioles, where CaCCs are particularly large and contribute to vascular contractility and thus to peripheral resistance.
Results

Disruption of TMEM16A eliminates CaCCs in VSMCs from the aorta. To overcome the limitations of the constitutive Tmem16a knockout mouse, we floxed exon 21 of the Tmem16a gene (Figure 1A), which encodes the most conserved part of the TMEM16 family (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI70025DS1). A CTDNA construct devoid of exon 21 transfected into HEK cells did not give rise to CaCCs, whereas large CaCCs were observed upon transfection with the wild-type cDNA (Supplemental Figure 1, B–E), illustrating that the part of the protein encoded by exon 21 is essential for channel function. The floxed (Tmem16afl/fl) line was subsequently mated with either a Cre-deleter line (23) to generate constitutive TMEM16A mutant mice or the tamoxifen-inducible SMMHC-Cre-ERT2 line, which allows induction of Cre recombinase activity in the contractile cells of blood vessels by administration of tamoxifen (24).

Tmem16a knockout mice with a homozygous deletion of exon 21 had malformations of the trachea (Supplemental Figure 1F) and died within the first 2 weeks of life, mirroring the phenotype previously reported for a constitutive Tmem16a knockout mouse model obtained by deletion of exon 12 (22). In VSMCs isolated from the aorta of non-induced conditional knockout mice, robust CaCCs were present, which depended on the presence of intracellular Ca2+ and could be blocked by niflumic acid, an inhibitor of CaCCs (Figure 1, B–D). CaCCs were absent in VSMCs isolated from mice after tamoxifen induction, indicating that this current is indeed mediated by TMEM16A (Figure 1, B–D).

Disruption of TMEM16A under the control of the smooth muscle myosin heavy chain promoter abolishes CaCCs in VSMCs of the aorta. (A) In the targeted Tmem16a locus, exon 21 is flanked by loxP sites (upper panel). After Cre-mediated excision, the floxed fragment is removed (lower). Numbered black squares represent exons and triangles, loxP sites; EcoRI restriction sites used for cloning/screening are indicated. (B) Typical currents recorded from VSMCs isolated from thoracic aortae of non-induced control mice (Cre+ no tamoxifen) and induced conditional Tmem16a knockout mice (Cre+ tamoxifen). (C) Mean I-V relationship was recorded after a 1 second test pulse from VSMCs isolated from thoracic aortae of WT, non-induced control (Cre+ no tamoxifen), and induced conditional Tmem16a knockout mice (Cre+ tamoxifen). CaCCs measured from controls were absent under Ca2+-free conditions (Cre+ no tamoxifen Ca2+ free). CaCCs of VSMCs were blocked by 300 µM niflumic acid (Cre+ no tamoxifen + 300 µM niflumic acid), n = 6–15 cells each. (D) Amplitude of various VSMC tail current densities measured after a voltage step to –80 mV from a 1 second test pulse at +80 mV (n = 6–15 cells each). Whereas no difference was detected between non-induced control and wild-type mice, non-induced control mice and mice under other conditions differed significantly. 2-way ANOVA; **P < 0.01.
excluded by monitoring blood pressure during tamoxifen induction in mice carrying the SMMHC-Cre-ER\textsuperscript{T2} transgene on a wild-type background (data not shown). Cardiac parameters such as fractional shortening, cardiac output, ejection fraction, and heart rate were unchanged 2 weeks after termination of tamoxifen treatment and did not differ between controls and mutant mice (Figure 2C). Importantly, the hypotensive effect of the disruption of TMEM16A was maintained and even increased in the presence of the vasopressor angiotensin II (Figure 2D).

We subsequently addressed whether the hypotensive effect of TMEM16A disruption was dietary salt dependent. Although the hypotensive effect of TMEM16A disruption was not further increased by a low-salt diet, the difference in systemic blood pressure was eliminated on a high-salt diet (Figure 2E).

Plasma aldosterone, renin, angiotensin II, K\textsuperscript{+}, and various blood gas parameters analyzed did not differ between genotypes, excluding a major renal compensation via the renin-angiotensin-aldosterone system (Table 1). We also determined creatinine clearance and fractional excretion for Na\textsuperscript{+}, Cl\textsuperscript{−}, and K\textsuperscript{+}, but did not detect any significant differences between groups (Table 1).

**Figure 2**
Disruption of TMEM16A in blood vessels decreases mean arterial blood pressure. (A) Blood pressure in three cohorts of floxed Tmem16a mice. Cre recombinase expressed under control of the SMMHC promoter was induced by feeding tamoxifen to the experimental cohort (Cre\textsuperscript{+} tamoxifen, n = 14). Littermates of the same genotype that did not receive tamoxifen served as a control (Cre\textsuperscript{+} no tamoxifen, n = 15). A Cre-negative control cohort received tamoxifen to exclude a gene-independent effect of tamoxifen on mean arterial blood pressure (Cre\textsuperscript{−} tamoxifen, n = 6). Induction with tamoxifen caused a significant decrease in systemic blood pressure in the Cre\textsuperscript{+} cohort (2-way ANOVA; **P < 0.01. (B) At the same time, the pulse pressure decreased (2-way ANOVA; **P < 0.01). (C) Fractional shortening, cardiac output, ejection fraction, and heart rate did not differ between cohorts before and 2 weeks after termination of the tamoxifen induction. Student’s t test. (D) The hypotensive effect of TMEM16A disruption on mean arterial blood pressure was enhanced in the presence of the vasopressor angiotensin II, which was continuously infused (1.5 ng•g\textsuperscript{−1}•min\textsuperscript{−1}) with osmotic mini pumps starting 2 weeks after tamoxifen induction (n = 6–7 for each cohort). 2-way ANOVA; **P < 0.01. (E) Mean arterial blood pressure did not further decrease on a low-salt diet. Switching to a high-salt diet eliminated the difference in blood pressure between induced and non-induced mice (n = 4 for each group). 2-way ANOVA; *P < 0.05.
Disruption of TMEM16A in VSMCs reduces contractility in aorta, but not in mesenteric arteries. We analyzed the wall-to-lumen ratio of blood vessels after disruption of TMEM16A, but did not observe any structural changes (Supplemental Figure 2, A and B). The response of aortic rings to various concentrations of external KCl did not differ between cohorts (Supplemental Figure 2, C and D). We measured the constriction of mesenteric arteries at 60 mM KCl, which was unchanged as well (Supplemental Figure 2, E and F). These results argue against a principal defect of voltage-gated Ca\(^{2+}\) channel–dependent vascular smooth muscle contraction upon disruption of TMEM16A. We next tested our hypothesis that vascular contractility is modulated by TMEM16A and compared the effects of various vasoconstrictors on aortic rings or rings isolated from first- and second-order mesenteric arteries of induced and non-induced mice. Changes in vessel tension of aortic rings from tamoxifen-induced mice in response to vasoconstrictor GPCR agonists such as angiotensin II (Figure 3G), U46619, a synthetic analog of prostaglandin PGH\(_2\) (Figure 3J), and phenylephrine (Supplemental Figure 2G) were attenuated. In accordance with smaller or even absent CaCCs in first- and second-order mesenteric arteries, contraction of isolated first- and second-order mesenteric artery rings to vasopressors as assessed by wire myography did not differ between genotypes (Figure 3, H and K, and Supplemental Figure 2H). Similarly, constriction of third- and fourth-order mesenteric arteries as assessed by videomicroscopy did not differ between cohorts (Figure 3, I and L, and Supplemental Figure 2I). These data are supported by previous reports showing strong CaCCs in large vessels such as pulmonary artery, aorta, and portal vein but not in medium-sized vessels (4).

CaCCs in pericytes of small arterioles from brain and retina are mediated by TMEM16A and regulate their contractility. Because large conduct arteries only marginally contribute to peripheral resistance (26), the difference in arterial blood pressure upon disruption of TMEM16A cannot be explained by the decreased contractility of the aorta. Instead, the regulation of peripheral resistance largely depends on the diameter of smaller arteries. During arborization of arterioles, VSMCs are gradually replaced by smooth muscle cell–like cells, which are termed intermediate cells or pericytes (27, 28). Pericytes had been reported to exhibit native currents (29) that are compatible with CaCCs. Indeed, microvessels in the brain, which are known to be densely packed with pericytes (30), showed marked immunoreactivity for TMEM16A (Figure 4A). Also, intermediate cells/pericytes of secondary and higher-order arterioles in the retina were intensely stained for TMEM16A, whereas expression in larger primary arterioles or veins was weak or absent (Figure 4, B and C). Notably, the TMEM16A signal appeared to be particularly intense in cells located at vascular branching points (Figure 4C, arrows). To examine whether our immunohistochemical results corresponded with the distribution of functional TMEM16A channels, we performed patch-clamp recordings on tissue prints of retinal microvascular complexes. While no CaCCs were found in VSMCs of primary arterioles in the retina (Figure 4, D and G), tail current densities recorded from dome-shaped intermediate cells or pericytes of secondary and higher-order arterioles of wild-type retina were more than 3 times larger than those in the aorta (Figure 4, E and G). Similar currents were recorded from pericytes from microvascular complexes isolated from WT brain and were absent in cells of the conditional knockout (Figure 4, F and G, and Supplemental Figure 3, A and B).

The special 2-dimensional structure of the retina also allowed us to quantify vasoconstriction of second-order retinal arterioles in response to U46619 using time-lapse microscopy. While no difference between vessels from control and conditional knockout mice was found for saturating drug concentrations, constriction was reduced at submaximal levels in preparations from conditional knockout mice (Figure 4H and Supplemental Figure 3C), indicating a regulatory role for TMEM16A in retina microcirculation.

**Table 1**

<table>
<thead>
<tr>
<th>Plasma hormone levels</th>
<th>No tamoxifen</th>
<th>n</th>
<th>Tamoxifen</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin (ng angiotensin I/ml/h)</td>
<td>25.25 ± 4.89</td>
<td>19</td>
<td>26.40 ± 4.57</td>
<td>21</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>4.27 ± 1.14</td>
<td>11</td>
<td>3.74 ± 1.49</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>22.51 ± 9.06</td>
<td>18</td>
<td>15.61 ± 4.84</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>ANP (ng/ml)</td>
<td>4.17 ± 0.23</td>
<td>14</td>
<td>4.20 ± 0.45</td>
<td>14</td>
<td>NS</td>
</tr>
</tbody>
</table>

| Plasma electrolyte levels | | |
|---------------------------| | |
| Na\(^+\) (mmol/l) | 149.15 ± 0.68 | 10 | 147.70 ± 1.22 | 10 | NS |
| K\(^+\) (mmol/l) | 5.37 ± 0.19 | 10 | 5.21 ± 0.15 | 10 | NS |
| Cl\(^-\) (mmol/l) | 119.30 ± 0.68 | 10 | 119.30 ± 1.07 | 10 | NS |

| Renal function | | |
|----------------| | |
| Creatinine clearance (ml/24 h) | 2.288.67 ± 1.139.78 | 9 | 2.023.66 ± 642.17 | 10 | NS |
| Na\(^+\) FE (%) | 0.174 ± 0.055 | 9 | 0.206 ± 0.051 | 10 | NS |
| K\(^+\) FE (%) | 5.48 ± 1.25 | 9 | 6.91 ± 1.47 | 10 | NS |
| Cl\(^-\) FE (%) | 0.0385 ± 0.0101 | 9 | 0.0545 ± 0.0146 | 10 | NS |

Data are shown as mean and SEM. FE, fractional excretion. Student's t test; P > 0.05.
The influence of TMEM16A on the resting membrane potential and U46619-induced depolarization of skeletal muscle pericytes was determined using gramicidin-perforated patch recordings. While no significant difference between the genotypes was found for the resting membrane potential (Figure 5D), U46619-induced depolarization of WT pericytes was almost absent in the mutant (Figure 5E). In some cells U46619 caused fluctuations of the membrane potential, which may be explained by Ca2+ oscillations (31). These cells were excluded from the analysis. Other TMEM16A-deficient pericytes hyperpolarized after agonist application, which suggests the presence of Ca2+-activated K+ channels. Replacing Cl– with methanesulfonate further increased the depolarization in WT, but not in mutant, pericytes (Figure 5E), indicating that Cl– ion flow is important for U46619-induced pericyte depolarization.

To address whether CaCCs are relevant for the contractility of not only arterioles in the CNS but also peripheral blood vessels, we...
perfused isolated legs of WT and mutant mice at a constant flow rate and recorded the perfusion pressure. Although the perfusion pressure did not differ between genotypes under steady-state conditions, the pressure response to a bolus injection of U46619 (Figure 5F) was attenuated in TMEM16A mutant mice (Figure 5G). This finding strongly supports a role of TMEM16A in the contractility of peripheral microvessels, e.g., in skeletal muscle or skin.

Discussion
CaCCs were substantial in VSMCs of large conduit arteries, but small or absent in VSMCs of medium-sized arteries and large arterioles. In contrast, intermediate cells/pericytes of small arterioles displayed particularly strong CaCCs. As disruption of TMEM16A in contractile cells along the vascular tree lowered systemic blood pressure, eliminated vascular CaCCs, and diminished GPCR vasoconstrictor responses, we propose that TMEM16A is an important determinant of peripheral vascular resistance and thereby contributes to systemic blood pressure.

Supporting that CaCCs are relevant in the aorta, aortic ring preparations from knockout mice were less contractile in response to various agonists. The relative difference was more prominent in response to angiotensin II and phenylephrine when compared with U46619. This might reflect differences in the excitation-contraction coupling. Apart from Ca$^{2+}$-dependent pathways,
U46619-mediated vasoconstriction also depends on the activation of Rho/Rho kinase signaling via G proteins Gα12-Gα13 (32), whereas phenylephrine and angiotensin II mainly act on Gαq-Gα11, which activate Ca2+/myosin light chain kinase–mediated signaling (24). Large-diameter blood vessels such as the aorta do not contribute significantly to systemic blood pressure. Their walls extend when the blood pressure rises during systole and recoil during diastole. The aorta thereby dampens the fluctuations in arterial blood pressure during the cardiac cycle (Windkessel effect; ref. 33). The relevance of CaCCs for the Windkessel effect is supported by the reduction of the pulse pressure in our mutant mice.

Key points:
- CaCCs in VSMCs of large- and medium-sized arteries were small or even absent, and the contractile responses of these blood vessels did not differ between genotypes.
- How can these data be reconciled with our observation that disruption of TMEM16A results in a decrease in systemic blood pressure?
- Whereas large- and medium-sized arteries only marginally contribute to peripheral resistance, peripheral resistance is mainly determined by small arterioles with diameters of 50 µm or even less (34). Importantly, CaCCs mediated by TMEM16A were particularly large in intermediate cells and pericytes of small brain and retinal arterioles compared with VSMCs of the aorta. In accordance with our electrophysiological data and compatible with a prominent role of CaCCs in the microcirculation, the contraction of second- and third-order arterioles of the retina was diminished in response to submaximal concentrations of U46619 in VSMC-specific Tmem16a knockout mice.

**Figure 5**
TMEM16A modulates peripheral resistance. (A) Double immunostaining for CD31 (left) and TMEM16A (center) of slices from skeletal muscle. The overlay is shown on the right. Scale bar: 50 µm. (B and C) I-V relationship (B) and tail current density (C) from isolated pericytes from skeletal muscle showed a drastic reduction of CaCCs upon disruption of TMEM16A (n = 13–16 cells each). **P < 0.01. (D) Resting membrane potentials of isolated skeletal muscle pericytes did not differ between genotypes. n = 6; Student’s t test; P > 0.05. (E) U46619-induced depolarization of WT pericytes was almost absent in the mutant. TMEM16A-deficient pericytes hyperpolarized after agonist application, which suggests the presence of Ca2+-activated K+ channels. Replacing Cl− with methanesulfonate further increased the depolarization in WT, but not in mutant pericytes, indicating that Cl− ion flow is important for U46619-induced pericyte depolarization. n = 6; Student’s t test; **P < 0.01. (F) Original tracing of the perfusion pressure of an isolated hind limb perfusion of a control mouse. The bolus injection of either 20 pmol or 60 pmol U46619 (arrows) caused a short pressure peak because of the injected volume and subsequently a protracted pressure increase. The peak responses are indicated by arrowheads. (G) The difference between peak and steady-state pressure at the two doses of U46619 analyzed. perf., perfusion. n = 20. Student's t test; *P < 0.05.
We asked whether TMEM16A might have a similar function in arterioles of other tissues such as skeletal muscle, which recruit approximately 20% of cardiac output during rest and up to 80% during exercise and thus significantly contribute to overall vascular resistance. As in brain and retina, small CaCCs in intermediate cells/pericytes of skeletal muscle arterioles were large in WT mice and absent upon disruption of TMEM16A. In isolated hind limb perfusions, where peripheral resistance is largely determined by the skeletal muscle and/or skin microvasculature, the vasopressor response to U46619 was attenuated upon disruption of TMEM16A. These findings fully support our assumption that TMEM16A plays a role in peripheral resistance and might explain the decrease in arterial blood pressure upon disruption of TMEM16A even in the absence of strong effects on the mesenteric circulation.

Our findings strengthen the emerging hypothesis that vascular contractility contributes to the long-term control of systemic blood pressure (35), which is also supported by some clinical studies (36). Evidence also comes from a recent mouse model with a VSMC-specific disruption of the mineralocorticoid receptor (37). Similar to our findings, these mice developed a reduced systemic blood pressure over time and showed a reduction in the vasopressor response to angiotensin II, without any defects in renal sodium handling or changes in vascular structure. Moreover, vasoconstriction to angiotensin II or thromboxane A2 was diminished. Further supporting this concept, a pronounced decrease in systemic blood pressure was observed upon VSMC-specific disruption of microRNAs (38), while overexpression of a myosin phosphatase inhibitory protein (CPI-17) in VSMCs increased systemic blood pressure (39).

To what extent TMEM16A might play a role in the vasculature of other tissues and to what degree long-term compensation of reduced blood pressure might occur needs to be tested in the future. Currently, we cannot exclude that TMEM16A disruption may also affect pericyte-rich vasa recta. Descending vasa recta arise from juxtamedullary efferent arterioles with a mean diameter of around 10 μm and are characterized by a continuous endothelium surrounded by contractile pericytes. Descending vasa recta isolated from outer medullary bundles have been shown to contract to various agonists including angiotensin II (40), an effect that is known to be modulated by Cl− (41, 42). Vascular disruption of TMEM16A may thus also have an effect on renal NaCl reabsorption and the regulation of the extracellular fluid compartment as another determinant of arterial blood pressure. Indeed, medullary blood flow was reported to be reduced in several models of experimental hypertension (43).

There are conflicting results regarding the possible role of CaCCs in renin-secreting cells, which are specialized VSMCs and where CaCCs have also been described (44). In renin-secreting cells, membrane depolarization by activation of chloride channels was associated with a reduction in renin release (45). However, nifluminic acid, which is considered to preferentially inhibit CaCCs, increased renin secretion (46).

Our observation that the renin-angiotensin-aldosterone system was not activated in conditional Tmem16a knockout mice, may argue against a major role of the kidney in the hypertensive response upon disruption of vascular CaCCs.

Notably, TMEM16A labeling appeared to be particularly strong at branching points of small arterioles, which is consistent with previous reports that pericytes at precapillary branching points are equipped with a specialized contractile apparatus (47). This suggests the idea that TMEM16A might play a role for the recruitment of downstream capillaries and, hence, the channeling of nutrient supply. TMEM16A may also play important roles in pathological conditions such as pulmonary hypertension and stroke. Large CaCCs most likely mediated by TMEM16A have been reported for VSMCs of the pulmonary artery (18). Experimental occlusion of the middle cerebral artery induced a sustained contraction of downstream capillary pericytes, which remained contracted for hours despite reopening of the occluded vessel (48). This impaired microcirculatory reflow phenomenon negatively affects tissue survival and may in part depend on CaCCs, which are prominently expressed in pericytes, as shown in our study. This could be also relevant for other tissues such as the heart or renal medulla, which are known to be very vulnerable to ischemia.

Whether TMEM16A proves to be a target for therapy in humans in arterial hypertension or other pathological settings will be an important question to address in the future.

Methods

Mice

A genomic clone of murine Tmem16a including exon 21 was isolated from a 129/Sv mouse genomic library (lFixII, Stratagene) and was cloned into the pK0 targeting vector. A diphtheria toxin A (DTA) cassette was introduced at the 3′ end of the targeting construct as a negative selection marker. A floxed neomycin selection cassette was cloned into the EcoRV restriction site 5′ of exon 21. A third loxP site was introduced into the Ajel restriction site 3′ of exon 21. This targeting vector was linearized via NotI and electroporated into R1 embryonic stem cells, which are derived from the Sj/S line. Neomycin-resistant clones were screened for homologous recombination by Southern blot analysis of a genomic EcoRI digest with an external probe exploiting a novel EcoRI restriction site introduced with the third loxP site. Neomycin-resistant clones that had undergone homologous recombination were transfected with a plasmid expressing Cre recombinase (49). Resulting clones were tested for the extent of the DNA excision. A clone with a deletion of the neomycin selection cassette was injected into C57BL/6 blastocysts and transferred into foster mice. The floxed line was backcrossed with C57BL/6. Mice were genotyped by PCR using primers loxp-fwd (5′-CTCGGTGTGGGACTATGAACC-3′) and loxp-rev (5′-CAGATAACACAGTGTTCCTCC-3′). The junction fragment upon deletion of exon 21 was amplified with the loxp-fwd primer and the primer KO-fwd (5′-CTCAGGCTCCCATGATCCTC-3′). Experiments were performed in F1 on a mixed Sjf/C57BL/6 background.

The SMMHC-CreERT2 (24) line was a gift from Stefan Offermanns, Max-Planck-Institut für Herz- und Lungenforschung, Bad Nauheim, Germany.

cDNA cloning. A Tmem16A-containing expressed sequence tag (IMAGE Consortium cDNA clone 30547439) was subcloned into the first multiple cloning site of the bicistronic pIRES vector (Clontech), containing GFP downstream of the IRES. cDNA corresponding to exon 21 was removed using standard PCR mutagenesis.

Antibodies

Polyclonal antibodies were raised in rabbits against the N-terminal mouse Tmem16a peptide RSVKQDHPLPGKGAQ coupled to keyhole limpet hemocyanin. The resulting antisera were affinity purified. One purified antiserum detected TMEM16A in tissue protein lysates and tissue sections.

Western blotting

Tissues were ground in liquid nitrogen and homogenized in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na3VO4, 1 mM NaF, 2.5 mM Na2HPO4, 2.5 mM Na2HPO4, 1 mM PMSF, and the Roche protease inhibitor cocktail. The samples were sonicated on
ice for 30 minutes and centrifuged at 3,100 g for 5 minutes to remove cell debris. The supernatant was collected, and the protein concentration was determined using the Pierce BCA Protein Assay Kit. 10 µg of protein was separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 3% blocking buffer (dry milk in TBS-T) and incubated with rabbit antibody against TMEM16A (1:500) in 1% blocking buffer or mouse anti-β-actin (1:2,000, Santa Cruz Biotechnology Inc.) or rabbit anti-α-tubulin (1:2,000, Abcam) in 3% blocking buffer overnight at 4°C. After the membranes were washed, they were incubated with HRP-conjugated anti-rabbit (1:2,000, Amersham Biosciences) or anti-mouse (1:2,000, Amersham Biosciences) secondary antibodies in 3% blocking buffer for 2 hours at room temperature (RT). Signals were detected with the ECL system (Millipore).

**Immunohistochemistry**

**Staining on cryosections.** Arteries were removed and postfixed with 4% paraformaldehyde in PBS overnight. After dehydration in 30% sucrose in PBS, the vessels were cut into 20-µm-thick cryosections. The slices were hydrated with PBS, blocked with 5% normal goat serum and 0.25% Triton X-100 in PBS, and incubated with our polyclonal TMEM16A antibody (1:250) and a monoclonal rat antibody against CD31 (1:1,000, BioLegend) in PBS overnight at 4°C. The slices were washed 3 times with PBS and incubated with Cy5-conjugated AffiniPure Goat anti-Rabbit IgG (1:1,000, Jackson ImmunoResearch Laboratories) and Alexa Fluor 555–labeled Goat Anti-Rat IgG (1:1,000, Molecular Probes, Life Technologies) in PBS overnight at 4°C. After a washing step with PBS, the nuclei were stained with 0.25% Triton X-100 in PBS, and incubated with our polyclonal TMEM16A antibody in blocking solution for 5 days at 4°C, rinsed, and Alexa Fluor 488–labeled Donkey Anti-Mouse IgG (1:500, Molecular Probes, Life Technologies) and Alexa Fluor 555–labeled Goat Anti-Rat IgG (1:1,000, Molecular Probes, Life Technologies) in PBS overnight. The slices were washed 3 times with PBS and incubated with Cy5-conjugated AffiniPure Goat anti-Rabbit IgG (1:1,000, Jackson ImmunoResearch Laboratories) and Alexa Fluor 555–labeled Goat Anti-Rat IgG (1:1,000, Molecular Probes, Life Technologies) in PBS. The sections were washed 3 times with PBS and mounted with Fluoromount-G (SouthernBiotech) and analyzed with a Leica TCS SP5 confocal scanning fluorescence microscope.

**Whole mount retina staining.** Eyes were removed postmortem and retinas isolated in Ringer solution (137 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, 5 mM HEPES, pH 7.4). Radial incisions were made to flat-mount retinas on black filter paper (Millipore AABP02500), photoreceptor side down, and samples were fixed for 20 minutes in 4% paraformaldehyde in 0.1 PBS at RT. Retinas were washed 3 times in 0.1% TN buffer (150 mM NaCl, Tris-HCl, pH 7.6) and blocked with 3% donkey serum and 1% BSA in TN buffer with 0.5% Triton X-100 and 0.02% NaN₃ overnight at RT. After incubation with our polyclonal rabbit antibody against TMEM16A (1:500) and a smooth muscle actin monoclonal antibody (1:500, Thermo Scientific) in blocking solution for 5 days at 4°C, retinas were washed for 1 hour in TN buffer and labeled with Cy5-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories) and Alexa Fluor 488–labeled Donkey Anti-Mouse IgG (1:500, Molecular Probes, Life Technologies) in blocking solution for 3 hours at RT, washed in TN buffer, and mounted in ProLong Gold antifade reagent (Life Technologies). For retina overview images, multiple individual images were taken using a Zeiss Observer Z1 epifluorescence microscope equipped with a CoolSNAP HQ² camera (Photometrics) and stitched together using MetaMorph acquisition software (Universal Imaging).

**Brain slices.** The brain was carefully removed, rinsed in ice-cold TN buffer, and glued to the stage of a vibrating blade microtome (Leica VT1200S). 300-µm-thick sagittal sections were fixed in 4% paraformaldehyde in 0.1 PBS for 20–30 minutes at RT, washed 3 times in TN buffer (pH 7.6), and processed as described for the retina.

**Skeletal muscle slices.** The paw of the hind limb of a WT mouse was cut open, and the extensor digitorum longus muscle was removed, embedded in 2.5% SeaPlaque GTG low-melting-point agarose (Cambrex), glued to the stage of the vibrating blade microtome, and processed as described for the retina.

**Histological analysis**

Wall-to-lumen ratios were determined in H&E-stained transversal sections of paraffin-embedded thoracic aortae and large and small mesenteric arteries.

**Radiotelemetric blood pressure measurement**

Mice with a minimum body weight of 23 g were anesthetized by intraperitoneal application of ketamine/xylazine adapted to body weight. Telemetric transmitters (PhysioTel PA-C10, Data Sciences International) were implanted subcutaneously, with the sensing tip placed in the aorta via the lef carotid artery. After 10 days of recovery from surgery interventions and/or recordings (Datquest A.R.T. software for acquisition and analysis) were started. Angiotensin II (1.5 ng·g⁻¹·min⁻¹) was infused for 14 days by subcutaneously implanted osmotic minipumps (Alzet, model 1002).

For investigation of the effects of salt intake, mice were fed with modified special diets (Altromin C1036) containing 0.2 g Na⁺/kg (low salt), 3 g Na⁺/kg (control), or 30 g Na⁺/kg (high salt).

**Heart ultrasound analysis**

Cardiac function and heart dimensions were assessed by transthoracic echocardiography using the Vevo 2100 System (VisualSonics) before and 2 weeks after termination of tamoxifen treatment. Echocardiography was performed during isoflurane anesthesia adapted to breathing frequency.

**Blood analysis and renal function**

For determination of plasma renin activity and plasma angiotensin II, aldosterone, and atrial natriuretic peptide (ANP) levels, mice were preconditioned to anesthesia for 9 days. One hour prior to the night period, mice were anesthetized with isoflurane, and blood was withdrawn by retro-orbital bleeding into EDTA-coated cups. Plasma was obtained by centrifugation.

For plasma renin activity, plasma taken right at the beginning of retro-orbital bleeding was diluted and incubated with substrate (plasma from bilaterally nephrectomized rats) for 90 minutes at either 4°C or 37°C. Angiotensin I formation was determined by RIA (Byk & DiaSorin Diagnostics), and specific formation (37°C values) was corrected by subtraction of unspecific interference (4°C values). Plasma aldosterone levels were determined by RIA (Siemens), plasma ANP levels by ELISA (Phoenix Pharmaceuticals), and plasma angiotensin II levels by ELISA (ENZO Life Science) after extraction as described by the manufacturer.

Plasma electrolytes were measured in blood collected from the retro-orbital plexus of isoflurane-anesthetized mice using an electrolyte analyzer with ion-selective electrodes (Spotchem EL SE-1520, Arkray).

For renal function studies, mice were housed in metabolic cages (Tecniplast metabolic cage for single mouse, type 304) for 4 days. Urine was collected on day 4 for 24 hours, and blood was collected at the end of day 4. The diet contained 3 g Na⁺/kg (ROD 16-R, LASvendi).

**Myography of isolated vessels**

Contraction of isolated aortae and mesenteric arteries was measured using a conventional small vessel wire myograph (DMT 610M, Danish Myo Technology). Briefly, thoracic aortae and mesenteric beds were removed, quickly transferred to cold (4°C) oxygenated (95% O₂, 5% CO₂) physiological salt solution (PSS), and dissected into 2 mm rings. Perivascular fat and connective tissues were removed. Each ring was dispensed
between two stainless steel wires (diameter, 0.0394 mm) in a 2 ml organ bath. The organ bath was filled with PSS. The composition of PSS was 119 mM NaCl, 4.7 mM KCl, 2 mM KH₂PO₄, 25 mM NaHCO₃, 1.6 mM CaCl₂, 1.2 mM MgSO₄, and 11 mM tris-glucose. The bath solution was continuously oxygenated with a gas mixture of 95% O₂ and 5% CO₂ and kept at 37 °C (pH 7.4). The aortic rings were placed under tension of 0.3 g. The mesenteric rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. The software Chart5 (AD Instruments Ltd.) was used for data acquisition and display. After an equilibration period of 60 minutes and before the start of the experiment, the vessels were contracted with 60 mM KCl to test viability. Subsequently, the vessels were contracted with phenylephrine, U46619, or angiotensin II at the concentrations indicated. For calibration and details, see ref. 50.

**Videomicroscopy of mesenteric vessels**

Mice were killed, and mesenteric beds were removed and transferred to cold (4°C) oxygenated (95% O₂, 5% CO₂) PSS. Mesenteric arteries were mounted onto glass cannulae on both sides, allowing application of hydrostatic pressure to the vessel. Perivascular fat and connective tissues were removed. For calibration and details, see ref. 50. Vessels were superfused continuously with Krebs-Henseleit solution (95% O₂, 5% CO₂; pH 7.4, 37°C) of the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.6 mM CaCl₂, 1.2 mM MgSO₄, 0.03 mM EDTA, and 11.1 mM tris-glucose. The vessels were pressurized stepwise to 20, 40, 60, 80, or 100 mmHg using a pressure servocontrol system (Living Systems Instrumentation). The inner diameter of the vessels was measured with a video microscope (Nikon Diaphot) connected to a personal computer for data acquisition and analysis (HsSoTec). The vessels were allowed to equilibrate for 45–60 minutes before the start of experiments. At 80 mmHg, the vessels were contracted with 60 mM KCl. Subsequently, the arteries were contracted with phenylephrine, U46619, or angiotensin II added to the bath solution. The myogenic tone was assessed by application of external Ca²⁺-free PSS.

**Contraction measurements of retinal arterioles**

Enucleated eyes were placed in Ca²⁺-free solution containing 135 mM NaCl, 5 mM NaH₂PO₄, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, 1 µM nifedipine; pH 7.4, adjusted to 300 mOsm with sucrose. Corneas and lenses were removed and the retinae separated from the pigment epithelium. Each retina was cut into 3–4 pieces and transferred to a solution comprising 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM Na₂HPO₄, 1.6 mM CaCl₂, 1.2 mM MgSO₄, 0.03 mM EDTA, and 11.1 mM tris-glucose. The vessels were pressurized stepwise to 20, 40, 60, 80, or 100 mmHg using a pressure servocontrol system (Living Systems Instrumentation). The inner diameter of the vessels was measured with a video microscope (Nikon Diaphot) connected to a personal computer for data acquisition and analysis (HsSoTec). The vessels were allowed to equilibrate for 45–60 minutes before the start of experiments. At 80 mmHg, the vessels were contracted with 60 mM KCl. Subsequently, the arteries were contracted with phenylephrine, U46619, or angiotensin II added to the bath solution. The myogenic tone was assessed by application of external Ca²⁺-free PSS.

**Perfused hind limb vasculature**

Isolated hind limb perfusion was performed as described previously for isolated mesenteric vascular bed preparations (52), with some modifications (53, 54). In brief, dissected hind legs of briefly perfused mice (PSS: 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) were placed on a heated (37°C) plate and kept moist. Femoral arteries were cannulated and perfused using a peristaltic pump at a constant flow with gassed (95% O₂, 5% CO₂) PSS. Perfusion pressure was continuously determined by a pressure transducer (Living Systems Instrumentation) and recorded on a polygraph. After an equilibration period of 10 minutes, the flow rate was gradually increased until a perfusion pressure of 80 mmHg was reached to induce a spontaneous myogenic tone (54). When the pressure had stabilized, a bolus of U46616 was injected (either 20 or 60 pmol in a total volume of 200 μl) and the changes in perfusion pressure recorded. Since flow was maintained at a constant rate, changes in peripheral vascular resistance resulted in changes in perfusion pressure.
Electrophysiology

Currents from HEK cells or SMCs/intermediate cells/pericytes were recorded in the whole-cell voltage-clamp mode, using an EPC-10 amplifier and PatchMaster software (HEKA). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 1.4 mM CaCl2, 10 mM HEDTA, 10 mM TEA, 10 mM HEPES; adjusted to pH 7.2. Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm O.D., 1.2 mm I.D.); pipette resistance was typically 3–4 MOhm. Patch pipettes were filled with a solution containing 140 mM CsCl, 1.2 mM MgCl2, 1.4 mM CaCl2, 10 mM HEDTA, 20 mM TEA, 10 mM HEPES; adjusted to pH 7.2 with CsOH. Free [Ca2+]i was 600 nM, determined using a NanoDrop 3300 fluorescence spectrometer (Thermo Fisher) and the ratiometric indicator Indo1 (Invitrogen). In some experiments, a calcium-free intracellular solution was used devoid of CaCl2 and supplemented with 10 mM EGTA. The osmolarity of all extra- and intracellular solutions was approximately 310 mOsm. Recordings were made at RT. The holding potential was –60 mV. To determine the voltage dependence of activation, a series of depolarization steps (1 second duration) from –80 to +100 mV was applied with 20 mV increments. Steady-state current amplitudes were measured at the end of the depolarization pulse. Tail currents were more appropriate for quantification because prolonged depolarization activated an unrelated outward rectifying conductance in some cells but had no effect on tail currents. The amplitude of tail currents was determined at ~80 mV after depolarization to ~+80 mV by fitting the tail current trace with a mono-exponential function using FitMaster software (HEKA). For patch-clamp recordings from retina pericytes, the extracellular solution contained 135 mM NaCl, 5 mM NaOH, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES; 300 mOsm, pH 7.4.

For recordings of brain and skeletal muscle pericytes, 0.5 ml of the tissue suspension was mixed with either 2.5 ml of brain preparation solution or PSS and placed in a glass petri dish. Microvessels were allowed to settle. Exchange of solutions was performed by a pressure-driven perfusion system (ALA-VM8; ALA Scientific Instruments).

In perforated patch-clamp recordings, pipette solution contained 130 mM KCl, 10 mM NaCl, 3 mM CaCl2, 1.2 mM MgCl2, 10 mM HEDTA, 10 mM HEPES, adjusted to pH 7.2 with KOH. Gramicidin (G5002, Sigma-Aldrich) was added at a final concentration of 5 µg/ml from a stock solution.

Statistics

Data are shown as mean ± SEM, and n represents the number of animals, specimen, or cells. Statistical analysis was performed by 2-tailed unpaired Student’s t test or 2-way ANOVA, if not stated otherwise. Mean arterial blood pressure was also compared by the Bonferroni post hoc analysis. A P value less than 0.05 was considered statistically significant.

Study approval

Animal experiments were performed according to and with approval of Thüringer Landesamt für Verbraucherschutz, Bad Langensalza, Germany, Landesamt für Gesundheit und Soziales, Berlin, Germany; and Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen, Hamburg, Germany.

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Address correspondence to: Christian A. Hübner, Institut für Humangenetik, Universitätsklinikum Jena, Kollegienstrasse 10, 07743 Jena, Germany. Phone: 0049.3641.935500; Fax: 0049.3641.935502; E-mail: christian.huebner@med.uni-jena.de. Or to: Björn C. Schroeder, Max-Delbrück-Centrum für Molekulare Medizin (MDC), Robert-Rösслle-Straße 10, 13125, Berlin, Germany. Phone: 0049.30.94062264; Fax: 0049.30.94063327; E-mail: bjorn.schroeder@mdc-berlin.de.
