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RESEARCH PAPER

Amplification of EDHF-type vasodilatations in TRPC1-deficient mice

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BACKGROUND AND PURPOSE

TRPC1 channels are expressed in the vasculature and are putative candidates for intracellular Ca^{2+} handling. However, little is known about their role in endothelium-dependent vasodilatations including endothelium-derived hyperpolarizing factor (EDHF) vasodilatations, which require activation of Ca^{2+}-activated K^+ channels (K_{Ca}). To provide molecular information on the role of TRPC1 for K_{Ca} function and the EDHF signalling complex, we examined endothelium-dependent and independent vasodilatations, K_{Ca} currents and smooth muscle contractility in TRPC1-deficient mice (TRPC1-/-).

EXPERIMENTAL APPROACH

Vascular responses were studied using pressure/wire myography and intravital microscopy. We performed electrophysiological measurements, and confocal Ca^{2+} imaging for studying K_{Ca} channel functions and Ca^{2+} sparks.

KEY RESULTS

TRPC1 deficiency in carotid arteries produced a twofold augmentation of TRAM-34- and UCL1684-sensitive EDHF-type vasodilatations and of endothelial hyperpolarization to acetylcholine. NO-mediated vasodilatations were unchanged. TRPC1-/- exhibited enhanced EDHF-type vasodilatations in resistance-sized arterioles *in vivo* associated with reduced spontaneous tone. Endothelial IK_{Ca}/SK_{Ca}-type K_{Ca} currents, smooth muscle cell Ca^{2+} sparks and associated BK_{Ca}-mediated spontaneous transient outward currents were unchanged in TRPC1-/-. Smooth muscle contractility induced by receptor-operated Ca^{2+} influx or Ca^{2+} release and endothelium-independent vasodilatations were unaltered in TRPC1-/-. TRPC1-/- exhibited lower systolic blood pressure as determined by tail-cuff blood pressure measurements.

CONCLUSIONS AND IMPLICATIONS

Our data demonstrate that TRPC1 acts as a negative regulator of endothelial K_{Ca} channel-dependent EDHF-type vasodilatations and thereby contributes to blood pressure regulation. Thus, we propose a specific role of TRPC1 in the EDHF–K_{Ca} signalling complex and suggest that pharmacological inhibition of TRPC1, by enhancing EDHF vasodilatations, may be a novel strategy for lowering blood pressure.

Abbreviations

ACh, acetylcholine; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; CA, carotid artery; EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; INDO, indomethacin; L-NA, N^G-nitro-L-arginine; MP, membrane potential; PE, phenylephrine; RyR, ryanodine receptors; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel; SMC, smooth muscle cell; SNP, sodium nitroprusside; STOCs, spontaneous transient outward currents; TRPC1, canonical transient receptor potential channel subtype 1; TRPC6, canonical transient receptor potential channel subtype 6

Introduction

The endothelium regulates vascular tone by secreting vasorelaxing autacoids and thereby pivotally contributes to blood pressure regulation (Furchgott and Zawadzki, 1980). Ca^{2+} channels provide endothelial Ca^{2+} influx, and thereby stimulate the synthesis of different vasorelaxing factors, including nitric oxide (NO) (Palmer *et al.*, 1987), prostacyclin (Moncada *et al.*, 1976) and the endothelium-derived hyperpolarizing factor (EDHF) (De Mey *et al.*, 1982; Feletou and Vanhoutte, 1988; 2009; Grgic *et al.*, 2009). EDHF candidates include cytochrome P450 epoxygenase (CYP)-derived metabolites of arachidonic acid (such as epoxyeicosatrienoic acids, EETs) (Li and Campbell, 1997; Fisslthaler *et al.*, 1999), K^+ ions (Edwards *et al.*, 1998) and hydrogen peroxide (H_2O_2) (Shimokawa and Morikawa, 2005; Hercule *et al.*, 2009). In addition, EDHF vasodilatations have been proposed to rely on the spread of endothelial cell hyperpolarization to adjacent vascular smooth muscle cells (SMC) through myoendothelial gap junctions (Griffith, 2004). In any case, calcium-activated potassium channels (K_{Ca}) expressed in the endothelium, specifically IK_{Ca} (encoded by the KCa3.1 gene) and SK_{Ca} (encoded by KCa2.3), provide the required endothelial hyperpolarization for EDHF-type vasodilatations (Köhler *et al.*, 2001a; Burnham *et al.*, 2002; Grgic *et al.*, 2009). Likewise smooth muscle BK_{Ca} [encoded by KCa1.1 (pore-forming α-subunit) and KCNMB1 (β1-subunit)], as targets of putative EDHFs (Nelson *et al.*, 1995; Li and Campbell, 1997), can also contribute to EDHF vasodilatations, which illustrates the need for Ca^{2+} influx and/or release in EDHF dilatation.

The molecular identity of endothelial Ca^{2+} channels is not resolved, although transient receptor potential (TRP) channels have been proposed to provide a Ca^{2+} influx pathway (Nilius *et al.*, 2003). TRPC4 and TRPV4 channels have been suggested to contribute to dilatations induced by NO and EDHF (Freichel *et al.*, 2001; Vriens *et al.*, 2005; Hartmannsgruber *et al.*, 2007; Saliez *et al.*, 2008; Mendoza *et al.*, 2010). Less is known about the roles of other TRP channels in endothelial function. Interestingly, TRPC1 is highly expressed in endothelial cells (Chang *et al.*, 1997; Köhler *et al.*, 2001b) and has been suggested to contribute to store-operated calcium influx (SOC) (Ahmmed *et al.*, 2004; Sundivakkam *et al.*, 2009). TRPC1 has been shown to modulate endothelial barrier function (Paria *et al.*, 2004). However, the role of TRPC1 in endothelium-dependent vasodilatation mediated by either NO or EDHF has not been defined so far, although TRPC1 are putative regulatory components in caveolae that act as key players in endothelium-dependent vasodilatation (Gratton *et al.*, 2004). Herein, the caveolin-1 scaffold domain interacts with TRPC1 channels and the inositol 1,4,5-triphosphate receptor (IP_3R) to regulate Ca^{2+} entry upon Ca^{2+} store release in endothelial cells (Sundivakkam *et al.*, 2009).

TRPC1 is also expressed in smooth muscle (Xu and Beech, 2001; Maroto *et al.*, 2005; Dietrich *et al.*, 2007) and seems to contribute to capacitative Ca^{2+} entry in conjunction with the stromal interaction molecule (STIM-1) in SMCs of pulmonary arteries (Ng *et al.*, 2009), but not in thoracic aortae and cerebral arteries (Dietrich *et al.*, 2007). Mechanosensitive currents and the myogenic response were unchanged in TRPC1-deficient mice indicating that TRPC1 does not act as a physiological stretch-activated channel in SMC (Dietrich *et al.*, 2007; Sharif-Naeini *et al.*, 2008). Instead, activation of TRPC6 induced by a conformational switch of G_q-coupled (angiotensin-II type-1) receptors after mechanical stress has been implicated in the myogenic response (Mederos y Schnitzler *et al.*, 2008). Very recently, it was suggested from experiments on cultured rat aortic SMC that TRPC1 and BK_{Ca} channels can form a functional complex in which Ca^{2+} influx through TRPC1 serves as a trigger for activation of BK_{Ca} (Kwan *et al.*, 2009), which may produce membrane hyperpolarization and vasorelaxation. However, BK_{Ca} are physiologically tightly controlled by calcium sparks originating from Ca^{2+} stores and opening of BK_{Ca} without calcium sparks does not contribute to the control of SMC $[Ca^{2+}]_i$, which regulates myogenic vasoconstriction (Gollasch *et al.*, 1998). Therefore, the physiological significance of the interaction of TRPC1 and BK_{Ca} is largely unclear and the role of TRPC1 in the regulation of arterial smooth muscle tone remains poorly understood.

Thus, in the present study we sought to define the role of endothelial TRPC1 in NO and EDHF-type vasodilatations by studying vascular responses in TRPC1-/- mice. In addition, we investigated the role of TRPC1 in SMC Ca^{2+} homeostasis, including store release, content and refilling, as well as possible interactions with BK_{Ca} and finally its role in overall smooth muscle contractility. Our data demonstrate that TRPC1 opposes vasodilatation by attenuating hyperpolarization of the endothelium and thus EDHF-type vasodilatation, but has little or no direct effect on NO-mediated vasodilatation, smooth muscle BK_{Ca} function and smooth muscle contractility. These data suggest that TRPC1 plays a specific functional role in endothelium, but not in smooth muscle, where it acts as a physiological suppressor of the EDHF-type vasodilatation and thereby is a newly identified regulator of systemic peripheral arterial resistance and blood pressure.

Methods

Animals

TRPC1-/- and TRPC6-/- mice of the same genetic background were bred and genotyped as described previously (Dietrich *et al.*, 2005; 2007). Littermates harbouring the respective wt allele were used and designated as controls. Animal protocols conformed to the guide for the care and use of laboratory animals (NIH Publication No. 85–23, revised 1996) and were approved by the local authorities.

Electrophysiology and measurement of Ca^{2+} sparks

KCa currents in freshly isolated carotid endothelial cells were recorded by patch-clamp (Brähler *et al.*, 2009). Membrane potentials were recorded in endothelial cell cluster (10–50 cells) by using the current-clamp mode of the amplifier. Freshly harvested cerebral SMC were loaded with fluo-3-AM and imaged using confocal laser scanning microscopy (Bio-Rad, Munich, Germany) (Essin *et al.*, 2007). Cells were scanned in line-scan mode and analysed using software written by K. Essin. Ca^{2+} sparks were defined as local fractional fluorescence increases (>1.2), spark width was determined at half-maximal amplitude, and decay was measured from peak to half-maximal amplitude. Spontaneous transient outward currents (STOCs) were recorded in SMC by the 'slow' whole-cell patch technique (Essin *et al.*, 2007).

Vessel myography

Carotid and cerebral arteries were mounted in a pressure myograph and diameter monitored as described previously (Si *et al.*, 2006; Dietrich *et al.*, 2007). After pressurization (80 mmHg) arteries were preconstricted using phenylephrine (PE, 1 μmol·L^{-1}) and acetylcholine was applied in the perfusate (ACh, 1 nmol·L^{-1}–10 μmol·L^{-1}). NO-synthase was blocked by N^G-nitro-L-arginine (L-NA, 100 μmol L^{-1}), cyclooxygenase by indomethacin (INDO, 10 μmol·L^{-1}) or the EDHF-type vasodilatation by using the IK_{Ca} and SK_{Ca} blockers, TRAM-34 and UCL1684 (each 1 μmol·L^{-1}), respectively, or adding 32 mmol·L^{-1} K^+ in exchange for Na^+. Constrictions were elicited by adding 60 mmol·L^{-1} K^+ or PE (1 nmol·L^{-1}–10 μmol·L^{-1}). Passive diameter changes were recorded in the presence of the NO-donor sodium nitroprusside (SNP, 10 μmol·L^{-1}) for carotid or 0 Ca^{2+} for cerebral arteries in response to stepwise increases of intraluminal pressure. Diameter changes to ACh are expressed as a percentage of the maximal vasodilatation induced by 10 μmol·L^{-1} SNP and constrictions to PE as percentage of the maximal constriction to 60 mmol·L^{-1} K^+. Mesenteric arteries and aortae were studied isometrically using wire myography (Lohn *et al.*, 2002; Fesus *et al.*, 2007) and stimulated using PE or caffeine, partially in the presence of Cd^{2+} (100 μmol·L^{-1}) to block $Ca_v1.2$ channels.

Intravital microscopy

Arteriolar dilatations were studied in the cremaster microcirculation of anesthetized mice using intravital microscopy (Wölfle and de Wit, 2005). Non-cumulative concentration–response curves were obtained by determination of inner arteriolar diameters before and during superfusion of ACh, SNP or adenosine (0.03–10 μmol·L^{-1}) in the presence of L-NA and INDO (30 and 3 μmol·L^{-1}). Vasodilatations were normalized to the maximal possible response to take into account different spontaneous constriction states (Wölfle and de Wit, 2005). Maximum response (E_{max}) and the concentration that produces half-maximal response (EC_{50}) were determined by non-linear regression analysis and significant changes determined as described previously (de Wit *et al.*, 1994).

Tail-cuff plethysmography

Systolic blood pressure was recorded in anesthetized male mice (0.04 mg·g^{-1} midazolam, i.p.) using a non-invasive tail-cuff monitor and the average of 7–15 measurements taken as the representative pressure for a single animal.

Statistics

Data are given as mean ± SEM. Differences between groups were assessed by Student's unpaired *t*-test and considered significant at $P < 0.05$.

Results

Augmented EDHF-mediated vasodilatation in carotid arteries of TRPC1-/- mice

The contribution of TRPC1 channels in EDHF-type vasodilatation was studied by pressure myography on isolated carotid arteries of TRPC1-/- mice and control mice in the presence of L-NA and INDO (100 and 10 μmol·L^{-1}). In carotid arteries of control mice, ACh produced concentration-dependent EDHF-type vasodilatation with amplitudes similar to wild-type mice studied by us previously (Brähler *et al.*, 2009). Surprisingly, EDHF-type vasodilatation to ACh was significantly larger in TRPC1-/- than in control arteries (Figure 1A, on left, for traces see Figure S1). At 100 nmol·L^{-1} ACh, vasodilatation amounted to 46 ± 12% in TRPC1-/- ($n = 5$) and to 23 ± 3% in control arteries ($n = 7$; $P < 0.05$). In both genotypes, this

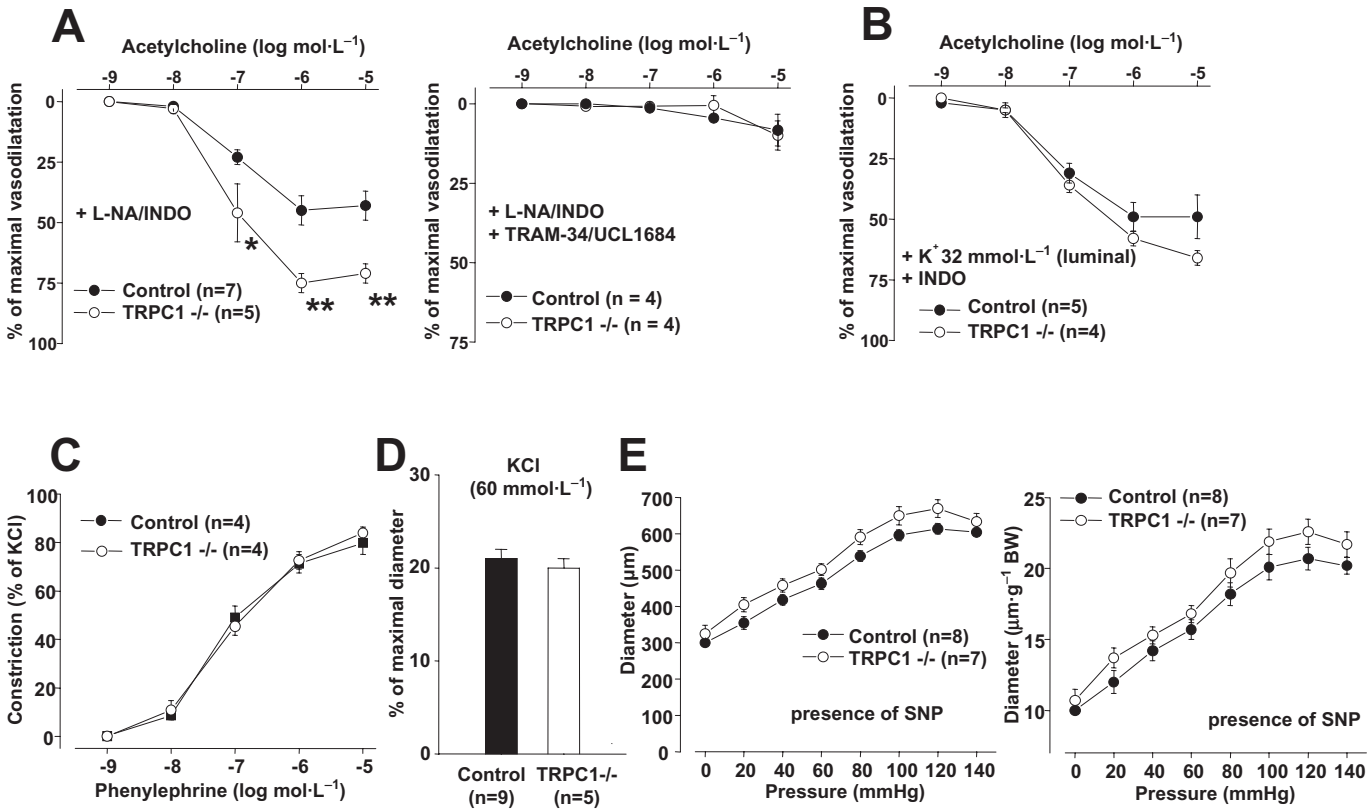

Figure 1

Responses in carotid arteries from TRPC1-/- mice. (A) On left: pressure myography revealed improved EDHF vasodilator responses of phenylephrine-precontracted arteries from TRPC1-/- mice (presence of L-NA and INDO, 100 and 10 µmol·L^{-1} respectively). On right: EDHF vasodilator responses were suppressed in both groups by TRAM-34 (1 µmol·L^{-1}) and UCL1684 (1 µmol·L^{-1}) in the perfusion buffer. (B) NO-mediated dilator responses [assessed in the presence of 32 mM KCl (luminal) and 10 µmol·L^{-1} INDO] were similar in -/- and control arteries. Phenylephrine (C)- and KCl (D)-induced constriction remained unaltered in -/- arteries. (E) Unaltered distensibility of -/- arteries. The plots show passive diameter changes (absolute and normalized to body weight) to stepwise increases of luminal pressure in the presence of SNP (10 µmol·L^{-1}). Data are given as mean ± SEM. *$P < 0.05$, **$P < 0.01$, Student's *t*-test.

EDHF-type vasodilatation was abolished by combined inhibition of endothelial IK_{Ca} (KCa3.1) and SK_{Ca} (KCa2.3) by addition of 1 µmol·L^{-1} TRAM-34 (IK_{Ca}) and 1 µmol·L^{-1} UCL1684 (SK_{Ca}) (Figure 1A, on right; for traces see Figure S1). In contrast, NO-mediated vasodilatations assessed in the presence of 10 µmol·L^{-1} INDO and 32 mmol·L^{-1} K^+ (to suppress any hyperpolarization and thus EDHF dilator responses) were not different between the groups (at 100 nmol·L^{-1} ACh: 36 ± 3% in TRPC1-/-, $n = 4$, vs. 31 ± 4% in control, $n = 5$; Figure 1B). These findings demonstrate that deficiency of TRPC1 amplifies selectively EDHF-type vasodilatation in carotid arteries.

In contrast to TRPC1-deficient arteries, the EDHF-type vasodilatations were not enhanced in carotid arteries from TRPC6-/- mice (Dietrich *et al.*, 2005), but instead exhibited a reduced EDHF-type vasodilatation at 100 nmol·L^{-1} ACh (Figure S2).

Vasoconstrictions to PE and 60 mmol·L^{-1} KCl were not altered in TRPC1-/- arteries (Figure 1C and D). Passive distension elicited by increasing intraluminal pressures (up to 140 mmHg) in the presence of SNP (10 µmol·L^{-1}) was not significantly different between the two genotypes (Figure 1E), although passive diameters of the carotid arteries tended to be larger in TRPC1-/- mice, which is perhaps due to the slightly larger size of the animals as indicated by an increased body weight (TRPC1-/-: 29 ± 1 g vs. controls: 25 ± 1 g; $P < 0.05$). Histological examination revealed no visible differences (i.e. number of smooth layers and elastic laminae, data not shown). Similarly, passive distensibility was unchanged in mouse cerebral arteries (Figure S3), which, as reported previously, also showed no differences in myogenic tone (Dietrich *et al.*, 2007). These findings suggest that smooth muscle contractility to an agonist and depolarization as well as compliance is preserved in carotid and cerebral arteries from TRPC1-/- mice.

We tested whether alterations of IK_{Ca} and SK_{Ca} currents underlie the increased EDHF-type

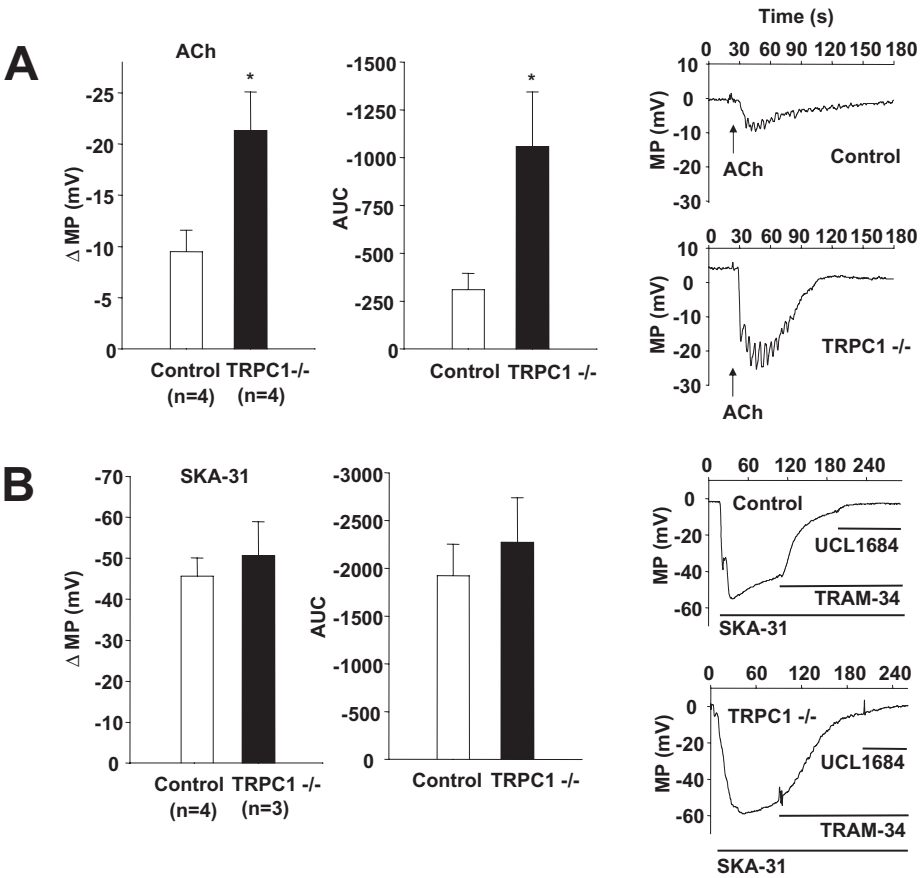

Figure 2

Membrane potential changes to acetylcholine in endothelial cell (EC) clusters from carotid arteries of TRPC1-/- and controls. (A) In current-clamp recordings of endothelial membrane potentials (MP), acetylcholine (100 nmol·L^{-1}) produced a transient shift of the MP to more negative values. The shift (ΔMP) and magnitude over time of the response (AUC) were stronger in EC cluster of TRPC1-/- (n = 4 animals, total of 14 experiments, 2–5 per animal), than in controls (n = 4 animals, total of 13 experiments, 2–5 per animal). Representative traces are shown on right. (B) Hyperpolarization responses to SKA-31 (1 μmol·L^{-1}) after ACh prestimulation were similar in TRPC1-/- (n = 3 animals, total of 11 experiments, 3–4 per animal) and in controls (n = 4 animals, total of 12 experiments, 1–5 per animal). AUC was calculated over the first minute of the response. Representative traces are shown on right. Note that TRAM-34 (1 μmol·L^{-1}) substantially reversed hyperpolarization and the combination of TRAM-34 and UCL1684 (1 μmol·L^{-1}) produced an almost complete suppression in both genotypes. None of the drugs changed interendothelial electrical coupling as indicated by similar capacitance values as at the beginning of the experiment. Data are given as mean ± SEM. *P < 0.05, **P < 0.01, Student's t-test.

vasodilatation in TRPC1-/-. However, our whole-cell patch-clamp studies on freshly isolated endothelial cells revealed no alterations of the functional expression and activity of endothelial K_{Ca} channels (TRPC1-/-: 27 ± 6 pA/pF at 0 mV, n = 4 cells; controls: 27 ± 3 pA/pF, n = 4 cells). In a second set of experiments, we measured membrane potentials in endothelial cells clusters (10–50 cells) by using the current-clamp mode. Endothelial cells were electrically coupled as indicated by high capacitance values of 214 ± 61 pF (n = 12 experiments) on four controls and of 225 ± 55 pF (n = 14 experiments) on four TRPC1-/- mice. Endothelial cell clusters were found to be depolarized with membrane potential values of 3 ± 2 mV in controls and of 4 ± 1 mV in TRPC1. Stimulation with 100 nmol·L^{-1} ACh resulted in a transient shift to negative potentials (lasting for 30 s–2 min). As shown in Figure 2A (on left), the amplitude of the response was significantly larger in TRPC1-/- (≈–21 mV) than in controls (≈–10 mV). Moreover, the magnitude over time (AUC) of the response was larger in TRPC1-/- than in controls (Figure 2A, on right). In contrast, subsequent stimulation with the IK_{Ca}/SK_{Ca} opener SKA-31 (1 μmol·L^{-1}) (Sankaranarayanan *et al.*, 2009) produced strong and sustained hyperpolarization responses, which were similar in both genotypes (controls: ≈–45 mV vs. TRPC1-/-: ≈–49 mV; Figure 2B). These responses were greatly reversed by TRAM-34 (controls: –11 ± 2 mV vs. TRPC1-/-: –9 ± 3 mV) and almost completely by further adding UCL1684 (controls: –1 ± 1 mV vs. TRPC1-/-:

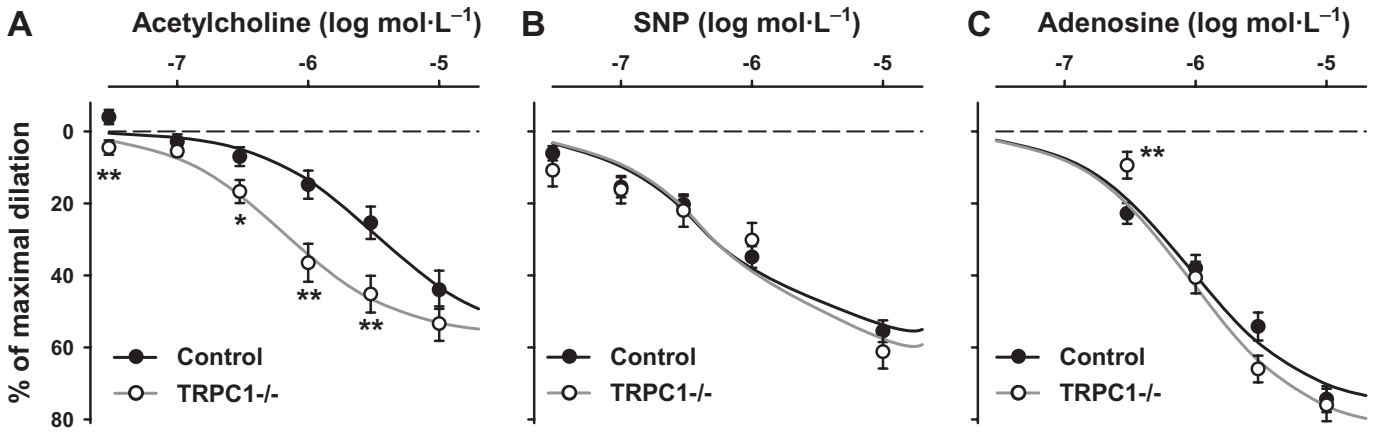

Figure 3

Endothelium-derived hyperpolarizing factor (EDHF)-type dilatations in the microcirculation are selectively enhanced in TRPC1-/- mice. Concentration-dependent dilatations in -/- and control mice in response to acetylcholine (A), sodium nitroprusside (SNP, B) and adenosine (C). The values measured were fitted using non-linear regression and the resulting curve is depicted for control and TRPC1-/-. ACh-induced dilatations were enhanced in -/- mice at low and intermediate concentrations resulting in a significant leftward shift of the concentration–response curve (A). In contrast, dilatations in response to the endothelium-independent dilators SNP and adenosine were similar. All experiments were performed in the presence of L-NA and INDO (30 and 3 μmol·L^{-1}), $n = 48$ in six animals each group (A), $n = 72$ in eight control and 35 in four -/- (B), $n = 53$ in seven control and 28 in three -/- (C). Data are given as mean ± SEM. *$P < 0.05$, **$P < 0.01$, Student's *t*-test.

–3 ± 1 mV; $n = 11$ experiments per genotype, for traces see Figure 2B). Likewise, Ca^{2+} sparks were indistinguishable between control and TRPC1-/- mice in SMCs freshly isolated from tibial artery (spark frequency; control: 0.6 ± 0.2 Hz, $n = 30$ cells; TRPC1-/-: 0.7 ± 0.1 Hz, $n = 40$ cells, $P > 0.05$).

Enhanced EDHF-mediated vasodilatations in resistance-sized arterioles in vivo *of TRPC1-/- mice*

We next investigated whether a deficiency of TRPC1 also affects EDHF-type vasodilatations in the cremaster muscle microcirculation by employing intravital microscopy (de Wit *et al.*, 2000). In this vascular bed, ACh-induced vasodilatations are mediated by EDHF (Koeppen *et al.*, 2004) and IK_{Ca} and SK_{Ca} channels play a critical role in this response (Brähler *et al.*, 2009). We studied a total of 205 arterioles, 122 in 14 control and 83 in 10 TRPC1-/- mice. Maximal diameters ranged from 14 to 56 μm (mean: 32.9 ± 0.8 μm) in control and from 12 to 57 μm in TRPC1-/- (mean: 35.8 ± 0.9 μm, $P < 0.05$). In the presence of L-NA and INDO, spontaneous diameter was significantly smaller in control (11.9 ± 0.5 μm) than in TRPC1-/- mice (14.2 ± 0.7 μm, $P < 0.01$) and these diameters expressed as a fraction of maximal diameter were higher in TRPC1-/- than in control mice (0.40 ± 0.02 vs. 0.36 ± 0.01, $P < 0.05$) indicating a reduced spontaneous tone in TRPC1-/- arterioles. Because tone depends on vascular size (Wölfle and de Wit, 2005), we analysed a subgroup of vessels with similar maximal diameters (maximal diameter >30 μm: control 38.3 ± 0.7, $n = 75$; TRPC1-/- 38.2 ± 0.8 μm, $n = 70$), but spontaneous tone was also attenuated in TRPC1-/- in this subgroup (0.40 ± 0.02 vs. 0.35 ± 0.01, $P < 0.05$).

Acetylcholine induced concentration-dependent EDHF-type vasodilatations in control and in TRPC1-/- mice. Maximal response was similar in both genotypes (E_{max}: 57 ± 4 vs. 57 ± 9%, TRPC1-/- and control respectively); however, the concentration–response curve was significantly shifted to lower ACh concentrations in TRPC1-/- (EC_{50}: 0.66 ± 0.18 vs. 3.25 ± 1.29 μmol·L^{-1} in control, $P < 0.01$, Figure 3A). Endothelium-independent vasodilatations to SNP and adenosine were similar in both genotypes, with the exception of a significant lower response to the lowest dose of adenosine in TRPC1-/- (Figure 3B and C).

Ca^{2+} sparks and BK_{Ca} activity in SMC of TRPC1-deficient mice

BK_{Ca} channels are activated by Ca^{2+} sparks, giving rise to STOCs and represent targets for epoxyeicosatrienoic acids acting as EDHF (Li and Campbell, 1997; Fisslthaler *et al.*, 1999). TRPC1 has been suggested to bind to and affect smooth muscle BK_{Ca} activity (Kwan *et al.*, 2009). We therefore determined whether a deficiency of TRPC1 alters Ca^{2+} sparks and resulting STOCs. Figure 4A shows representative confocal line-scan images of freshly isolated fluo-3-loaded SMC. Ca^{2+} sparks were observed only in close proximity to the cell surface. Ca^{2+} sparks in control cells had an amplitude of 2.1 ± 0.1

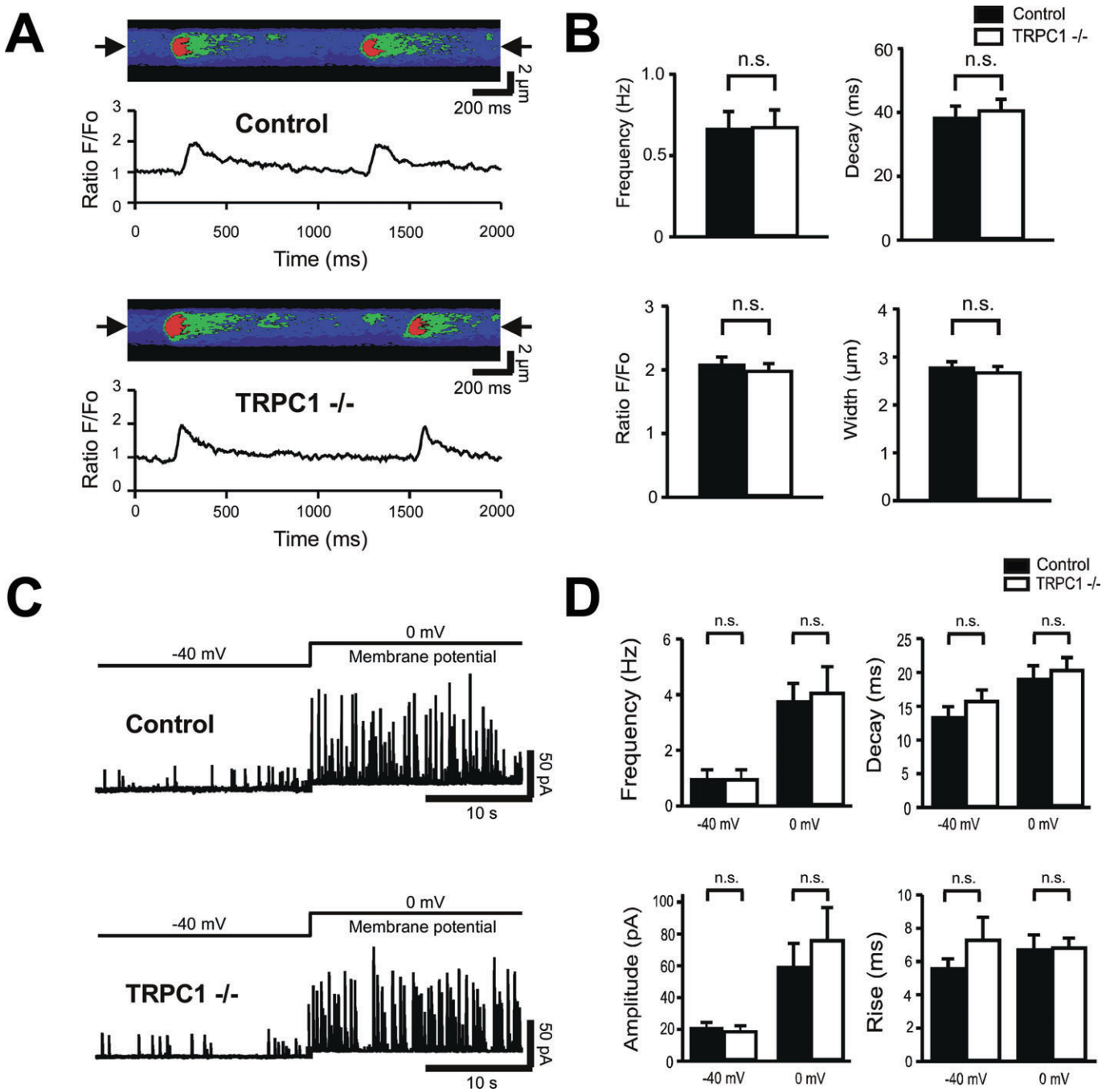

Figure 4

Ca^{2+} sparks and spontaneous transient outward currents (STOCs) in TRPC1-/- smooth muscle cells (SMC) isolated from cerebral arteries. **(**A) Confocal line-scan images of fluo-3-loaded SMC, in which fluorescence levels are colour-coded for a certain distance within the cell (ordinates) over time (abscissae). The change of the fluorescence (F/F_o) with time was determined over a single line as indicated by arrows and is depicted below each image. Ca^{2+} sparks appear as red areas in line-scan images and are defined as fluorescence increases greater than 1.2. (B) Frequency, amplitude, decay and width of Ca^{2+} sparks was similar in both genotypes. Control: $n = 30$, -/-: $n = 58$ SMC; each cell was scanned for 10 s. (C) Representative original recordings of STOCs in isolated cerebral SMC. The holding potential was increased from –40 to 0 mV. (D) Frequency, amplitude and temporal characteristics of STOCs at –40 or 0 mV. Data are mean ± SEM, $n = 7$–10 cells each genotype, n.s. not significant, Student's *t*-test.

(measured as F/F_o), a width of 2.8 ± 0.1 μm, a rise time of 22 ± 2 ms and decayed to half amplitude after 39 ± 3 ms. Their frequency was 0.67 ± 0.1 Hz (n = 30 cells). Ca^{2+} sparks were not different in SMC (n = 58) obtained from TRPC1-/- mice for any of these characteristic parameters (Figure 4B).

Because of the close proximity of Ca^{2+} sparks to the cell surface and to BK_{Ca}, they serve as a local signal for BK_{Ca} activation and thus Ca^{2+} sparks result in the generation of STOCs (Nelson *et al.*, 1995). Figure 4C shows representative traces of STOCs in SMC from control and TRPC1-/- mice at a test potential of –40 mV and after an increase to 0 mV. At –40 mV, STOCs were observed at a frequency that corresponded to the frequency of Ca^{2+} sparks in both genotypes. At –40 mV, STOCs in control cells occurred with a frequency of 1.0 ± 0.3 Hz, had an amplitude of 19.4 ± 2.9 pA, a rise time of 7.4 ± 1.3 ms and decayed within 16.0 ± 1.5 ms to half of their amplitude (n = 9 cells, Figure 4D). At 0 mV, the frequency of STOCs increased to 4.1 ± 0.9 Hz and their amplitude to 60 ± 14 pA (n = 10 cells), consistent with the voltage dependence of Ca^{2+} spark frequency and BK_{Ca} currents. STOCs in cells from TRPC1-/- mice were not different from those in control cells and likewise voltage dependence was preserved [Figure 4C (representative trace) and Figure 4D (statistics)]. These data indicate that a deficiency of TRPC1 does not alter the physiological BK_{Ca} channel activity induced by Ca^{2+} sparks in SMC.

Endothelium-independent contractions are preserved in TRPC1-/-

Isometric contraction of small mesenteric artery rings to spasmogenic 60 mmol·L^{-1} K^+, ryanodine receptor (RyR) activation by caffeine, to IP_3R activation by PE did not differ between control and TRPC1-/- arteries (Figure S4). Moreover, TRPC1 deficiency did not alter contractions in the absence of functional $Ca_v1.2$ L-type channels (in the presence of 100 μmol·L^{-1} Cd^{2+}, Figure S4). These results indicate TRPC1 channels are not required for smooth muscle contraction initiated by Ca^{2+} release from the sarcoplasmic reticulum via IP_3R and RyR.

Blood pressure in TRPC1-/- mice

The finding that EDHF-type vasodilatations are enhanced in TRPC1-/- arteries and there is a lower spontaneous tone in cremaster muscle arterioles suggests that systemic blood pressure is altered, and we therefore conducted tail-cuff measurements of systolic blood pressure. We found that systolic blood pressure was lower in TRPC1-/- mice (109 ± 11 mmHg, n = 4) compared with control mice (148 ± 4 mmHg, n = 5; $P < 0.01$).

Discussion

In the present study, we assessed the role of TRPC1 channels in endothelium-dependent and independent vasodilatations as well as in smooth muscle contractility. Our study provides the first evidence that TRPC1 channels contribute to endothelium-dependent vasodilatation by specifically influencing endothelial hyperpolarization and EDHF-type vasodilatation and therefore it is most likely that TRPC1 channels affect arteriolar tone and systemic blood pressure. This conclusion is based on the following observations: (i) TRPC1 deficiency substantially improved the EDHF-type vasodilatation that required IK_{Ca}/SK_{Ca} channel activity in isolated conduit arteries and resistance-sized arterioles of skeletal muscle *in vivo* and augmented ACh-induced hyperpolarization of carotid artery endothelial cells; (ii) TRPC1 deficiency resulted in a lower spontaneous tone of resistance-sized arterioles and was associated with systolic hypotension; and (iii) TRPC1 deficiency did not affect either NO-mediated vasodilatation or smooth muscle responses to various stimuli, which highlights a selective impact of TRPC1 on endothelial function during EDHF signalling.

TRPC1 channels have been shown to be expressed in both, endothelial and SMC (Köhler *et al.*, 2001b; Nilius *et al.*, 2003; Dietrich *et al.*, 2007). In smooth muscle, TRPC1 channels have been proposed to mediate store-operated Ca^{2+} currents (SOC) after agonist-induced store depletion; concluded from studies using a blocking antibody (Xu and Beech, 2001; Ng *et al.*, 2009). However, a genetic approach using TRPC1-/- mice revealed that store-operated Ca^{2+} influx does not crucially rely on TRPC1 in arterial smooth muscle (Dietrich *et al.*, 2007). In the present study, we confirmed and extended these findings, in that TRPC1 deficiency had no sizeable impact on constriction of various arteries to spasmogenic K^+ depolarization, PE or caffeine, even after pharmacological blockade of $Ca_v1.2$ L-type channels. Thus, smooth muscle TRPC1 is not essential for constrictions elicited by either membrane depolarization (activation of $Ca_v1.2$ L-type channels), by α-adrenoceptor stimulation (involving Ca^{2+} influx and Ca^{2+} release from internal Ca^{2+} stores via IP_3R) or by Ca^{2+} release mediated via sarcoplasmic RyR (caffeine). Moreover, the refilling of Ca^{2+} stores upon depletion remained likewise intact, excluding a crucial role of TRPC1 in SOC in arterial SMC. Endothelium-independent vasodilatations to

a NO-donor (SNP) and to adenosine were equally preserved in TRPC1-deficient mice. In contrast to the apparently non-essential roles of TRPC1-/- in smooth muscle functions, TRPC1 appears to play a role in endothelial functions as deficiency of TRPC1 channels substantially improved EDHF-type vasodilatations in a conduit vessel (carotid artery) and also in resistance-sized arterioles in the cremaster muscle microcirculation. This augmented EDHF-type vasodilatation seems to rely on an enhanced hyperpolarization response of the endothelium to ACh. As concluded from our electrophysiological measurements, this higher hyperpolarization response was not associated with an altered functional expression of endothelial K_{Ca} channels (KCa3.1 and KCa2.3), the crucial molecular mediators of endothelial hyperpolarization and the resulting EDHF response (Feletou and Vanhoutte, 2006; Grgic *et al.*, 2009), as K_{Ca} functions as well as hyperpolarizations to the opener SKA-31 were unchanged. Moreover, the recently suggested binding of TRPC1 to smooth muscle BK_{Ca} channels (Kwan *et al.*, 2009) does not appear to affect the physiological BK_{Ca} channel – a putative target of diffusible EDHFs – and its regulation by Ca^{2+} sparks, as the extensive data presented in conjunction with previous results (Dietrich *et al.*, 2007) did not reveal any functional changes of Ca^{2+} sparks and BK_{Ca} activity (measured as STOCs) in TRPC1-/- SMC.

The improved endothelial functions appeared to be restricted to the EDHF-type vasodilatation as NO-mediated vasodilatation, which has been shown to account for a large part of endothelium-dependent vasodilatation to ACh in the murine carotid artery, was unchanged in TRPC1-/-. As endothelial NO-mediated vasodilatations to ACh are also Ca^{2+}-dependent, the normal NO-mediated vasodilatations in TRPC1-/- further suggest that endothelial Ca^{2+} signalling and eNOS activation are unchanged following muscarinic receptor stimulation in TRPC1-/- mice. This indicates that TRPC1 channels do not play an essential role in endothelial Ca^{2+} release but provide a depolarizing current, which counteracts the hyperpolarizing K_{Ca} activity after receptor stimulation. Regardless of these findings, the significance of the selectively improved EDHF vasodilatations in the regulation of arterial tone in TRPC1-/- is indicated by the lower spontaneous arteriolar tone in the microcirculation in the absence of NO signalling and the lower systolic blood pressure in these mice, although the mechanisms of this effect remain unclear.

The observed cardiovascular alterations seem to be specific for TRPC1 as mice deficient of TRPC6 channels (Dietrich *et al.*, 2005) exhibited, in contrast to TRPC1-deficient mice, a slightly reduced EDHF response and have been shown to be hypertensive (Dietrich *et al.*, 2005). The latter effect has been attributed to an overexpression of smooth muscle TRPC3 (Dietrich *et al.*, 2005). Whether similar adaptive changes in TRPC expression levels and in improved Ca^{2+} influx pathways (perhaps linked to activation of co-localized KCa channels) account for the improved endothelial hyperpolarization and EDHF-type vasodilatations in TRPC1-deficient mice, remains to be clarified. At least in smooth muscle of TRPC1-/- mice, expression levels of other TRPC channels were found to be unchanged (Dietrich *et al.*, 2007).

In conclusion, our study demonstrates a functional role of TRPC1 channels in the control of EDHF-type vasodilatations in conduit and resistance-sized arterioles *in vivo*. TRPC1 appears to act as a moderate suppressor of endothelial hyperpolarization and resultant EDHF-type vasodilatation, and possibly thereby facilitate arterial vascular tone and increase systemic blood pressure. Our data suggest that TRPC1 channels specifically inhibit EDHF responses, but have little or no effect on NO-mediated vasodilatations and vasorelaxing actions, or Ca^{2+} handling in SMCs required for constriction. Although still not available at present, small molecule blockers of TRPC1 may be used to lower blood pressure by selectively augmenting EDHF vasodilator responses.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative recordings of EDHF-type vasodilatation to increasing concentrations of acetylcholine (ACh, 10^{-9}–10^{-5} mol·L^{-1} as 300 µL bolus) in carotid arteries from controls (upper panel) and TRPC1-/- mice (lower panel) and in the presence of L-NA (300 µmol·L^{-1}) and INDO (10 µmol·L^{-1}). Carotid arteries were preconstricted with 1 µmol·L^{-1} (or first with 0.1 µmol·L^{-1} and then 1 µmol·L^{-1}) PE. 'Flow' indicates an increase of perfusion from 30–90 µL to 600–900 µL. At the end of the experiment, a saline buffer with 60 mmol·L^{-1} KCL (K60) was added to the bath to induce maximal constriction. After washout, a standard saline buffer containing 1 µmol·L^{-1} sodium nitroprusside (SNP) was used to induce maximal vasodilatation. Traces on the right show suppression of EDHF-type vasodilatation in the continuing presence of TRAM-34 (IK_{Ca} blocker) and UCL1684 (SK_{Ca} blocker; each 1 µmol·L^{-1}) in the perfusion buffer. Note that vasodilator responses to increased vessel perfusion were also inhibited, which indicates a role of particularly SK_{Ca} as reported previously by us (Hartmannsgruber *et al*., 2007; Brähler *et al*., 2009).

Figure S2 EDHF-type vasodilatations of carotid arteries from TRPC6-/- and control mice. Note that EDHF-type vasodilatations to 100 nM ACh were significantly reduced in TRPC6-/- mice ($n = 5$). Data are

given as mean ± SEM, values of controls are replotted from Figure 1 for comparison. * $P < 0.05$, Student's *t*-test.

Figure S3 Unchanged passive properties of TRPC1-/- cerebral arteries. (A) Diameter of isolated cerebral arteries from -/- and control mice at 10 mmHg and vasoconstriction to 60 mmol·L^{-1} KCl. (B) Passive distension in response to transluminal pressure steps as indicated in mmHg. With the exception of the two columns on the right, arteries were studied in normal Ca^{2+}-containing bath solution. Data are given as mean ± SEM.

Figure S4 Endothelium-independent contractions are preserved in TRPC1-/-. Isometric contraction of small mesenteric artery rings to spasmogenic 60 mmol·L^{-1} K^+ did not differ between control and TRPC1-/- arteries (A). Also the ryanodine receptor (RyR) activator caffeine (10 mmol·L^{-1}) produced similar transient contractions in both genotypes (B). The strength of the constriction in response to a secondary caffeine application depended on refilling of Ca^{2+} stores as indicated by the gradual recovery of this second caffeine-induced contraction with time (C). This recovery was similar in control and TRPC1-/- mice (C), demonstrating that Ca^{2+} store refilling and Ca^{2+} release from RyR-sensitive Ca^{2+} pools in sarcoplasmic reticulum is not affected by lack of TRPC1. The physiological IP_3R activator PE induced similar contractions in control and TRPC1-/- mesenteric arteries (D) and aortae (E). To unmask a possible role of TRPC1 channels in the absence of functional $Ca_v1.2$ L-type channels, we performed experiments in the presence of the inorganic $Ca_v1.2$ channel blocker Cd^{2+} (100 µmol·L^{-1}). However, force generated by exposure to either caffeine (B) or PE (D) was not different between mesenteric arteries from control and TRPC1-/- mice in the presence of Cd^{2+}. As contractions rely under these conditions on RyR- (caffeine) and IP_3R-dependent (PE) intracellular Ca^{2+} release, respectively, these results indicate TRPC1 channels are not required for smooth muscle contraction initiated by Ca^{2+} release from the sarcoplasmic reticulum via IP_3R and RyR. Data are given as mean ± SEM. n.s., not significant, Student's *t*-test.

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