

The role of cGMP/cGKI signalling and Trpc channels in regulation of vascular tone

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Aims	Signalling via cGMP-dependent protein kinase I (cGKI) is the major pathway in vascular smooth muscle (SM), by which endothelial NO regulates vascular tone. Recent evidence suggests that canonical transient receptor potential (Trpc) channels are targets of cGKI in SM and mediate the relaxant effects of cGMP signalling. We tested this concept by investigating the role of cGMP/cGKI signalling on vascular tone and peripheral resistance using <i>Trpc6</i> ^{-/-} , <i>Trpc3</i> ^{-/-} , <i>Trpc3</i> ^{-/-} / <i>6</i> ^{-/-} , <i>Trpc1</i> ^{-/-} / <i>3</i> ^{-/-} / <i>6</i> ^{-/-} , and SM-specific cGKI ^{-/-} (sm-cGKI ^{-/-}) mice.
Methods and results	α -Adrenergic stimulation induced similar contractions in L-NG-nitroarginine methyl ester (L-NAME)-treated aorta and comparably increased peripheral pressure in hind limbs from all mouse lines investigated. After α -adrenergic stimulation, 8-Br-cGMP diminished similarly aortic tone and peripheral pressure in control, <i>Trpc6</i> ^{-/-} , <i>Trpc3</i> ^{-/-} , <i>Trpc3</i> ^{-/-} / <i>6</i> ^{-/-} , and <i>Trpc1</i> ^{-/-} / <i>3</i> ^{-/-} / <i>6</i> ^{-/-} mice but not in sm-cGKI ^{-/-} mice. In untreated aorta, α -adrenergic stimulation induced similar contractions in the aorta from control and <i>Trpc3</i> ^{-/-} mice but larger contractions in sm-cGKI ^{-/-} , <i>Trpc6</i> ^{-/-} , <i>Trpc3</i> ^{-/-} / <i>6</i> ^{-/-} , and <i>Trpc1</i> ^{-/-} / <i>3</i> ^{-/-} / <i>6</i> ^{-/-} mice, indicating a functional link between cGKI and Trpc6 channels. Trpc3 channels were detected by immunocytochemistry in both isolated aortic smooth muscle cells (SMCs) and aortic endothelial cells (ECs), whereas Trpc6 channels were detected only in ECs. Phenylephrine-stimulated Ca ²⁺ levels were similar in SMCs from control (Ctr) and <i>Trpc6</i> ^{-/-} mice. Carbachol-stimulated Ca ²⁺ levels were reduced in ECs from <i>Trpc6</i> ^{-/-} mice. Stimulated Ca ²⁺ levels were lowered by 8-Br-cGMP in Ctr but not in <i>Trpc6</i> ^{-/-} ECs.
Conclusions	The results suggest that cGKI and Trpc1,3,6 channels are not functionally coupled in vascular SM. Deletion of Trpc6 channels impaired endothelial cGKI signalling and vasodilator tone in the aorta.
Keywords	Phenylephrine • Smooth muscle • Relaxation • Nitric oxide • Endothelium

1. Introduction

The dilator action of endothelium-derived NO contributes to the control of basal and stimulated regional blood flow.¹ The cellular effects of NO are mainly mediated by a rise in intracellular cGMP levels in the target cells.^{2,3} The major target for cGMP in vascular smooth muscle (SM) is the cGMP-dependent protein kinase I (cGKI).⁴ cGKI relaxes SM through interaction with the myosin-interacting subunit of myosin phosphatase 1,⁵ the regulator of G-protein signalling 2,⁶ and the IP₃R-associated cGMP kinase substrate (IRAG⁷). Recent studies added new potential targets by showing that cGKI inhibits the activity of the canonical transient receptor potential (Trpc) channels in SM.^{8–11}

Trpc channels (Trpc1–7) are a subfamily of the TRP channel family that is expressed in many cells including vascular SM and vascular endothelium cells.^{9,12} Trpc channels have been invoked to be essential for the Bayliss effect.¹³ Evidence for the control of Trpc channels by cGKI, especially of Trpc1, 3, and 6 channels, comes from *in vitro* and *in vivo* experiments. For instance, inhibition of Trpc1/Trpc3 by cGKI has been shown to contribute to NO-mediated vasorelaxation in freshly isolated smooth muscle cells (SMCs) from the rat carotid artery.⁸ In line with this finding, Trpc3 channels were directly phosphorylated by cGKI at position T11 and S263 when expressed in HEK293 cells.¹⁰ Further, the NO-cGMP-cGKI pathway negatively regulated Trpc6 channels in cultured vascular smooth muscle cells (VSMCs).¹¹ In addition, a functional

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link between TRP channels and cGKI signalling has been proposed to explain the effects of atrial natriuretic peptides on trans-vascular fluid exchange.¹⁴

In the present study, we tested the concept of a functional coupling between Trpc3/6 channels and cGMP/cGKI in SM *in vivo* using the respective knockout mice. To rule out an effect of the reported over-expression of Trpc3 channels in *Trpc6*^{-/-} mice,¹⁵ we also used Trpc3/Trpc6-double knockout mice and, as a further control, Trpc1/Trpc3/Trpc6-triple knockout mice. We measured vascular contraction in aortic rings and pressure in the hind limb system. The hind limb system represents a more confident model for vessels involved in blood pressure regulation than the aorta. Surprisingly, we found no functional coupling between cGMP/cGKI and Trpc3/6 channels in murine vascular SM. We further investigated the expression of Trpc channels in vascular SMC and EC and measured α -adrenergic- or muscarinic-induced Ca²⁺ levels in these cells. We present evidence for the existence of a functional coupling between cGMP/cGKI signalling and Trpc6 channels in ECs.

2. Methods

2.1 Mice

All procedures relating to animal care and treatment conformed to the institutional and governmental guidelines (Directive 2010/63/EU of the European Parliament) and were approved by local authorities (Regierung von Oberbayern). The following mouse lines were generated and used for the experiments: wild-type (WT) mice, mice lacking cGKI selectively in SM (*sm-cGKI*^{-/-} mice¹⁶), mice lacking Trpc3 channels (*Trpc3*^{-/-} mice¹⁷), mice lacking Trpc6 channels (*Trpc6*^{-/-} mice¹⁵), mice lacking both Trpc3 and Trpc6 channels (*Trpc3*^{-/-}/*6*^{-/-} mice), and mice lacking Trpc1,¹⁸ Trpc3, and Trpc6 channels (*Trpc1*^{-/-}/*3*^{-/-}/*6*^{-/-} mice). Control (Ctr) mice were heterozygous with respect to the respective knockout (see Supplementary material online, Figure S1A). Since none of the Ctr mice shows any differences in the effects investigated to those observed in WT mice (see Supplementary material online, Figure S1A), data obtained in the Ctr mouse lines were pooled with those obtained in WT mice. Mice of either sex were used for the experiments at the age of 3–6 months and sacrificed by decapitation.

2.2 Tension recordings

Segments of the thoracic aorta were taken from mice and placed in buffer solution at 37°C. Connective tissue and adhering fat were removed. Aortic rings were mounted into organ baths (Myograph 601, www.dmt.dk). Tension was recorded isometrically at 37°C. Rings were pre-stretched by 3 mN/mm. Contraction was measured after establishment of steady-state conditions with respect to the baseline.

2.3 Hind limb perfusion

After sacrifice, the aorta abdominalis was prepared and a 1 mm catheter was introduced, advanced to the bifurcation of the iliac arteries, and tied with a 6/0 silk suture. The inferior caval vein was slit open longitudinally to prevent venous congestion. Pressure was measured at 37°C using the universal perfusion set-up UP-100 (www.hugo-sachs.de). The hind limbs were perfused with buffer solution at a constant flow rate of 5–8 mL/min to give a resting pressure of ~40–70 mmHg. The resting pressure was not different between the heterozygous and the respective knockout mice (see Supplementary material online, Figure S1B). Substances were applied by changing the buffer solution to a buffer containing the substances at the concentrations indicated. Responses were measured as changes with respect to the resting pressure.

2.4 Immunocytochemistry

VSMCs and ECs were isolated as described.¹⁹ The following primary antibodies were used: anti-cGKI (1:100 dilution²⁰), anti-CD31 (2.5 μ g/mL, www.bdbiosciences.com), anti- α -actin (0.5 μ g/mL (rabbit, www.abcam.com) or 1:100 dilution (mouse, www.millipore.com)), anti-Trpc3, and anti-Trpc6 (1:200 dilution). Primary antibody/antigen complexes were detected with secondary antibodies conjugated to fluorescent dyes (1:300 dilutions for Alexa Fluor 488 and Alexa Fluor 594, www.lifetechnologies.com; 1:500 dilutions for Cy2 and Cy3, www.dianova.com). The fluorescence signals were visualized by a confocal laser scanning microscope using fixed adjustments for each condition (LSM510, www.zeiss.de).

2.5 Ca²⁺ measurements

Intracellular [Ca²⁺] was monitored in freshly isolated aortic SMC and EC using Fura-2/AM at room temperature as described.²¹ [Ca²⁺]_i was recorded as the fluorescence intensity (at 510 nm) at alternating 350 (F350) and 380 nm (F380) excitation wavelengths and their respective ratios (F350/F380) using a TILLvision device (www.till-photonics.de). Stimulation was performed by local application of phenylephrine (PE; 30 μ M), carbachol (CCh; 30 μ M), and 8-Br-cGMP (300 μ M) via a syringe device.

2.6 RNA isolation

Total RNA isolation was performed on intact and endothelium-denuded aortas from 12 to 15 WT mice as described by the supplier (peqGOLD RNAPure, PEQLAB Biotechnologie, Erlangen, Germany).

2.7 PCR analysis

Total RNA (1 μ g) was utilized as a template for semi-quantitative PCR to amplify primers against Trpc3, Trpc6, α -SMA (alpha smooth muscle actin), PECAM-1 (platelet endothelial cell adhesion molecule 1), and HPRT (hypoxanthine-guanine phosphoribosyltransferase). The analysis was performed using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and using a peqSTAR thermocycler (PEQLAB Biotechnologie, Erlangen, Germany).

2.8 Substances

All salts and substances were used as pure as commercially available and purchased from Sigma (www.sigmaaldrich.com) unless otherwise indicated.

2.9 Statistical analysis

Results are presented as photos and original recordings or expressed as means \pm SEM. Effects of substances were analysed in steady-state conditions. Statistical comparisons of data sets were performed by Student's *t*-test or by ANOVA followed by a Bonferroni *post hoc* test using Prism 5 (www.graphpad.com). Differences were considered significant at *P* < 0.05.

An expanded Method section is presented in the Supplementary material online.

3. Results

Trpc channels, especially the Trpc6 channel, have been proposed to play a central role in the control of vascular SM contraction.^{9,13,22} Consequently, we investigated the magnitude of vascular contraction in various mouse models including Ctr, *Trpc3*^{-/-}, *Trpc6*^{-/-}, *Trpc3*^{-/-}/*6*^{-/-}, *Trpc1*^{-/-}/*3*^{-/-}/*6*^{-/-}, and *sm-cGKI*^{-/-} mice. Contraction was induced by stimulation with the α -adrenergic agonist PE. Contraction was measured directly as an increase in tension using intact aortic rings and evaluated indirectly as an increase in pressure using the hind limb perfusion system. In aortic rings, the contraction by PE (3 μ M) was assessed in the presence of the NO synthase inhibitor L-NG-nitroarginine methyl ester (L-NAME) (100 μ M) to exclude the vasodilator tone provided by the NO generation in the vascular

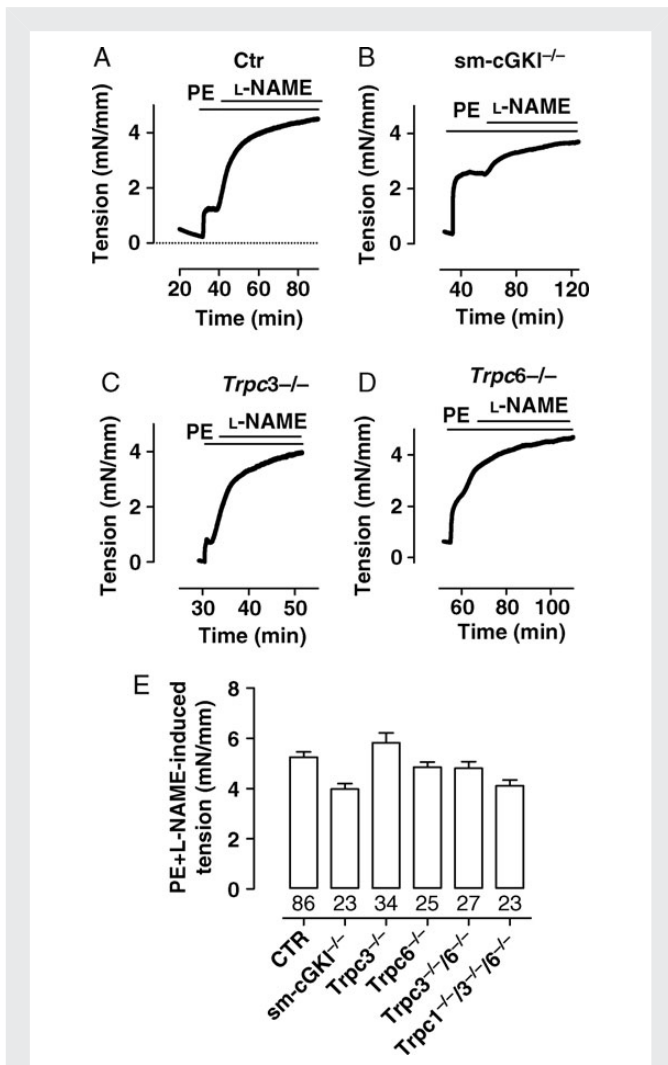


Figure 1 Magnitudes of PE-induced contractions in the murine aorta in the presence of L-NAME. (A–D) Original recordings of PE-induced contraction in aortic rings from a CTR (A), sm-cGKI^{-/-} (B), Trpc3^{-/-} (C), and Trpc6^{-/-} (D) mouse. Bars indicate the presence of PE (3 μ M) and L-NAME (100 μ M), respectively. (E) Magnitudes of PE-induced contractions in aortic rings from the indicated mouse line in which NO synthases were inhibited by L-NAME. Columns represent mean \pm SEM. Numbers represent the number of experiments. Data were analysed using ANOVA followed by the Bonferroni *post hoc* test, which indicated no statistically significant differences between Ctr and the respective mouse strain.

endothelium.^{3,23} In the presence of L-NAME, PE elicited contractions of similar magnitude in aortic rings from all mouse lines investigated (Figure 1A–E, see Supplementary material online, Figure S2). These findings suggest that, in the presence of an NOS inhibitor, α -adrenergic contraction of the murine aorta does not rely on the presence of Trpc1,3,6 channels.

In agreement with the above experiments, PE (10 μ M) induced similar increases in pressure in all mouse lines using the hind limb perfusion system (Figure 2A–E, see Supplementary material online, Figure S2). L-NAME (100 μ M) had no major influence on this effect, indicating that the vessels of the hind limb system do not exhibit a significant relaxing tone due to spontaneous NO production, at least if they were stimulated by an α -adrenergic agonist (see Supplementary material online,

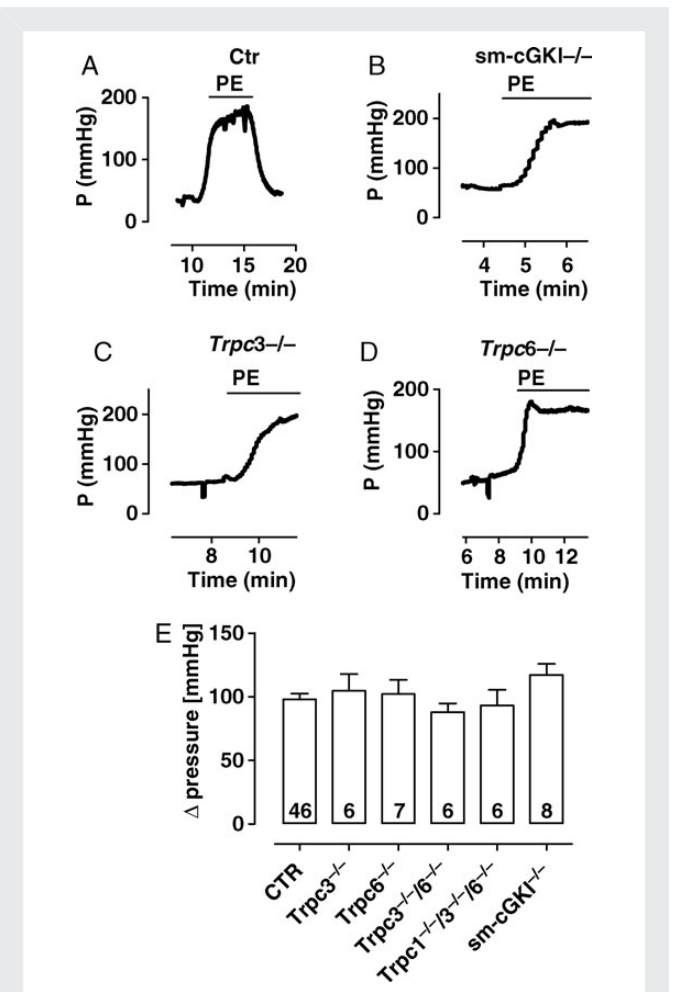


Figure 2 Magnitudes of PE-induced pressure increase in the murine hind limb. (A–D) Original recordings of the increase in PE-induced pressure in the hind limb from a CTR (A), sm-cGKI^{-/-} (B), Trpc3^{-/-} (C), and Trpc6^{-/-} (D) mouse. Bars indicate the presence of PE (10 μ M). (E) Magnitudes of the increase in PE-induced pressure in the indicated mouse line. Columns represent means \pm SEM. Numbers represent the number of experiments. Data were analysed using ANOVA followed by the Bonferroni *post hoc* test, which indicated no statistically significant differences between Ctr and the respective mouse strain.

Figure S3). These results indicate that, in the absence of an NO-dependent relaxing tone, Trpc1,3,6 channels are not involved in α -adrenergic-mediated contraction of vascular SM.

Recent evidence indicates that NO-mediated relaxation partially involves cGKI-dependent inhibition of Trpc channel activity.⁸ Therefore, we tested the significance of a functional coupling between cGMP/cGKI and the presence of Trpc channels, in particular the Trpc1, Trpc3, and Trpc6 channels, using aortic rings and hind limbs from Ctr, Trpc3^{-/-}, Trpc6^{-/-}, Trpc3^{-/-}/Trpc6^{-/-}, Trpc1^{-/-}/Trpc3^{-/-}/Trpc6^{-/-}, and sm-cGKI^{-/-} mice. Aortic rings were contracted by PE (3 μ M) and L-NAME (100 μ M) and then relaxed by the membrane-permeable analogue of cGMP, 8-Br-cGMP (300 μ M). 8-Br-cGMP similarly reduced these contractions in aortic rings from all mouse lines investigated except rings from sm-cGKI^{-/-} mice (Figure 3A–E, see Supplementary material online, Figure S2). Likewise, 8-Br-cGMP (300 μ M) comparably reduced PE-induced pressure increases in hind limbs from all mouse lines

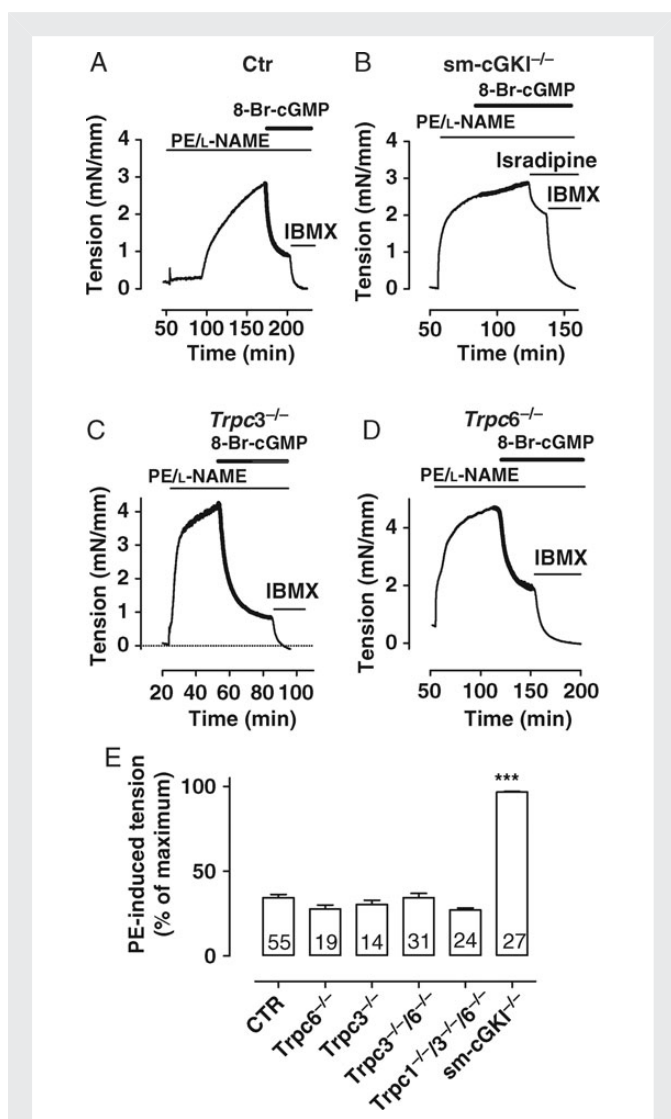


Figure 3 Magnitudes of cGMP-mediated relaxation in pre-contracted murine aorta. (A–D) Original recordings of 8-Br-cGMP-mediated relaxation in PE-contracted aorta from a CTR (A), sm-cGKI^{-/-} (B), Trpc3^{-/-} (C), and Trpc6^{-/-} (D) mouse. Bars indicate the presence of PE (3 μ M), L-NAME (100 μ M), 8-Br-cGMP (300 μ M), IBMX (100 μ M), and isradipine (1 μ M), respectively. (E) Magnitudes of tension in the presence of 8-Br-cGMP (300 μ M) in PE-contracted aortic rings from the indicated mouse line. Values are calculated as the % of maximal contraction with respect to the baseline in the presence of IBMX. Columns represent means \pm SEM. Numbers represent the number of experiments. Data were analysed using ANOVA followed by the Bonferroni *post hoc* test. Asterisks indicate a significant difference from Ctr with *** $P < 0.001$.

investigated except hind limbs from sm-cGKI^{-/-} mice (Figure 4A–E, see Supplementary material online, Figure S2). These results demonstrate (i) that cGKI is essential for the effect of 8-Br-cGMP in aortic rings and hind limb vessels, and (ii) that the effect of 8-Br-cGMP does not require Trpc1, Trpc3, or Trpc6 channels.

In parallel experiments, the effect of PE on contraction was assessed in intact aorta in which a relaxing tone exists partially due to the presence of spontaneous endothelial NO production.^{3,23} In the absence of L-NAME, PE (3 μ M) induced similar contractions in aortic rings from Ctr and Trpc3^{-/-} mice but a significantly larger contraction in rings

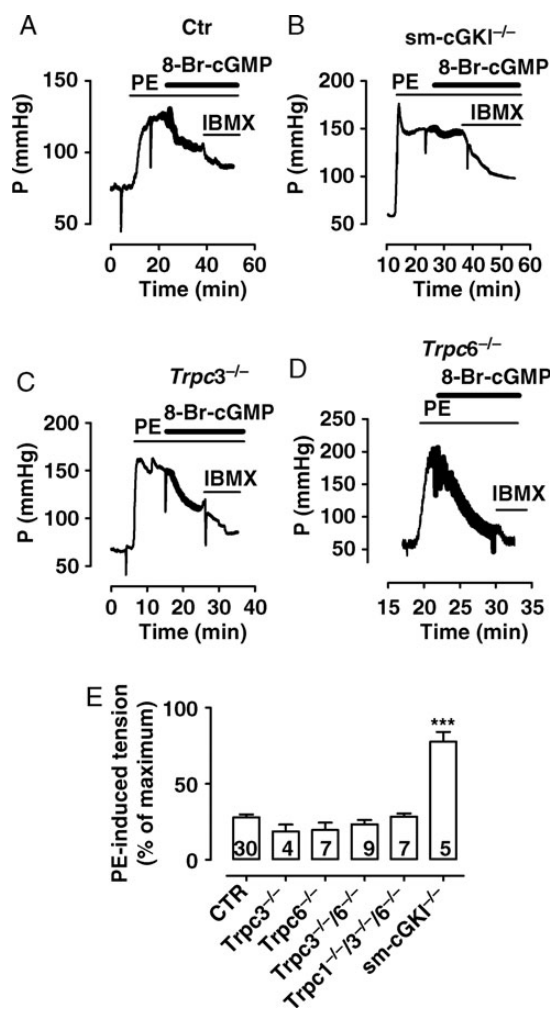
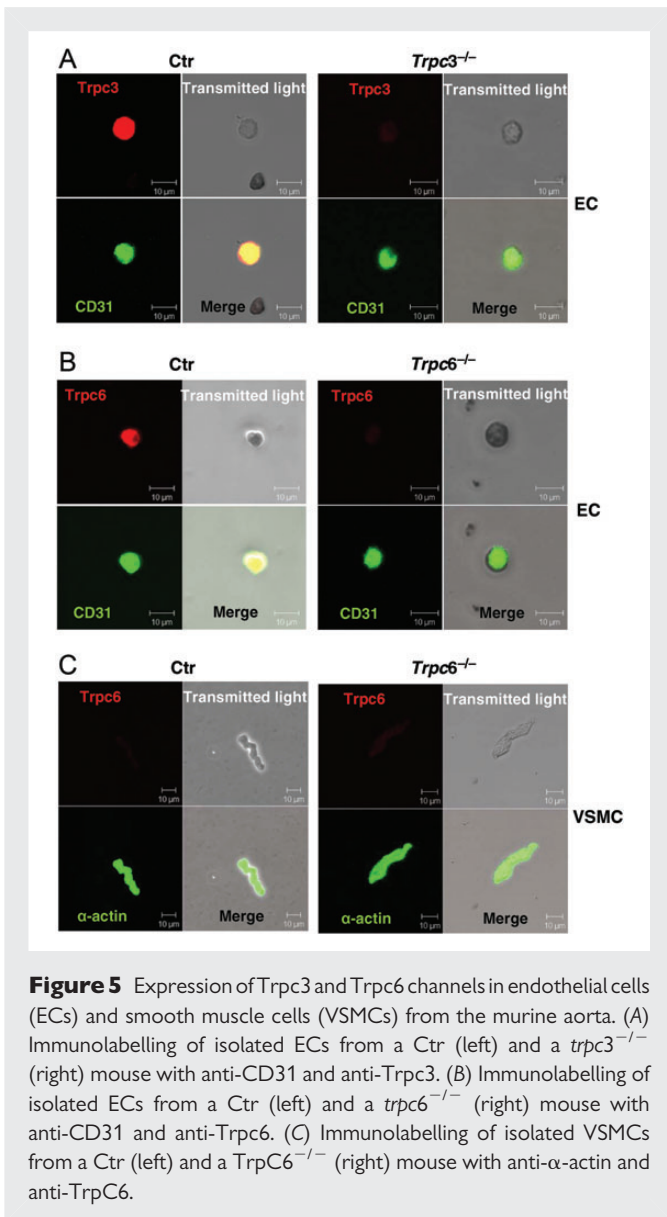


Figure 4 Magnitudes of decrease in cGMP-induced pressure in the murine hind limb. (A–D) Original recordings of increase in PE-induced pressure in the hind limb from a CTR (A), sm-cGKI^{-/-} (B), Trpc3^{-/-} (C), and Trpc6^{-/-} (D) mouse. Bars indicate the presence of PE (10 μ M), 8-Br-cGMP (300 μ M), and IBMX (100 μ M). (E) Magnitudes of pressure in the presence of 8-Br-cGMP (300 μ M) in PE-treated hind limbs from the indicated mouse line. Values are calculated as the % of maximal pressure with respect to the baseline in the presence of IBMX. Columns represent means \pm SEM. Numbers represent the number of experiments. Data were analysed using ANOVA followed by the Bonferroni *post hoc* test. Asterisks indicate a significant difference from Ctr with *** $P < 0.001$.

from sm-cGKI mice (see Supplementary material online, Figure S4A–E). PE also induced significant larger contractions in rings from Trpc6^{-/-} mice (see Supplementary material online, Figure S4D and E). The larger PE-induced contractions were likewise observed in rings from Trpc3^{-/-}/I6^{-/-}, and Trpc1^{-/-}/I3^{-/-}/I6^{-/-} mice (see Supplementary material online, Figures S2 and S4E) challenging the view that the larger contractility observed in the aorta from Trpc6^{-/-} mice is due to an up-regulation of Trpc3 channels.¹⁵ Equal results were obtained if the effects of PE in intact aorta were expressed in relation to the contraction stimulated by both PE and L-NAME (paired conditions; see Supplementary material online, Figure S5) or to an analysis per mouse basis (see Supplementary material online, Figure S5). Together, these results indicate



that there exists a relaxing component in intact aorta that depends on the presence of sm-cGKI and that may represent the NO-dependent relaxing tone reported previously.³ Further, the enhanced α-adrenergic-mediated contraction in intact aortic rings from *Trpc6*^{-/-}, *Trpc3*^{-/-}/*16*^{-/-}, and *Trpc1*^{-/-}/*3*^{-/-}/*16*^{-/-} mice suggests that Trpc6 channels are partially involved in the maintenance of the NO-mediated relaxing tone.

So far, our results indicate that cGKI and Trpc6 channels are necessary to observe a fully activated vasodilator tone in intact aortic rings but that they are not functionally coupled in SM. To further elucidate this issue, we performed semi-quantitative PCR analysis on intact aorta and endothelium-denuded aorta using primer pairs against Trpc3 and Trpc6 and, as a reference, against HPRT, α-SMA (acting as a marker for SM), and PECAM-1 (acting as a marker for the endothelium). Amplification products for α-SMA and PECAM-1 were detected in intact aorta, whereas only products for α-SMA were detected in endothelium-denuded aorta, confirming the absence of the endothelium in the latter preparation. Semi-quantitative RT-PCR analysis revealed signals for Trpc3 in intact and endothelium-denuded aorta, whereas signals for

Trpc6 were found in intact but not in endothelium-denuded aorta (see Supplementary material online, Figure S6). This result indicates that Trpc3 is expressed in both aortic SM and endothelium, whereas Trpc6 is only expressed in aortic endothelium but not in aortic SM.

To confirm this finding, we performed immunocytochemistry to study more directly the expression of Trpc3 and Trpc6 channels in isolated VSMCs and, for comparison, in vascular endothelium cells. For this purpose, ECs and SMCs were freshly isolated from the murine aorta. Cells were identified using anti-CD31 as a marker for ECs and anti-α-actin as a marker for SMCs. The markers showed a specific signal for the respective cell type (see Supplementary material online, Figure S7). Co-staining of ECs with anti-Trpc3 and anti-Trpc6 showed that both Trpc3 and Trpc6 channels were present in ECs (Figure 5A and B). Co-staining of SMCs with anti-cGKI, anti-Trpc3, and anti-Trpc6 revealed a signal for cGKI and Trpc3 channels in SMCs, but not for Trpc6 channels (Figure 5C, see Supplementary material online, Figure S8A–C). No signals of anti-cGKI, anti-Trpc3, and anti-Trpc6 were detected in cells from *sm-cGKI*^{-/-}, *Trpc3*^{-/-}, and *Trpc6*^{-/-} mice, respectively, confirming the specificity of the used antibodies (Figure 5, see Supplementary material online, Figures S8 and S9). These findings indicate that Trpc3 channels are expressed in both freshly isolated vascular endothelial and SMCs, whereas Trpc6 channels are expressed in freshly isolated endothelial but not in freshly isolated aortic SMCs.

From these findings, we speculated that Trpc6 channels are involved in regulating intracellular Ca²⁺ homeostasis in ECs. Ca²⁺ modulates endothelial NO production by endothelial NO synthase.²⁴ Consequently, we studied agonist-induced changes in intracellular [Ca²⁺] in aortic SMCs and ECs from Ctr, *Trpc3*^{-/-}, and *Trpc6*^{-/-} mice. In addition, we tested again the possibility that Trpc6 channels are functionally coupled to cGMP/cGKI signalling by measuring the effect of 8-Br-cGMP on agonist-induced changes in intracellular [Ca²⁺]. PE (30 μM) similarly increased intracellular Ca²⁺ levels in aortic SMCs from Ctr, *Trpc3*^{-/-}, and *Trpc6*^{-/-} mice (Figure 6A, see Supplementary material online, Figure S10). Application of 8-Br-cGMP (300 μM) equally reduced the PE-stimulated increases in intracellular Ca²⁺ levels in the SMCs from all three mouse lines investigated (Figure 6A, see Supplementary material online, Figure S10), indicating that Trpc3 and Trpc6 have no major impact on α-adrenergic-induced Ca²⁺ signals and their attenuation by cGKI signalling in SM. CCh (30 μM) increased intracellular Ca²⁺ levels in ECs from Ctr, *Trpc3*^{-/-}, and *Trpc6*^{-/-} mice but the Ca²⁺ levels in the cells from *Trpc6*^{-/-} mice were significantly lower than those found in cells from Ctr and *Trpc3*^{-/-} mice (Figure 6B, see Supplementary material online, Figure S10). After the activation of cGKI signalling by 8-Br-cGMP (300 μM), the CCh-induced Ca²⁺ levels were reduced in ECs from Ctr and *Trpc3*^{-/-} mice but not in those cells from *Trpc6*^{-/-} mice (Figure 6B, see Supplementary material online, Figure S10). These results suggest that CCh-induced Ca²⁺ signals in ECs depend partially on the presence of Trpc6 channels. The missing effect of 8-Br-cGMP on CCh-induced Ca²⁺ signals in ECs from *Trpc6*^{-/-} mice points to a possible coupling of cGMP/cGKI signalling to Trpc6 channels in this cell type. To strengthen this view, we performed immunocytochemistry to clarify the expression of cGKI in isolated aortic endothelium cells. Co-staining of ECs with anti-cGKI and anti-CD31 showed that cGKI is present in ECs from Ctr but not in those from *cGKI*^{-/-} mice (see Supplementary material online, Figure S11).

To further support the hypothesis that Trpc6 channels are involved in the generation of an endothelial-mediated relaxing tone, we assessed the effects of CCh on PE-induced contraction in intact aortic rings. Stimulation of muscarinic receptors on the endothelium induces

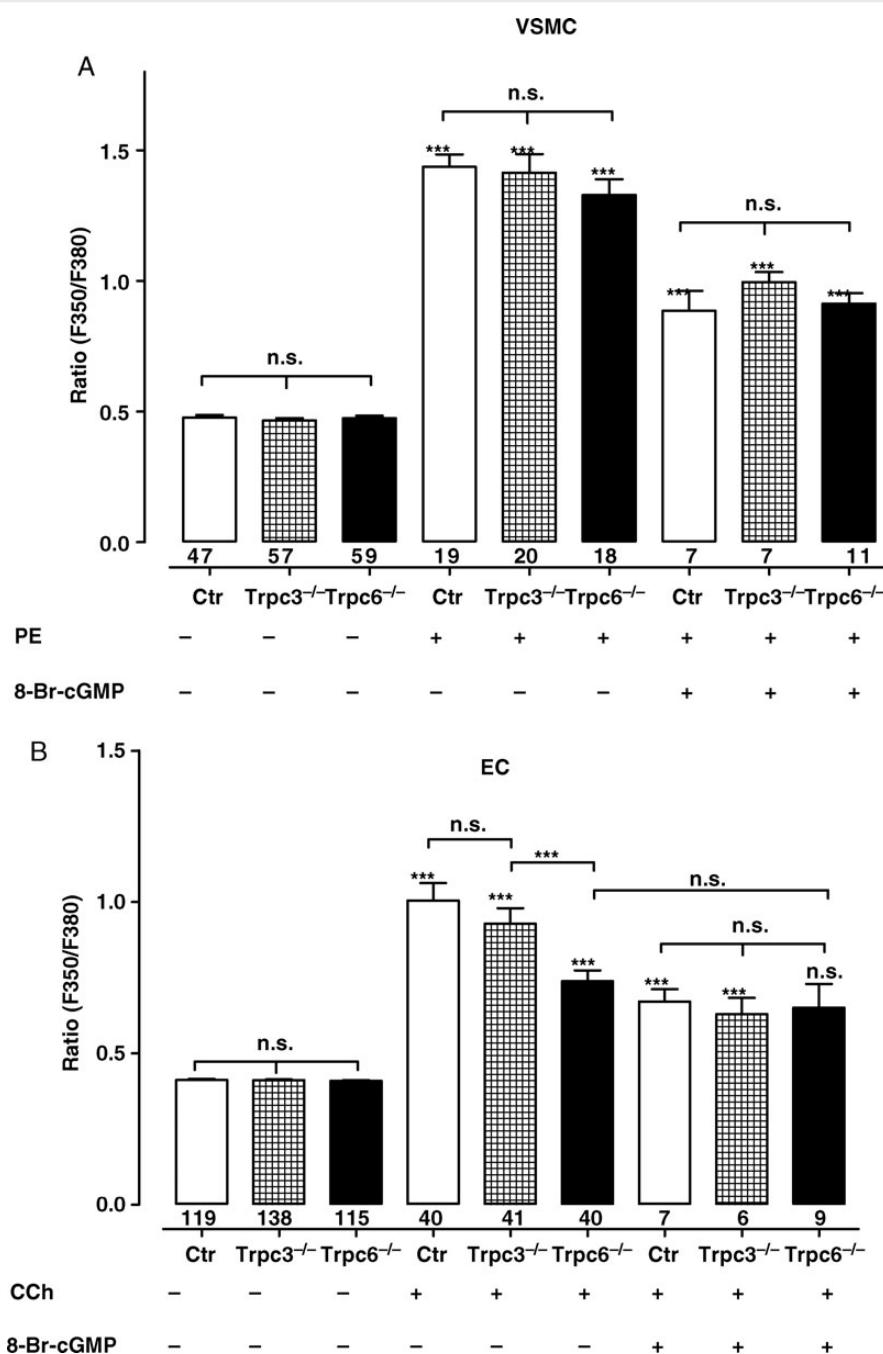


Figure 6 Intracellular Ca^{2+} levels in SMCs and ECs from the aorta. Intracellular Ca^{2+} levels are expressed as the ratio of the fluorescence intensity (at 510 nm) at alternating 350 (F350) and 380 nm (F380) excitation wavelengths. Please note that corresponding original recordings are presented in see Supplementary material online, Figure S7. (A) Intracellular Ca^{2+} levels in vascular SMCs from Ctr, $\text{Trpc3}^{-/-}$, and $\text{Trpc6}^{-/-}$ mice under control conditions, in the presence of phenylephrine (PE, 30 μM), and in the presence of PE and 8-Br-cGMP (300 μM). (B) Intracellular Ca^{2+} levels in ECs from Ctr, $\text{Trpc3}^{-/-}$, and $\text{Trpc6}^{-/-}$ mice under control conditions, in the presence of carbachol (CCh, 30 μM), and in the presence of CCh and 8-Br-cGMP (300 μM). Columns represent means \pm SEM. Ca^{2+} levels represent the maximum in the presence of the respective agonist (reached within 2 min) and the minimum in the presence of 8-Br-cGMP (reached within 5 min). Numbers represent the number of cells. Cells were obtained from 5 to 9 mice. Data were analysed using ANOVA followed by the Bonferroni *post hoc* test. Asterisks on top of the columns represent statistically significant differences of the values in the presence of PE and CCh to the control values, respectively, or of the values in the presence of 8-Br-cGMP to the values in the presence of the respective agonist. $***P < 0.001$; n.s., non-significant.

partially relaxation by the synthesis of nitric oxide.²³ Contractions by 3 μ M PE were relaxed by CCh to a smaller extent in intact aortic rings from *Trpc6*^{-/-} and *Trpc3*^{-/-}/*16*^{-/-} mice than in those from Ctr and *Trpc3*^{-/-} mice (see Supplementary material online, Figure S12). This result suggests that endothelial *Trpc6* channels are partially involved in the CCh-induced relaxation of vascular SM.

4. Discussion

The present study shows that α -adrenergic-induced contraction and relaxation of this contraction by cGMP/cGKI signalling does not depend on the presence of *Trpc1,3,6* channels in vascular SM. The data support the notion that cGKI and *Trpc3* or *Trpc6* channels are not functionally linked in the aorta and hind limb smooth muscles, because cGMP/cGKI-mediated relaxation in the aorta and hind limb vessels was not impaired in *Trpc3*^{-/-}, *Trpc6*^{-/-}, and *Trpc3*^{-/-}/*6*^{-/-} mice. Instead, our data suggest that *Trpc6* channels are involved in the maintenance of the NO-derived vasodilator tone in intact vessels. This notion is supported by the following findings: (i) contraction after α -adrenergic stimulation in intact aorta from *Trpc6*^{-/-} mice was larger than that observed in Ctr mice but identical if NO production was blocked by L-NAME; (ii) immunocytochemistry revealed no significant signal for *Trpc6* channels in aortic SMCs but in aortic ECs; (iii) PCR analysis showed no significant signal for *Trpc6*-coding mRNA in endothelium-denuded aorta; (iv) hormone-induced Ca^{2+} signalling was diminished in endothelial but not in SMCs from *Trpc6*^{-/-} mice; (v) muscarinic-induced relaxation was attenuated in PE-contracted intact aorta from *Trpc6*^{-/-} mice; (vi) cGMP/cGKI did not attenuate the intracellular Ca^{2+} level in endothelial cells from *Trpc6*^{-/-} mice. Thus, we propose that, at least in aorta, endothelial *Trpc6* channels are involved in the generation of the NO-mediated vasodilator tone. A similar involvement has been described for the endothelial *Trpc4* channel.²⁵ In the latter study, ACh-induced Ca^{2+} levels were attenuated in cultured mouse vascular EC from *Trpc4*^{-/-} mice as well as ACh-induced vasorelaxation of aortic rings from *Trpc4*^{-/-} mice. Thus, it seems possible that the remaining muscarinic-induced vasorelaxation and Ca^{2+} signals in EC from *Trpc6*^{-/-} are due to the activity of *Trpc4* channels.

Contraction of vascular SM has been proposed to depend partially on *Trpc* channels that are either mediating receptor-operated currents and/or store-operated currents to provide Ca^{2+} for contraction and/or refilling of intracellular Ca^{2+} stores, respectively.²⁶ Receptor-activated currents mediated by *Trpc1, 3, and 6* channels have been recorded in some vascular SMs including mesenteric artery myocytes,²⁷ cerebral artery myocytes,²⁸ and portal vein myocytes.²⁹ However, some of these results were not repeated in studies using knockout mice. PE-induced contraction of aortic SM was normal in *Trpc1*^{-/-} mice.³⁰ PE-induced contraction of aortic SM was not reduced but enhanced in *Trpc6*^{-/-} mice.¹⁵ We did not observe a reduced but a normal contractile response in PE-stimulated aortic rings and hind limb vessels from *Trpc3*^{-/-}, *Trpc6*^{-/-}, *Trpc3*^{-/-}/*16*^{-/-}, and *Trpc1*^{-/-}/*3*^{-/-}/*16*^{-/-} mice in conditions in which the vasodilator tone, induced by endothelial NO production, was inhibited. A vasodilator tone was only observed in PE-stimulated aortic rings but not in the PE-stimulated murine hind limb. This difference may be related to the experimental design: contractility in aortic rings is usually studied at the maximum of the length-tension relation to obtain maximal responses to agonist stimulation but may also provide mechanical stretch that stimulates endothelial NO production.³¹ In agreement with the hind limb perfusion results, it was reported that an endothelium-derived hyperpolarizing factor

distinct from NO and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of WT mice.³²

This study supports the notion that *Trpc1, Trpc3, and Trpc6* channels do not significantly participate in α -adrenergic-induced contraction of vascular SM in aorta and hind limb vessels. We cannot exclude the possibility that overexpression of an additional TRP channel may functionally substitute the deleted *Trpc* channels in SM from *Trpc3*^{-/-}, *Trpc6*^{-/-}, *Trpc3*^{-/-}/*16*^{-/-}, and *Trpc1*^{-/-}/*3*^{-/-}/*16*^{-/-} mice although no up-regulation of *Trpc2-7* channels was observed in *Trpc1*^{-/-} mice.³³ In addition, we cannot exclude effects of cGMP/cGKI signalling on *Trpc* channel activity in cell culture of SMCs in which the pattern of *Trpc* channel expression may have been changed. For example, *Trpc1* expression is up-regulated in cerebral arteries following organ culture.³⁴

Endothelium-derived NO contributes to a vasodilator tone that counteracts hormone-induced contraction of vascular SM.^{3,23} In aortic rings with intact endothelium, e.g. in the presence of the NO-mediated vasodilator tone, α -adrenergic stimulation induced larger contractions in the rings from sm-cGKI^{-/-} mice than in rings from Ctr mice. Similar results were reported for the α -adrenergic stimulation in the rings from eNOS^{-/-} mice.³⁵ These findings confirm that NO and SM cGKI are the main mediators of the vasodilator tone.

A number of studies showed that the activity of *Trpc* channels is controlled by cGMP/cGKI signalling in SM.^{8,10,11,15} We challenged this mechanism in a more physiological setting by measuring the effects of cGMP on α -adrenergic stimulated aorta and hind limbs from *Trpc* knockout mice. We found no differences in the relaxant effects of cGMP in preparations from *Trpc3*^{-/-}, *Trpc6*^{-/-}, *Trpc3*^{-/-}/*16*^{-/-}, or *Trpc1*^{-/-}/*3*^{-/-}/*16*^{-/-} mice. Thus, we suggest that the relaxant effects of cGMP/cGKI signalling on vascular SM do not depend on *Trpc1, Trpc3, or Trpc6* channels, supporting no functional coupling between cGKI and these *Trpc* channels in vascular SM. However, cGMP/cGKI did attenuate CCh-induced Ca^{2+} levels in ECs of Ctr but not of *Trpc6*^{-/-} mice, supporting a functional link between *Trpc6* and cGKI in the endothelium. Another endothelial TRP channel, TRPV4, has likewise been proposed to participate in endothelial-dependent muscarinic receptor signalling.^{25,36}

In summary, the present study suggests that the absence of *Trpc1,3,6* does not influence α -adrenergic-mediated contraction and attenuation of this contraction by cGMP/cGKI signalling in vascular SM, indicating no physiological link between cGKI and *Trpc6* channels in this murine tissue. Instead, the data indicate that the absence of cGKI impairs the vasodilator tone by disrupting signalling in SMCs, whereas the absence of *Trpc6* impairs the vasodilator tone by interfering with endothelial Ca^{2+} signalling and probably NO production.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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