British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 150–162 150

RESEARCH PAPER

Do $K_V7.1$ channels contribute to control of arterial vascular tone?

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Received 29 October 2015; **Revised** 11 October 2016; **Accepted** 28 October 2016

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BACKGROUND AND PURPOSE

$K_V7.1$ voltage-gated potassium channels are expressed in vascular smooth muscle cells (VSMC) of diverse arteries, including mesenteric arteries. Based on pharmacological evidence using R-L3 ($K_V7.1$ channel opener), HMR1556, chromanol 293B ($K_V7.1$ channel blockers), stimulation of these channels has been suggested to evoke profound relaxation in various vascular beds of rats. However, the specificity of these drugs *in vivo* is uncertain.

EXPERIMENTAL APPROACH

We used *Kcnq1*$^{-/-}$ mice and pharmacological tools to determine whether $K_V7.1$ channels play a role in the regulation of arterial tone.

KEY RESULTS

R-L3 produced similar concentration-dependent relaxations (EC_{50} ~ 1.4 μM) of arteries from wild-type (*Kcnq1*$^{+/+}$) and *Kcnq1*$^{-/-}$ mice, pre-contracted with either phenylephrine or 60 mM KCl. This relaxation was not affected by 10 μM chromanol 293B, 10 μM HMR1556 or 30 μM XE991 (pan-K_V7 channel blocker). The anti-contractile effects of the perivascular adipose tissue (PVAT) were normal in *Kcnq1*$^{-/-}$ arteries. Chromanol 293B and HMR1556 did not affect the anti-contractile effects of (PVAT). Isolated VSMCs from *Kcnq1*$^{-/-}$ mice exhibited normal peak K_V currents. The $K_V7.2$–5 channel opener retigabine caused similar relaxations in *Kcnq1*$^{-/-}$ and wild-type vessels.

CONCLUSION AND IMPLICATIONS

We conclude that $K_V7.1$ channels were apparently not involved in the control of arterial tone by α_1-adrenoceptor agonists and PVAT. In addition, R-L3 is an inappropriate pharmacological tool for studying the function of native vascular $K_V7.1$ channels in mice.

Abbreviations

4-AP, 4-aminopyridine; ADRF, adipocyte-derived relaxing factor; HMR1556, N-(6-cyano-3-hydroxy-2,2-dimethyl-3,4-dihydrochromen-4-yl)-N-methylethanesulfonamide; ML277, (2R)-N-[4-(4-methoxyphenyl)-1,3-thiazol-2-yl]-1-(4-methylphenyl)sulfonylpiperidine-2-carboxamide; PVAT, perivascular adipose tissue; VSMC, vascular smooth muscle cells

DOI:10.1111/bph.13665

Tables of Links

TARGETS
Voltage-gated ion channels[a]
K_V7.1 potassium channels
K_V7.2 potassium channels
K_V7.3 potassium channels
K_V7.4 potassium channels
K_V7.5 potassium channels
L type Ca_V1.2 calcium channels
GPCRs[b]
α_{1A}-adrenoceptor
α_{1B}-adrenoceptor
α_{1D}-adrenoceptor

LIGANDS
4-AP
ACh
Chromanol 293B
HMR1556
ML277
Phenylephrine
Retigabine
R-L3
XE991

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

Recent data suggest that the KCNQ family of voltage-gated K^+ (K_V7) channels represents a new therapeutic target in cardiovascular disease (Mackie and Byron, 2008; Greenwood and Ohya, 2009; Gurney *et al.*, 2010; Tano *et al.*, 2014). The K_V7 channel family is composed of five different isoforms, namely K_V7.1–5. Among them, K_V7.1 channels are highly expressed at the mRNA and protein level in different types of vessels in humans and animals (Yeung *et al.*, 2007; Ng *et al.*, 2011; Chadha *et al.*, 2012). Based on the effects of pharmacological drugs, stimulation of K_V7.1 channels has been suggested to cause profound relaxation in various vascular tissues of rats, including the aorta, mesenteric and pulmonary arteries (Chadha *et al.*, 2012). Immunohistochemical studies using anti-KCNQ1 antibodies indicated *Kcnq1* expression in murine arteries (aortic artery), including the endothelial and smooth muscle layers, at late embryonic and fetal stages (E14.5 to E16.5) (de Castro *et al.*, 2006) and in adult arteries (Yeung *et al.*, 2007). Of note, blood pressure is enhanced in *Kcnq1*$^{-/-}$ mice (Takagi *et al.*, 2007). In humans, mutations in the *KCNQ1* gene cause Jervell and Lange–Nielsen syndrome (Wang *et al.*, 1996; Goldenberg *et al.*, 2008). A recent meta-analysis suggests a possible causative role of the *KCNQ1* gene in type 2 diabetes (Liu *et al.*, 2013). However, it is unknown whether *KCNQ1* gene products are related to vascular pathologies and/or blood pressure regulation in humans.

K_V7.1 channels are expressed in vascular smooth muscle cells (VSMCs) of thoracic aorta, carotid, femoral and mesenteric arteries in mice (Yeung *et al.*, 2007; Schleifenbaum *et al.*, 2014) and rats (Chadha *et al.*, 2012). Although these channels presumably do not contribute to resting vascular tone, K_V7.1 channel activators, such as R-L3 (L-364373), were shown to be effective vasorelaxants (Chadha *et al.*, 2012). R-L3 responses were inhibited by blockers of K_V7.1 channels, such as HMR1556 or chromanol 293B, which suggests that KCNQ1 activation is an important mechanism underlying vascular relaxation (Chadha *et al.*, 2012). However, the specificity of the drugs used to target native K_V7.1 channels is unknown, since the pore-forming K_V7.1 subunit can interact with several accessory KCNE subunits, which are known to modulate the biophysical properties of K_V7 channels *in vivo* (Jespersen *et al.*, 2005). Second, these accessory subunits may influence the pharmacological properties of native, complex, multimeric K_V7.1 channels (MacVinish *et al.*, 2001). Therefore, the aim of this study was to test the contribution of K_V7.1 channels in the regulation of arterial vascular tone by using *Kcnq1*$^{-/-}$ mice and pharmacological tools, such as R-L3, chromanol 293B and HMR1556. We also used a novel K_V7 channel opener, ML277, which has recently been shown to activate K_V7.1 channels in cardiomyocytes with an EC_{50} of 260 nM (Mattmann *et al.*, 2012).

Methods

Mouse model

All animal care and experimental procedures followed American Physiological Society guidelines and were approved by the local authorities (Landesamt für Gesundheit und Soziales Berlin, LAGeSo). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Animals were housed in individually ventilated cages under standardized conditions with an artificial 12 h dark–light cycle with free access to water and food.

We used *Kcnq1*$^{-/-}$ mice (C57BL/6 background) with targeted disruption of exon 2 of the *Kcnq1* gene (Casimiro *et al.*, 2001). Heterozygous mice were used for breeding to obtain homozygous knockout (*Kcnq1*$^{-/-}$) mice. Littermate (12–16 weeks old) male wild-type mice ($^{+/+}$) were used as

controls (Figures 1C,D; 4C; 5 and Figures S4A; S5C,D; S6; S7); as everywhere else age-matched (12–16 weeks old) male C57BL/6 mice purchased from Charles River, Sulzfeld, Germany. For experiments involving rat mesenteric arteries, male Sprague Dawley rats (Charles River, Sulzfeld, Germany, age 10 weeks) were used. Animals were randomly assigned to the experimental procedures in accordance with the German legislation on protection of animals.

Genotyping of Kcnq1$^{-/-}$ and Kcnq1$^{+/+}$ mice

Tail samples from 4-week-old mice were digested in 150 μL tail lysis buffer (75 μL 25 mM NaOH; 75 μL 40 mM TrisHCl, pH 5.0) for 30 min at 95°C. Genotyping PCR was carried out on 3.5 μL tail lysate with Go Taq ® G2 Green Master mix according to manufacturer's specifications in 15 μL reactions. The following PCR programme was used: 94°C 6 min, repeat 40 times 94°C for 45 s, 58°C for 45 s, 72°C for 50 s followed by 72°C for 8 min then holding at 4°C. Following primers were used: forward primer 5′- CCAGGAGTGGGTGGTTCTAC-3′, reverse primer 5′-GCCAGCACTAAAGATCTTGC-3′, Neo forward primer 5′-CGCTTCCTCGTGCTTTACG-3′, as previously described (Casimiro *et al.*, 2001). PCR reactions were analysed on 2% agarose gels containing ethidium bromide, visualized by exposure to ultraviolet light with DNA ladder 100 BP manufactured by PeqLab.

Quantitative real-time PCR

Experiments were performed according to MIQE guidelines (Bustin *et al.*, 2009). Briefly, total RNA was isolated from either

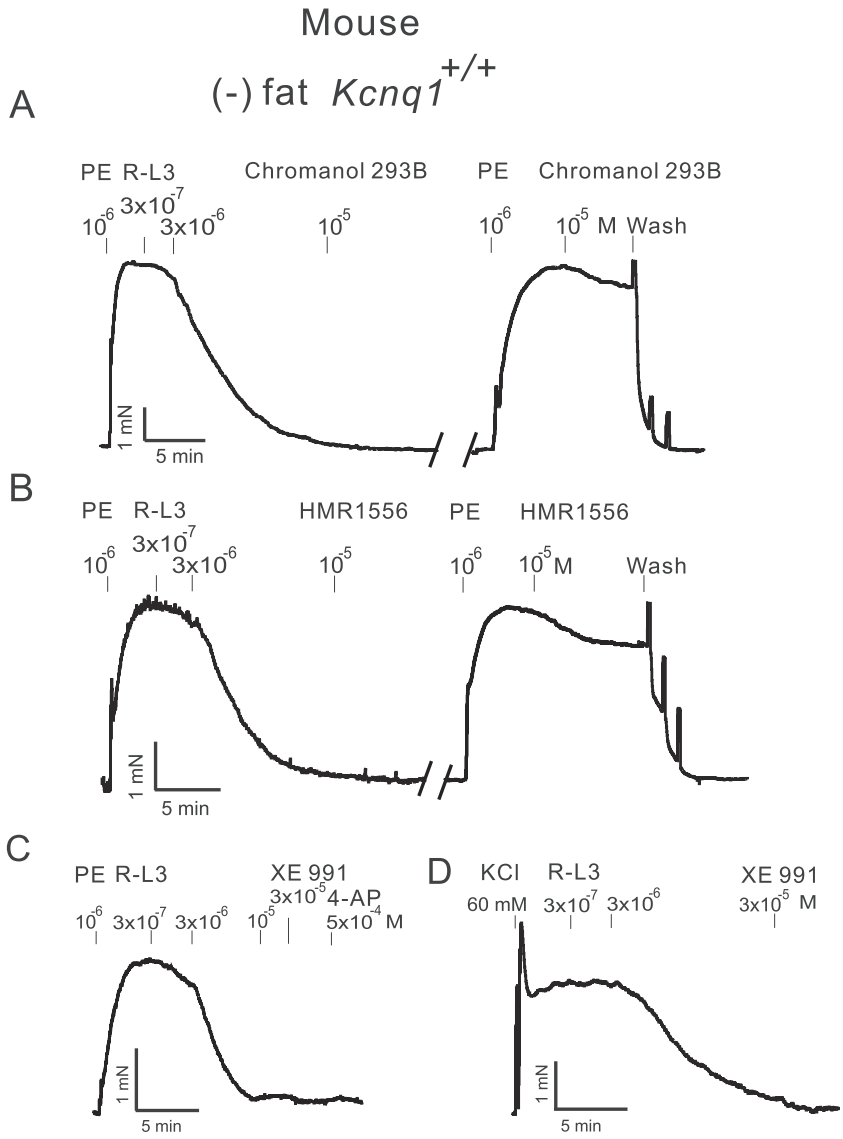

Figure 1

Original recordings showing the effects of 0.3-3 μM R-L3, 10 μM chromanol 293B, 10 μM HMR1556, 0.5 mM 4 aminopyridine (4-AP) and 10–30 μM XE991 on arterial tone of isolated mesenteric artery rings without PVAT (−) fat. Mesenteric arteries were either precontracted with 1 μM phenylephrine (PE) (A,B,C) or with 60 mM KCl (D). Effects of chromanol 293B (A) and HMR1556 (B) on arterial tone induced by PE. All vessels were from wild-type (*Kcnq1*$^{+/+}$) mice.

Kcnq1$^{+/+}$ or *Kcnq1*$^{-/-}$ mesenteric arteries (first branches) by using the RNeasy RNA isolation kit (Qiagen, Hamburg, Germany) according to the manufacturer's instruction. Isolated RNA concentration was measured and RNA quality was tested by NanoDrop-1000 spectrophotometer (PeqLab, Erlangen, Germany). For the synthesis of cDNA, equivalent amounts of RNA (2 μg) were used and processed by a TaqMan® Reverse Transcription Reagents (Life Technologies GmbH, Darmstadt, Germany, catalogue number: N8080234). Real-time PCR were done using 2.0 μL of cDNA in a total volume of 25 μL using the Faststart Universal SYBR green Master Mix (Roche, catalogue number: 04913850001). Experiments were run on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). Primers were designed using Primer 3 software on different exons to exclude any DNA contamination. Specificity of amplified products was validated *in silico* (BLAST) and empirically with gel electrophoresis and analysis of melt curves. Primers were synthesized by BioTez (Berlin, Germany); the sequences are provided below. The cycling conditions were the following: initial activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Samples and negative controls were run in parallel. The expression level of the target genes was normalized by the expression of 18 s. Under our experimental conditions, expression of 18 s as a reference gene did not differ between *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ tissues. The fold change in gene expression between *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ was calculated using 2 ΔΔCt method (Livak and Schmittgen, 2001). The following primers were used:

18s: F: 5′-ACATCCAAGGAAGGCAGCAG-3′; R: 5′-XTTTTCGTCACTACCTCCCCG-3′

Kcnq3: F: 5′-CAGTATTCGGCCGGACATCT-3′; R: 5′-GAGACTGCTGGGATGGGTAG-3′

Kcnq4: F: 5′-CACTTTGAGAAGCGCAGGAT-3′; R: 5′-CCAGGTGGCTGTCAAATAGG-3′

Kcnq5: F: 5′-CCTCACTACGGCTCAAGAGT-3′; R: 5′-TTAAGTGGTGGGGTGAGGTC-3′

Wire myography

Mesenteric or renal arteries were removed immediately after killing the mice with isoflurane anaesthesia, quickly transferred to cold (4°C), oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS) containing (in mmol L^{-1}) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, 1.6 $CaCl_2$ and dissected into 2 mm rings. The perivascular fat (PVAT) and connective tissue was either intact "(+) fat" or removed "(−) fat" from each ring without damaging adventitia. For experiments involving endothelium-denuded arteries, a 1 mL air bubble was used to disrupt the endothelium and removal of a functional endothelium was confirmed by absence of a vasodilatory response to 10 μM ACh. Each ring was positioned between two stainless steel wires (diameter 0.0394 mm) in a 5 mL organ bath of a Mulvany Small Vessel Myograph (DMT 610 M; Danish Myo Technology, Denmark). The organ bath was filled with PSS. The bath solution was continuously oxygenated with a gas mixture of 95% O_2 and 5% CO_2 and kept at 37°C (pH 7.4) (Fésüs *et al.*, 2007). The mesenteric and renal rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mm Hg by stepwise distending the vessel using LabChart DMT Normalization module. This normalization procedure was performed to obtain the passive diameter of the vessel at 100 mm Hg (Fésüs *et al.*, 2007). The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display. After 60 min, incubation arteries were pre-contracted either with isotonic external 60 mM KCl or 1–3 μM phenylephrine until a stable resting tension was acquired. The composition of 60 mM KCl (in mmol L^{-1}) was 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose and 1.6 $CaCl_2$. Drugs were added to the bath solution if not indicated otherwise. Tension is expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60 mM KCl.

Isolation of arterial VSMCs

VSMCs from mesenteric arteries were isolated as described (Gollasch *et al.*, 1998; Plüger *et al.*, 2000; Schleifenbaum *et al.*, 2014). Briefly, the first order of mesenteric or main renal arteries was removed and quickly transferred to cold (4°C) oxygenated (95% O_2–5% CO_2) PSS. The arteries were cleaned, cut into pieces and placed into a Ca^{2+}-free Hank's solution (mM): 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 $MgCl_2$, 1 mg·mL^{-1} BSA (Sigma, Taufkirchen), 10 glucose and 10 HEPES (pH 7.4 with NaOH) containing 0.5 mg·mL^{-1} papain (Sigma) and 1.0 mg·mL^{-1} DTT for 50 min at 37°C. The segments then were placed in Hank's solution containing 1 mg·mL^{-1} collagenase (Sigma, type F an H, ratio 30 and 70%) and 0.1 mM $CaCl_2$ for 10 min at 37°C. Following several washes in Ca^{2+}-free Hank's solution (containing 1 mg·mL^{-1} BSA), single cells were dispersed from artery segments by gentle triturating. Cells were then stored in the same solution at 4°C.

Electrophysiology

Voltage dependent potassium (K_V) currents were measured in the conventional whole-cell configuration of the patch-clamp technique at room temperature as previously described (Gollasch *et al.*, 1996; Essin *et al.*, 2007; Schleifenbaum *et al.*, 2014). Patch pipettes (resistance, 3–5 MΩ) were filled with a solution containing (in mM): 130 KCl, 1 $MgCl_2$, 3 Na2-ATP, 0.1 Na3-GTP, 10 HEPES and 5 EGTA (pH 7.2; Yeung and Greenwood, 2005). The external bath solution contained (in mM): 126 NaCl, 5 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 11 Glucose (pH 7.2; Yeung and Greenwood, 2005). Holding potential was −60 mV. Whole cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) or an EPC 7 amplifier (List, Darmstadt, Germany) and digitized at 5 kHz, using a Digidata 1440A digitizer (Axon CNS, Molecular Devices) and pClamp software versions 10.1 and 10.2.

Data and statistical analysis

These studies comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are presented as mean ± SEM. EC_{50} values were calculated using a Hill equation: $T = (B_0 - Be)/(1 + ([D]/EC_{50})^n) + Be$, where T is the tension in response to the drug (D); Be is the maximum response induced by the drug; B_0 is a constant; EC_{50} is the concentration of the drug that elicits a half-maximal response (Bychkov *et al.*, 1998). Curve fittings and data analysis were done by GraphPad6 (Software, La Jolla California USA) using nonlinear

regression. Statistical significance was determined by *t*-test or ANOVA with multiple comparison by Holm–Sidak method. The *post hoc* tests were run only if F achieved $P < 0.05$, and there was no statistical significant variance in homogeneity. Extra sum-of-squares F test was performed for comparison of concentration–response curves. *P* values <0.05 were considered statistically significant; *n* represents the number of animals used; data from multiple rings, multiple cells from the same animal were averaged and treated as a single *n*. The data analyst was blinded, whereas blinding for the operator was not possible due to the shaker-waltzer phenotype of mice (Video S8). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Materials

All salts and other chemicals were obtained from Sigma-Aldrich (Germany) or Merck (Germany). All drugs were freshly dissolved in the day of each experiment accordingly to the material sheet. When DMSO was used as solvent, maximal DMSO concentration after application did not exceed 0.5%. The following concentration of drugs were used: phenylephrine (Sigma Aldrich) ranged from 0.1 to 100 μM, retigabine (Valeant Research North America) from 0.01 to 100 μM, R-L3 (Merck) from 0.3 to 10 μM, ML277 (Sigma Aldrich) from 0.1 to 10 μM, 30 μM XE991 (Tocris), 10 μM chromanol 293B (Sigma Aldrich), 0.5 mM 4-aminopyridine (4-AP; Sigma Aldrich). KCNQ1/KCNE1 subunits are blocked by chromanol 293B with EC_{50} ~ 7 μM; whereas homomeric KCNQ2, KCNQ3, KCNQ4 and heteromeric KCNQ2/KCNQ3 channels are only slightly blocked by 100 μM (Lerche *et al.*, 2007). Chromanol 293B is also known as (3*R*,4*S*)-293B or 3*S*,4*R*-293B (Seebohm *et al.*, 2001). XE991 was found to inhibit either $K_V7.1$ homomeric or $K_V7.1$/KCNE channels with IC_{50} ~ 0.8 μM and 11.1 μM respectively (Wang *et al.*, 2000). As a pan KCNQ inhibitor, XE991 also inhibits KCNQ2/3 channels (EC_{50} ~ 1 μM) (Wang *et al.*, 1998), KCNQ4 (EC_{50} ~ 5.5 μM) (Søgaard *et al.*, 2001) and KCNQ5 (EC_{50} ~ 65 μM) (Schroeder *et al.*, 2000). 10 μM HMR1556 is known to inhibit 97% of I_{Ks} current (mainly composed of $K_V7.1$/KCNE channels) (Bosch *et al.*, 2003). ML277 is a potent activator of KCNQ1 with EC_{50} 260 nM and >100-fold selective versus KCNQ2 and KCNQ4 channels (Mattmann *et al.*, 2012). Retigabine is an activator of the KCNQ2/3 (EC_{50} 5.2 μM) (Main *et al.*, 2000) and KCNQ3/KCNQ5 channels (EC_{50} 1.4 μM) (Wickenden *et al.*, 2001).

Results

Failure of $K_V7.1$ channel blockers to reverse arterial relaxation induced by R-L3

First, we tested the vasodilatory effects of R-L3, which supposedly acts through activation of $K_V7.1$ channels. R-L3 produced concentration-dependent relaxation of murine mesenteric arteries pre-contracted with 1 μM phenylephrine (Figures 1A,B,C and 2A,B). Both chromanol 293B and HMR1556 ($K_V7.1$ channel blockers) on the contrary did not affect contractions induced by 1 μM phenylephrine (Figure 1A,B) (increase by 8.4 ± 4.3%, $n = 5$, $P > 0.05$, paired *t*-test; increase by −7.3 ± 3.8%, $n = 5$, $P > 0.05$, paired *t*-test, respectively). Neither 10 μM chromanol 293B (Figure 1A), 10 μM HMR1556 (Figure 1B), 30 μM XE991 nor 0.5 mM 4-AP (Figure 1C) reversed R-L3-induced relaxations of the murine mesenteric rings (but see Figure 3 for rat mesenteric arteries). Pretreatment of the murine mesenteric arteries with 10 μM chromanol 293B did not prevent relaxation by R-L3 (Figure S1A). Similar results were obtained in rat mesenteric arteries (Figure S1B). Interestingly, the resting tone of murine mesenteric arteries was not affected by $K_V7.1$ channel blockade, that is neither by 10 μM chromanol 293B (increase by −0.4 ± 0.2%, $n = 5$, $P > 0.05$, paired *t*-test) nor by 10 μM HMR1556 (increase by −0.2 ± 0.1%, $n = 5$, $P > 0.05$, paired *t*-test). Additionally and similarly to the mice, the resting tone of rat mesenteric arteries was not affected by $K_V7.1$ channel blockade with 10 μM chromanol 293B (increase by −0.5 ± 0.3%, $n = 5$, $P > 0.05$, paired *t*-test).

Next, we aimed to determine whether the R-L3 effects rely on K^+ channel activation through experiments using 60 mM KCl-induced contractions, which are largely determined by Ca^{2+} influx through L-type $Ca_v1.2$ channels (Moosmang *et al.*, 2005; Essin *et al.*, 2007). Importantly, R-L3 produced similar concentration-dependent relaxation in mesenteric rings regardless of whether they were pre-contracted with KCl or PE (Figures 1D and 2A). Similar results were also obtained in renal arteries (Figure S2). A total of 30 μM XE991 was unable to reverse R-L3-induced relaxations of rings pre-contracted with KCl (Figure 1D). R-L3 produced normal relaxations in arterial rings, from *Kcnq1*$^{-/-}$ mice and the EC_{50} values were ~1.4 μM in rings from both *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ mice (Figure 2A,B). The relaxant effects of R-L3 were independent of the endothelium (Figure S3A) (see also Figure S3B for similar results in rat mesenteric arteries). Our data suggests that R-L3, a supposed $K_V7.1$ channel activator, did not induce relaxations of murine mesenteric arteries through activation of $K_V7.1$ channels.

Effects of ML277, another activator of $K_V7.1$ channels

In order to better understand the possible contribution of $K_V7.1$ channels in vasoregulation, we studied the vasodilatory effects of ML277, a potent activator of $K_V7.1$ channels EC_{50}, (260 nM) (Mattmann *et al.*, 2012). Studies suggest that it changes the conformational dynamics of the $K_V7.1$ pore and/or global motions in the channel, including regions critical for K_V7 gating transitions (Xu *et al.*, 2015). In our experiments, ML277 induced relaxation of mesenteric arteries only at high concentrations, EC_{50} ~100 μM (Figure 2D, Figure S4). These effects were not different between arterial rings from wild-type and *Kcnq1*$^{-/-}$ mice and may have occurred due to activation of other K_V7 channels (Mattmann *et al.*, 2012).

Anti-contractile effects of PVAT

The PVAT produces endothelium-independent relaxation by opening VSMC K_V channels, which can be blocked by the pan K_V7 channel blocker XE991 and depends on the release of adipocyte-derived relaxing factor (ADRF) (Gollasch, 2012; Tano *et al.*, 2014). To test whether $K_V7.1$ channels play a role in these effects, we performed a series of experiments using $K_V7.1$ channel inhibitors and arteries from *Kcnq1*$^{+/+}$ and

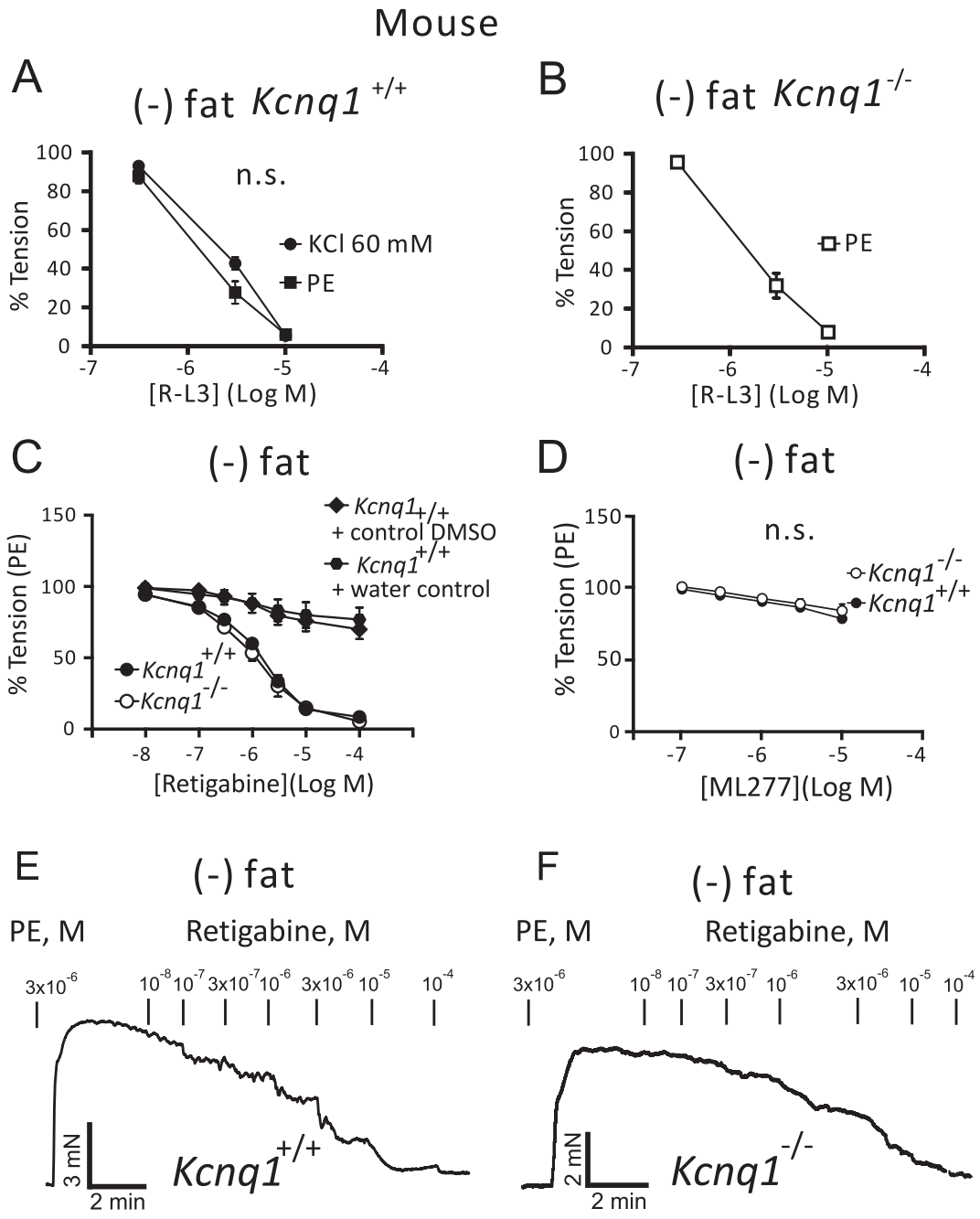

Figure 2

Relaxation of (−) fat mesenteric artery rings by 0.3-3 μM R-L3, 0.1-10 μM ML 277 or 0.01-10 μM retigabine. Vessels were isolated from wild-type (*Kcnq1*$^{+/+}$) mice (A) or *Kcnq1*$^{-/-}$ mice (B). Mesenteric arteries were precontracted by phenylephrine (PE) 1 μM (A,B) or KCl 60 mM (A). Representative traces showing relaxation induced by retigabine in rings from *Kcnq1*$^{+/+}$ (E) and *Kcnq1*$^{-/-}$ mice (F) and average values compared with vehicle (DMSO) and water controls (C). ML277 effects on rings from *Kcnq1*$^{-/-}$ and *Kcnq1*$^{+/+}$ mice (D). Tension is expressed as a percentage of KCl or PE-induced contractions. n = 5 per group.

Kcnq1$^{-/-}$ mice (Figure 4). Mesenteric arteries were prepared either (+) fat or (−) fat (with or without PVAT). Chromanol 293B (10 μM) did not diminish the anti-contractile effects of PVAT (Figure 4A). Also, HMR1556 (10 μM) had no effect on phenylephrine-induced contractions of both (−) fat rings and (+) fat rings isolated from *Kcnq1*$^{+/+}$ mice. Moreover, HMR1556 did not diminish the anti-contractile effects of PVAT (Figure 4B, see also Figure S5A,B for data analysis by curve fitting using nonlinear regression). Similarly, the anti-contractile effects of PVAT were normal in arteries isolated from *Kcnq1*$^{-/-}$ mice (Figure 3C, see also Figure S5C,D for data analysis by curve fitting using non-linear regression). Interestingly, the activator of K_V7.2–5 channels, retigabine, induced normal concentration-dependent relaxations of

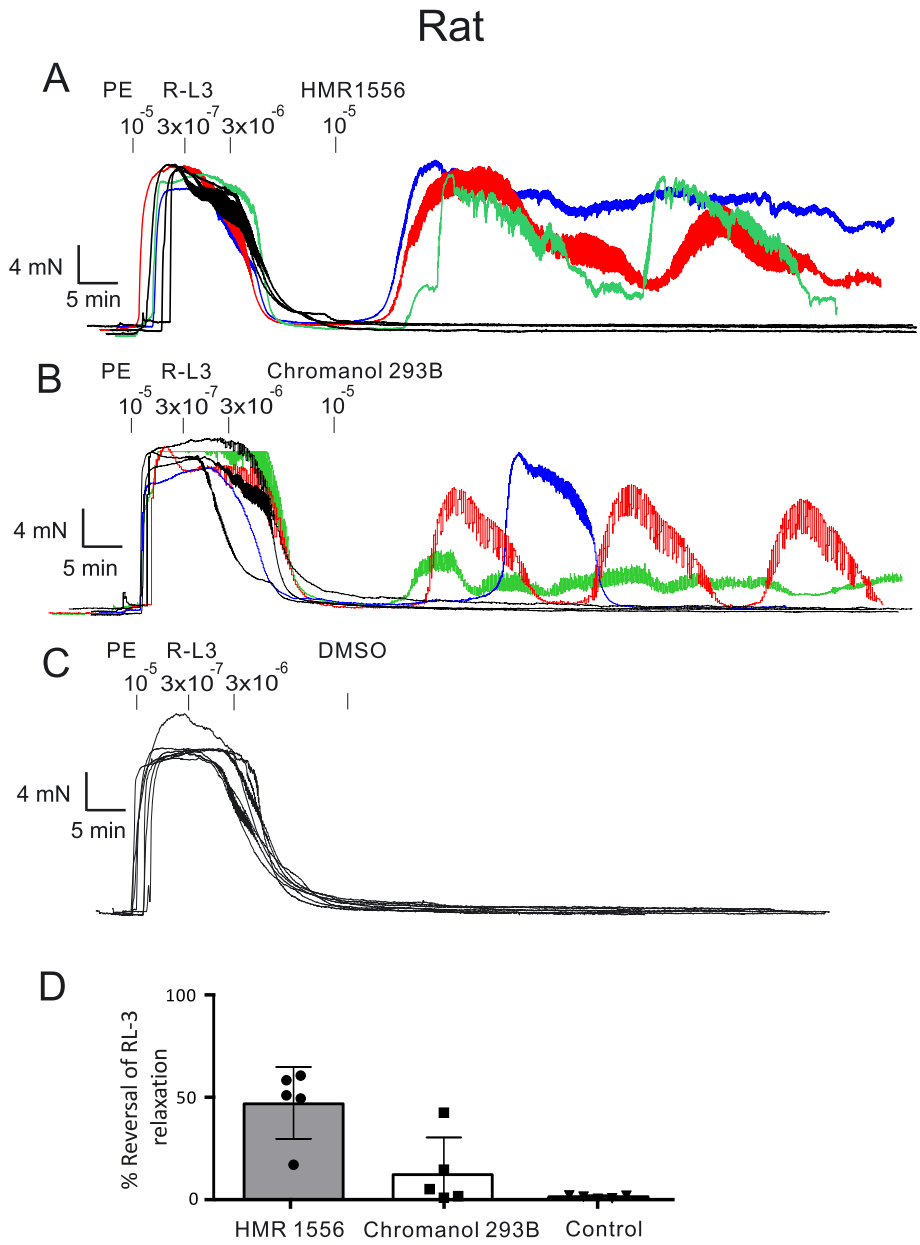

Figure 3

R-L3-induced relaxation of (−) fat mesenteric arteries from rats and subsequent exposure to K_V7.1 channel blockers 10 μM HMR 1556 (A), 10 μM chromanol 293B (B), vehicle control DMSO (C). Percentage reversal of R-L3-induced relaxation by HMR 1556, chromanol 293B (D). The reversal was caused by inducing long-lasting oscillatory contractions in ~50% of vessel rings. *n* = 5 per group.

Kcnq1$^{-/-}$ arteries (EC_{50} ~1 μM) (Figure 2C,E,F). Taken together, our data indicate that K_V7.1 channels did not contribute to the anti-contractile effects of PVAT in our arterial rings.

Electrophysiology

Consistent with the above data indicating that K_V7.1 channels do not contribute to resting vascular tone, we found that outward voltage-dependent K_V currents were normal in VSMCs isolated from *Kcnq1*$^{-/-}$ mesenteric arteries (Figure 5). There were no differences in normalized peak K_V currents (*Kcnq1*$^{+/+}$, 11.3 ± 1.5 pA/pF; *Kcnq1*$^{-/-}$, 13.5 ± 1.8 pA/pF, panel B), cell capacities (*Kcnq1*$^{+/+}$, 23.5 ± 1.8 pF; *Kcnq1*$^{-/-}$, 23.3 ± 1.5 pF, panel C) and block of K_V currents by XE991 (30 μM) (*Kcnq1*$^{+/+}$, 56.8 ± 12%; *Kcnq1*$^{-/-}$, 62.5 ± 9% panels D,E,F) between *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ cells. Moreover, we did not observe statistically significant differences in steady state current, fast and slow time constants of current activation at +20 and +40 mV [steady state current +20 mV: *Kcnq1*$^{+/+}$: 13.4 ± 1.7 pA/pF (*n* = 5); *Kcnq1*$^{-/-}$: 15.9 ± 1.6 pA/pF (*n* = 5); +40 mV: *Kcnq1*$^{+/+}$ 23.5 ± 3.1 pA/pF (*n* = 5); *Kcnq1*$^{-/-}$: 24.7 ± 3.3 pA/pF (*n* = 5); slow time constant +20 mV: *Kcnq1*$^{+/+}$: 70.7 ± 19.3 ms (*n* = 5); *Kcnq1*$^{-/-}$: 138.9 ± 55.6 ms (*n* = 5); +40 mV: *Kcnq1*$^{+/+}$: 51.2 ± 25.1. ms (*n* = 5); *Kcnq1*$^{-/-}$: 49.5 ± 15.9 ms (*n* = 5); fast time constant

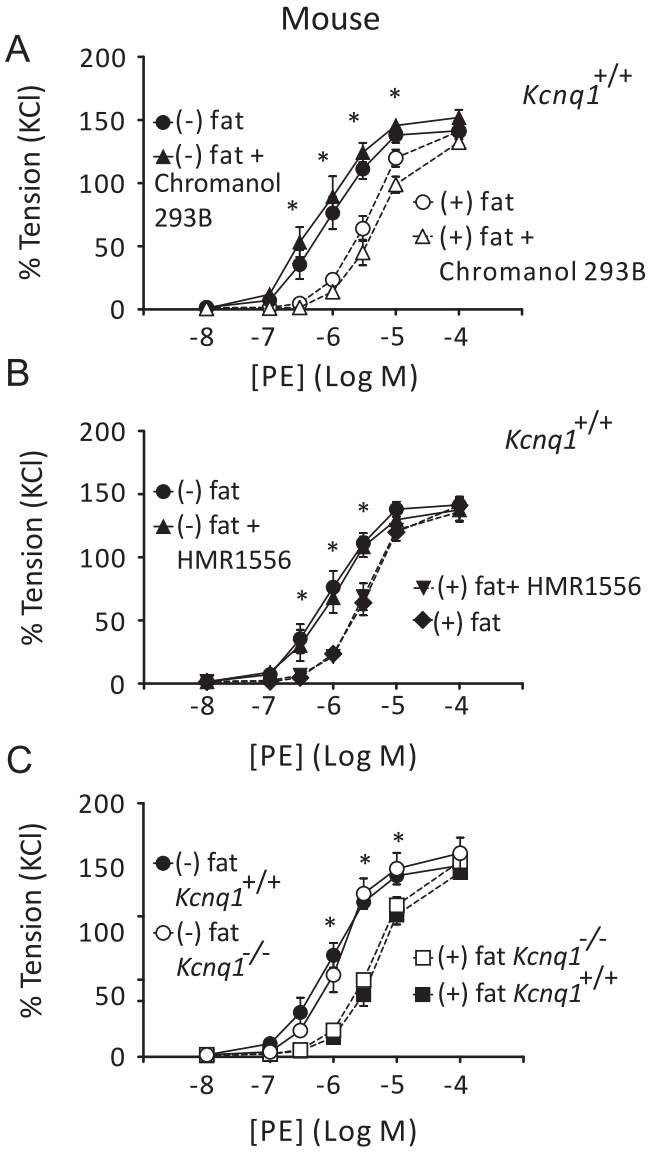

Figure 4

Effects of 10 μM chromanol 293B or 10 μM HMR1556 on dose–response relationships for phenylephrine (PE)-induced contractions of arterial mesenteric rings with [(+) fat] (n = 5 per group) and without (−) PVAT [(−) fat] (n = 5 per group) (A,B). Dose–response relationships for PE-induced contractions of (+) fat and (−) fat rings isolated from wild-type ($Kcnq1^{+/+}$) [(+) fat; (−) fat, n = 7 per group] and $Kcnq1^{-/-}$ mice [(+) fat; (−) fat, n = 7 per group] (C). Tension is expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60 mM KCl. *$P < 0.05$, significant difference between (−) fat and (+) fat rings.

+20 mV: $Kcnq1^{+/+}$: 45.1 ± 4.6 ms (n = 5); $Kcnq1^{-/-}$: 56.3 ± 13.0 ms (n = 5); +40 mV: $Kcnq1^{+/+}$: 23.3 ± 3.2 ms (n = 5); $Kcnq1^{-/-}$: 34.7 ± 5.2 ms (n = 5)].

Discussion

Our findings provide compelling evidence that K_V7.1 channels do not contribute to vascular contraction and to the anti-contractile effects of PVAT in mouse mesenteric arteries. We observed no functional role for K_V7.1 channels in R-L3-induced relaxations. R-L3-induced relaxations fully persisted after genomic deletion of *Kcnq1* and after pharmacological blockade of K_V7.1 channels by two compounds, namely HMR1556 or chromanol 293B. These conclusions were supported by findings obtained using another potent K_V7.1 channel opener, ML277. Our experiments rule out a major role for anticipated downstream targets of ADRF signalling in mesenteric vessels, namely the *Kcnq1* channel gene family. Instead, our genetic mouse model revealed an unappreciated role of R-L3 in antagonizing high KCl-induced L-type Ca_V1.2 channel-dependent vascular contractions. This relaxation occurred independent of the endothelium and opening of K^+ channels, including K_V7.1 channels. We thus conclude that R-L3 is an inappropriate pharmacological tool for studying native vascular K_V7.1 channels in mice.

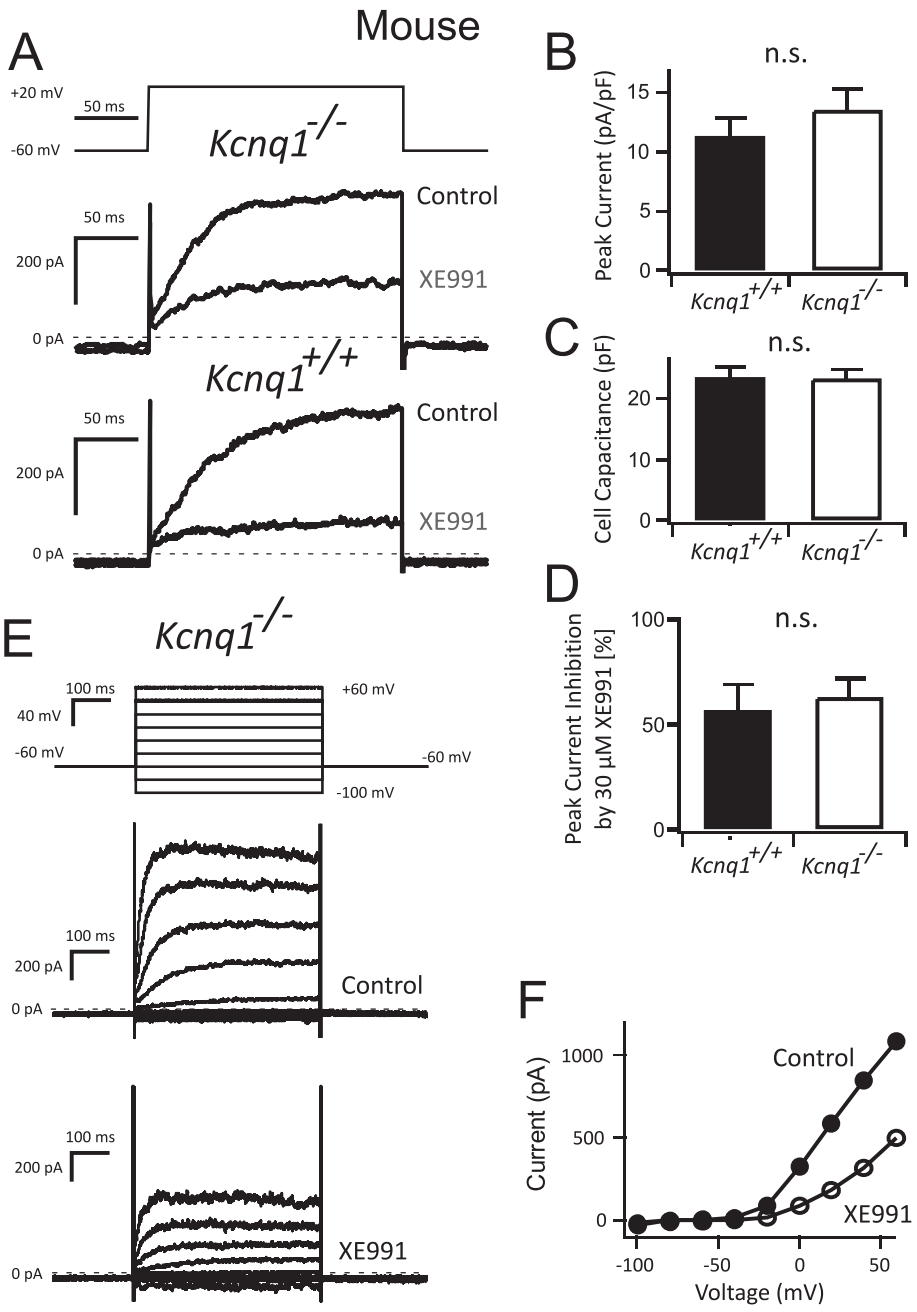

Figure 5

Voltage-dependent potassium K_V currents in freshly isolated *Kcnq1*$^{-/-}$ VSMCs. Original recordings of K_v currents in VSMCs from *Kcnq1*$^{-/-}$ and wild-type (WT) (*Kcnq1*$^{+/+}$) mice (A). Normalized peak K_v currents (B), cell capacitance (C) and relative inhibition of K_V currents by 30 μM XE991 (D). Original recordings of whole-cell K_V currents in a *Kcnq1*$^{-/-}$ VSMC in the absence (Control) and presence of 30 μM XE991 (E). Voltage clamp protocol included command voltage steps (500 ms) ranging from −100 to +60 mV in 20 mV increments. Holding potential was −60 mV; test pulse frequency was 1·20 s^{-1}. Current–voltage (I–V) relationship of the currents from (E) (F). n = 5 per group.

K_V7.1 channels and vascular tone

According to our experimental data obtained using chromanol 293B, HMR1556 and genomic deletion, the K_V7.1 isoform is unlikely involved in the control of vascular tone of murine mesenteric arteries. Based on the effects of putative K_V7.1 channel blockers (HMR1556 and L-7) in reversing R-L3-induced relaxation, Chadha *et al.* proposed that K_V7.1 channels can be functionally relevant in rat mesenteric arteries (Chadha *et al.*, 2012). These results were reproduced in this study (Figure 3), as the relaxant effect of R-L3 was reversed by the K_V7.1 channel blockers chromanol 293B and HMR1556 in rings from rat mesenteric arteries. However, since the observation periods in our experiments were longer than in Chadha *et al.*, we noticed that HMR1556 produced a

sustained reversal of R-L3-induced relaxations only in one out of six vascular rings. Instead, HMR1556 and chromanol 293B produced rather long-lasting oscillations. This phenomenon was observed only in ~50% of the vessels. In extension to the studies of Chadha *et al.*, we explored whether blockade of K_V7.1 channels (by HMR1556) could prevent R-L3 relaxations. Our results demonstrate that K_V7.1 channel blockade (chromanol 293B) failed to prevent R-L3 relaxations (Figure S1), raising doubts that R-L3 vasodilation in rat mesenteric arteries is indeed primarily caused by K_V7.1 channel openings. Finally, the K_V7.1 channel activator ML277 (EC_{50} 260 nM in cardiomyocytes) even at 10 μM, failed to induce relaxation in murine mesenteric arteries. Together, the data support the notion that stimulation of K_V7.1 channels cannot evoke profound relaxation in mesenteric arteries of mice.

Recent data suggest a major role of K_V family of K^+ channels as putative downstream targets of ADRF and possibly other relaxing factors released by PVAT (Schleifenbaum *et al.*, 2010; Tano *et al.*, 2014). This hypothesis is supported by the blockade of the anti-contractile effects of PVAT by XE991 in rat aortas (Köhn *et al.*, 2012). Similar to the aorta, smaller visceral and skeletal muscle arteries, which are important in the regulation of peripheral vascular resistance, rely on opening of XE991-sensitive K_V channels to mediate the paracrine effect of ADRF (Schleifenbaum *et al.*, 2010). In this study, we found that both chromanol 293B and HMR1556 did not affect the anti-contractile effects of PVAT. The anti-contractile effects of PVAT were normal in *Kcnq1*$^{-/-}$ vessels. We therefore conclude that K_V7.1 channels are not the putative downstream K^+ channel targets of ADRF that we seek. It is likely that the ADRF effects are mediated by opening of other K_V channels, for example K_V7.2–5 (Figure 6).

A limitation of our study is that we cannot unequivocally exclude protein overexpression of other K_V channels in *Kcnq1*$^{-/-}$ arteries to functionally compensate for the loss of *Kcnq1*. However, we found that the pan K_V7.2–5 channel activator retigabine (Yeung *et al.*, 2007) produced normal relaxations in *Kcnq1*$^{-/-}$ arteries. In addition, we also found similar expression of *Kcnq3*, *Kcnq4* and *Kcnq5* at the mRNA levels between *Kcnq1*$^{-/-}$ and *Kcnq1*$^{+/+}$ mesenteric arteries (Figure S6). *Kcnq2* mRNA is not detectable in this preparation, either in *Kcnq1*$^{+/+}$ (Schleifenbaum *et al.*, 2014) or in *Kcnq1*$^{-/-}$ (our data, not shown). These results argue against a significant up-regulation of K_V7.2–5 channels in these arteries. Furthermore, we found that K_V currents and their block by XE991 were normal in *Kcnq1*$^{-/-}$ VSMCs, which also argues against a contribution of functionally up-regulated K_V7.2–5 channels in the vessels of this mouse model. Moreover, another K_V7.1 channel inhibitor (L-735821) has been tested for inhibition of I_{Ks} current in the *Kcnq1*$^{-/-}$ mice used in our study (Knollmann *et al.*, 2004). Notably, L-735821 inhibited I_{Ks} and prolonged action potential duration in *Kcnq1*$^{+/+}$ but not *Kcnq1*$^{-/-}$ hearts. These data are also consistent with the idea that K_V7.2–5 channels are not up-regulated in mice deficient in K_V7.1 channels.

Specificity of R-L3

R-L3 has been used to study the functional role of K_V7.1 channels in various vascular beds and species (Seebohm *et al.*, 2003; Chadha *et al.*, 2012). The results indicate that K_V7.1 channels may play a functional role in mesenteric (Chadha

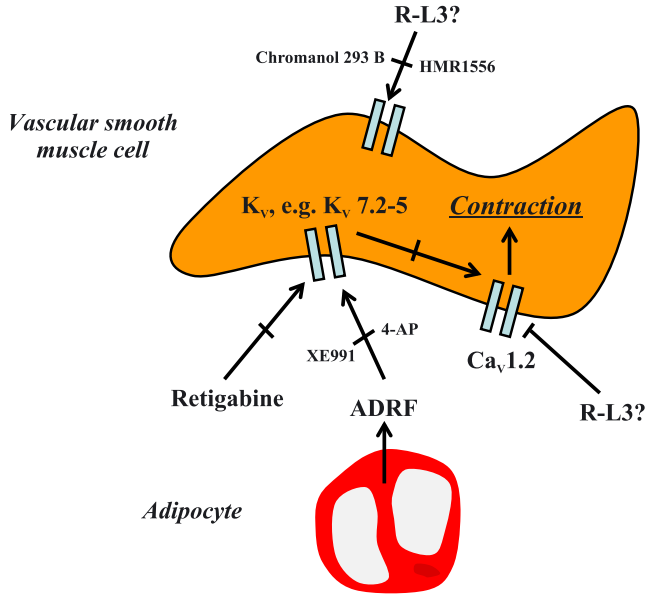

Figure 6

Schematic representation of voltage-dependent potassium (K_V) channels involved in vasoconstriction and ADRF-dependent relaxation. K^+ efflux through K_V7.1 channels does not seem to be involved in either process and the compound R-L3 is not specific for these channels in mice. L-type Ca_V1.2 channels play a dominant role in depolarization-induced contraction. The focus for future studies should be other K_V channels and the use of selective pharmacological agents to identify the channels involved in this process. 4-AP, 4-aminopyridine.

et al., 2012), but not coronary arteries of rats (Khanamiri *et al.*, 2013). However, it is worth stressing that results with R-L3 are difficult to interpret because the enantiomeric ratio of the drug can, at least in heart (Corici *et al.*, 2013), affect activation kinetics of native K_V7.1 channels. Furthermore, R-L3 has been suggested to be a potent inhibitor of native vascular L-type Ca_V1.2 channels in mouse aorta (Yeung *et al.*, 2007), but not in rat arteries (Chadha *et al.*, 2012). Our genetic model supports this idea for being particularly relevant in murine mesenteric arteries (Figure 6). Our conclusion is also based on similar cumulative response curves for R-L3 in both *Kcnq1*$^{-/-}$ and *Kcnq1*$^{+/+}$ arteries. Moreover, the relaxant effects of R-L3 were inhibited neither by chromanol 293B, HMR1556, XE991 nor by 4-AP. Furthermore, R-L3 produced similar concentration-dependent relaxation in mesenteric rings regardless of whether they were pre-contracted with phenylephrine or KCl. Of note, tamoxifen-induced smooth muscle-specific inactivation of the L-type Ca_V1.2 Ca^{2+} channel gene revealed a dominant role of these channels in KCl contractions (Moosmang *et al.*, 2005; Essin *et al.*, 2007). R-L3 is thus not suitable for the study of native vascular K_V7.1 channels in mice.

R-L3 may represent a more appropriate tool to study K_V7.1 channels in the rat vasculature (Figure 3). However, it is not clear why we observed R-L3 effects, which were inhibited by HMR1556 or chromanol 293B only in 50% of the arteries. One possibility is that the enantiomeric ratio of the drugs used could play an important role. It is also well

known that KCNE subunits influence the pharmacology of $K_V7.1$ channels (Melman *et al.,* 2002, 2004), which may explain drug, tissue and species differences *in vivo*. As R-L3 enantiomers have opposing effects on cardiac I_{Ks} (KCNQ1) currents (Corici *et al.,* 2013), $K_V7.x$ channel subunits can hetero-oligomerize, and KCNE subunits can influence the pharmacology of KCNQ channels (Melman *et al.,* 2002, 2004). Further studies should clarify the putative role of KCNE subunits/isoforms in enantiomeric enrichment of R-L3 enabling the stimulatory effects of R-L3 on native $K_V7.1$ channels *in vivo*. New studies are needed to clarify a possible contribution of $K_V7.1$ channels in producing oscillatory contractions in the rat vasculature and the specificity of KCNQ1 modulators in these effects.

Clinical relevance

One of the most dangerous potential side effects of a drug is its pro-arrhythmic action due to cardiac QTc prolongation, in particular by KCNQ1 channel inhibition. Recently, arterial KCNQ1 ($K_V7.1$) channels have attracted particular attention as putative novel drug targets for regulating arterial vascular tone and systemic blood pressure. Our genetic mouse model revealed novel insights into the specificity of pharmacological drugs commonly used to characterize KCNQ1 channel function *in vivo*. Our data indicate that $K_V7.1$ channels are apparently not involved in the control of mesenteric and renal arterial tone in mice. Our studies highlight the importance of KCNQ1 channels in playing a critical role in cardiac arrhythmias, such as long QT syndrome, with negligible impact on KCNQ1 in the peripheral arteries, at least in mice.

In conclusion, our study demonstrated that $K_V7.1$ channels were not required for the control of arterial tone by α_1-adrenoceptor agonists and PVAT in mesenteric and renal arteries of mice. Furthermore, R-L3, recently described as a specific activator of $K_V7.1$ channels, did not meet this criterion in our hands in the murine vasculature. R-L3-induced relaxations persisted after genomic deletion of *Kcnq1* and following pharmacological blockade by two $K_V7.1$ channel blockers, HMR1556 or chromanol 293B.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to M.G. and the Deutsche Akademische Austauschdienst (DAAD) to M.G. and D.T. We thank Dr Iain A. Greenwood for providing HMR1556. D.T. is recipient of the ERA/EDTA Fellowship and J.Y.T. is an Alexander von Humboldt Fellow. We thank Atakan Aydin and Lajos Markó for help and advice in RT-qPCR and Kornelia Buttke for expert technical assistance.

Author contributions

All authors planned and designed experimental studies. D.T. and C.L. performed the wire myography experiments. M.K. and J.Y.T. performed the electrophysiological experiments. D.T. and M.G. drafted the article, and all authors contributed to its completion.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://doi.org/10.1111/bph.13665

Figure S1 Pretreatment of (−) fat murine (panel A) and (−) fat rat (panel B) mesenteric arteries with 10 μM chromanol 293B [(+) chromanol 293B] and subsequent exposure to R-L3. (−) Chromanol 293B; control, non-treated vessels. n.s., $P > 0.05$. $n = 5$ per group.

Figure S2 Relaxation of (−) fat murine renal artery rings by R-L3. Vessels were isolated from wild-type (*Kcnq1* +/+) mice. Arteries were precontracted by 1 μM phenylephrine (PE) or 60 mM KCl. Tension is expressed as a percentage of KCl or PE induced contractions. *, $P < 0.05$. $n = 5$ per group.

Figure S3 Relaxation of endothelium-denuded murine (panel A) and rat (panel B) mesenteric artery rings by R-L3. (−) Endothelium: (−) fat, endothelium-denuded vessels; (+) Endothelium: (−) fat, endothelium-intact vessels. Arteries were precontracted by 1 μM phenylephrine (PE). Murine mesenteric arteries were isolated from *Kcnq1*$^{+/+}$ mice. Tension is expressed as a percentage of PE-induced contractions. n.s., $P > 0.05$. $n = 5$ per group.

Figure S4 Original traces showing relaxation induced by the Kv7.1 channel opener ML277 in (−) fat murine mesenteric artery rings isolated from *Kcnq1*$^{+/+}$ (panel A) and *Kcnq1*$^{-/-}$ mice (panel B) preconstricted with 1 μM phenylephrine.

Figure S5 Dose response curves and EC_{50} values for phenylephrine (PE)-induced contractions of (−) fat and (+) fat murine mesenteric artery rings isolated from *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ mice. Nonlinear regression model of dose–response curves to PE in the presence or absence of HMR1556 with or without fat ($n = 5$ per group) (panel A) and their corresponding EC50 values (panel B). Nonlinear regression model of dose–response curves to PE in *Kcnq1*$^{+/+}$ ((+) fat; (−) fat, $n = 7$ per group) and *Kcnq1*$^{-/-}$ arteries ((+) fat; (−) fat, $n = 7$ per group) (panel C) and their corresponding EC50 values (panel D). *, $P < 0.05$.

Figure S6 Relative expression of Kcnq3–5 channels at RNA levels in (−) fat mesenteric arteries from *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ mice normalized to *18 s*. Relative mRNA levels for *Kcnq3* (panel A) ($n = 6$ for *Kcnq1*$^{+/+}$; $n = 5$ for *Kcnq1*$^{-/-}$), Kcnq4 (panel B) ($n = 6$ for *Kcnq1*$^{+/+}$; $n = 6$ for Kcnq1−/−) and Kcnq5 expression (panel C) ($n = 5$ for *Kcnq1*$^{+/+}$; $n = 5$ for *Kcnq1*$^{-/-}$). n.s., $P > 0.05$, unpaired *t*-test.

Figure S7 PCR genotyping of *Kcnq1*$^{+/+}$ and *Kcnq1*$^{-/-}$ mice. Amplification by using the forward and reverse primers gives a 240-bp product specific to the wild-type (+/+) allele. Amplification by using the Neo forward and reverse primers gives a 370-bp product specific to the null allele (−/−) (Casimiro *et al.*, 2001).

Video S8 Demonstration of shaker-waltzer phenotype (hyperactivity, head shaking, and/or circling), due to abnormality of the vestibular apparatus in *Kcnq1*$^{-/-}$ mice. The video shows the typical behaviour of lack of $K_V7.1$ channel function in the mouse.