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RESEARCH PAPER

Pharmacological dissection of $K_v7.1$ channels in systemic and pulmonary arteries

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BACKGROUND AND PURPOSE

The aim of this study was to characterize the functional impact of KCNQ1-encoded voltage-dependent potassium channels ($K_v7.1$) in the vasculature.

EXPERIMENTAL APPROACH

Mesenteric arteries, intrapulmonary arteries and thoracic aortae were isolated from adult rats. $K_v7.1$ channel expression was established by fluorescence immunocytochemistry. Wire myography determined functionality of these channels in response to selective blockers and activators. *Xenopus* oocytes expressing $K_v7.1$ channels were used to assess the effectiveness of selective $K_v7.1$ channel blockers.

KEY RESULTS

$K_v7.1$ channels were identified in arterial myocytes by immunocytochemistry. $K_v7.1$ blockers HMR1556, L-768,673 (10 μM) and JNJ39490282 (JNJ282; 1 μM) had no contractile effects in arteries, whereas the pan-K_v7 channel blocker linopirdine (10 μM) evoked robust contractions. Application of two compounds purported to activate $K_v7.1$ channels, L-364 373 (R-L3) and mefenamic acid, relaxed mesenteric arteries preconstricted by methoxamine. These responses were reversed by HMR1556 or L-768,673 but not JNJ282. Similar effects were observed in the thoracic aorta and intrapulmonary arteries.

CONCLUSIONS AND IMPLICATIONS

In contrast to previous assumptions, $K_v7.1$ channels expressed in arterial myocytes are functional ion channels. Although these channels do not appear to contribute to resting vascular tone, $K_v7.1$ activators were effective vasorelaxants.

Abbreviations

HMR1556, N-(6-cyano-3-hydroxy-2,2-dimethyl-3,4-dihydrochromen-4-yl)-N-methylethanesulfonamide; JNJ39490282, 2-(2-bromo-4-chlorophenoxy)-2-methyl-N-{5 [(methylsulfonyl)amino]tricyclo[3.3.1.13,7]dec-2-yl}propanamide; L-768, 6732-[2,4-bis(trifluoromethyl)phenyl]-N-[(3R)-2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-3H-1,4-benzodiazepin-3-yl]acetamide; RL-3, [(3-R)-1, 3-dihydro-5-(2-fluorophenyl)-3-(1H-indol-3-ylmethyl)-1-methyl-2H- 1,4-benzodiazepin-2-one]

Introduction

KCNQ1 is a member of the KCNQ gene family (1–5) that encode for the pore-forming subunits of voltage-dependent potassium (K^+) channels (termed $K_v7.1$–7.5). KCNQ1 is expressed abundantly in the heart and epithelia where it has well-established roles. In the heart, $K_v7.1$ channels co-assemble with the protein product of KCNE1 (*minK*)

(Barhanin *et al.*, 1996; Kang *et al.*, 2008) to constitute the slow component of the delayed rectifier K^+ current (IKs; reviewed, Jespersen *et al.*, 2005). Mutation of KCNQ1 and KCNE1 underlie a large number of hereditary arrhythmias leading to long QT syndrome (Herbert *et al.*, 2002; Jespersen *et al.*, 2005). In colonic epithelia, $K_v7.1$ associates with the protein encoded by KCNE3 to form a constitutively active channel that regulates fluid flux (Schroeder *et al.*, 2000). More recently, KCNQ1 expression has also been shown to be abundant in rodent and human vascular smooth muscle cells along with KCNQ4 and KCNQ5 (Ohya *et al.*, 2003; Yeung *et al.*, 2007; 2008; Greenwood and Ohya, 2009; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Ng *et al.*, 2011), but in contrast to its well-defined role highlighted earlier in non-vascular tissues, the functional impact of $K_v7.1$ in blood vessels remains enigmatic.

XE991 and linopirdine, which block all K_v7 channel isoforms, cause smooth muscle depolarization and contraction of rodent and human arteries (Yeung *et al.*, 2007; 2008; Mackie *et al.*, 2008; Joshi *et al.*, 2009; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Ng *et al.*, 2011). However, in no smooth muscle study has chromanol 293B, a preferential $K_v7.1$ blocker (Bett *et al.*, 2006; Lerche *et al.*, 2007), been shown to mimic the effects of linopirdine or XE991. In addition, activation of K_v7 channels by compounds such as retigabine and the acrylamide S-1 have been shown to hyperpolarize smooth muscle cells and cause relaxation of blood vessels (Yeung *et al.*, 2007; 2008; Mackie *et al.*, 2008; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Ng *et al.*, 2011). These effects are mediated through $K_v7.2$–7.5 channels but not $K_v7.1$, which lacks the tryptophan residue in the fifth transmembrane domain that is required for the actions of these drugs (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005; Bentzen *et al.*, 2006). The lack of efficacy of chromanol 293B and the $K_v7.1$-independent effects of K_v7 channel activators has led to the assumption that $K_v7.1$ channels in blood vessels are most likely redundant and do not influence vascular tone. However, this conclusion relies heavily on the selectivity and responses of one agent, chromanol 293B, which may affect other ion channels. Indeed, the obvious expression of $K_v7.1$ channels in vascular tissue (Yeung *et al.*, 2007; 2008; Mackie *et al.*, 2008; Zhong *et al.*, 2010; Jepps *et al.*, 2011) as well as their critical function in cardiac myocytes leads to persisting questions regarding their role in smooth muscle. To address this issue, in the present study we utilized three structurally different IKs blockers [HMR1556, L-768,673 and JNJ39490282 (abbreviated to JNJ282)] that have greater potency than chromanol 293B (IC_{50}: 74, 6 and 1 nM for HMR1556, L-768,673 and JNJ282, respectively, compared with 6.9 μM for chromanol 293B) (Lerche *et al.*, 2000; 2007; Towart *et al.*, 2009), to confirm the impact of $K_v7.1$ channels on vascular tone. In addition, the effectiveness of activating $K_v7.1$ channels as an anti-contractile mechanism was assessed using two identified $K_v7.1$ activators, mefenamic acid (Busch *et al.*, 1994; 1997) and L-364373 (abbreviated to R-L3) (Salata *et al.*, 1998; Seebohm *et al.*, 2003).

Methods

All genes are shown as KCNQ and all proteins as K_V7 in agreement with the nomenclature in Alexander *et al.* (2011).

Tissue isolation

In accordance with the UK Animal (Scientific Procedures) Act 1986, male Wistar rats (200–225 g) (Charles River UK Ltd., Kent, U.K.), were killed by cervical dislocation. Thoracic aorta, third-order mesenteric arteries and intrapulmonary arteries were isolated and cleaned of all fat and connective tissue.

Isolation of arterial myocytes

Mesenteric artery and thoracic aorta smooth muscle cells were isolated as previously described (Yeung *et al.*, 2008; Jepps *et al.*, 2011). Briefly, third-order branches of mesenteric artery were cleaned of adherent connective tissue and transferred to Ca^{2+}-free physiological saline solution (PSS, in mM: 6.0 KCl, 120 NaCl, 1.2 $MgCl_2$, 10.0 D-glucose and 10.0 HEPES, pH was adjusted to 7.3 with NaOH), supplemented with protease type X (0.5 $mg{\cdot}mL^{-1}$) and collagenase type IA (1.5 $mg{\cdot}mL^{-1}$), and incubated at 37 °C for 15 min. The thoracic aorta was cleaned of adherent connective tissue, cut into strips and transferred to Ca^{2+}-free PSS supplemented with BSA (10 $mg{\cdot}mL^{-1}$), DTT (10 $mg{\cdot}mL^{-1}$) and papain (5 $mg{\cdot}mL^{-1}$). The tissue was incubated for 20 min at 37°C before being transferred to Ca^{2+}-free PSS supplemented with BSA (10 $mg{\cdot}mL^{-1}$), and collagenase type IA (10 $mg{\cdot}mL^{-1}$) and incubated for a further 20 min at 37°C. Single cells were obtained by gentle agitation with a wide-bore pipette in PSS containing 0.1 mM Ca^{2+}. The cells were gently spun down, the supernatant removed and replaced with 0.75 mM Ca^{2+} PSS, transferred to glass coverslips and kept for 20 min at room temperature to allow adherence of the cells.

Immunocytochemistry

Similar to previous experiments (Davis *et al.*, 2010), enzymatically isolated smooth muscle cells were fixed in 4% paraformaldehyde solution for 1 min at room temperature and then permeablized for 5 min with antibody diluent (PBS containing 0.1% Triton X-100). Cells were subsequently co-incubated with anti $K_v7.1$ (1:50; AB5932 Millipore, Watford, UK) and anti sodium potassium ATPase (1:100; AB7671 Abcam Ltd., Cambridge, UK) antibodies in antibody diluent overnight in a humidified chamber at 4°C. For control experiments, primary antibodies were omitted. The following day, cells were incubated with TRITC donkey anti-rabbit IgG (1:100; 711-025-152 Jackson ImmunoResearch Europe Ltd., Suffolk, UK) and FITC sheep anti-mouse IgG (1:100; 515-095-062 Jackson ImmunoResearch Europe Ltd.) for 60 min at room temperature. Unbound secondary antibody was removed, and cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Single cells were imaged using a laser scanning confocal microscope (model LSM 510, Zeiss, Welwyn Garden City, UK). Images for each cell type were gain-matched to ensure accuracy between samples. The $K_V7.1$ antibody had been validated by Western blot using KCNQ1 knockout mice (Knollmann *et al.*, 2007).

Isometric tension myography

Segments (~2 mm) of artery were mounted in a Mulvany-Halpern myograph (Danish Myo Technology, Aarhus, Denmark) containing Krebs solution (in mM: 125 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 15.4 $NaHCO_3$, 1 Na_2HPO_4, 0.6 $MgSO_4$, 10

D-glucose) constantly aerated with 95% O_2/5% CO_2 at 37°C. Following a 30-min equilibration period, arteries were placed under a tension equivalent to 90% of the diameter of the vessel at a distending pressure of 100 mmHg (thoracic aorta and mesenteric artery) or 20 mmHg (intrapulmonary artery). After a further equilibration period, vessels were exposed to an external solution containing 60 mM KCl to assess vessel viability. Measurement of contractions and relaxations to K_v7 blockers and activators were made following a plateau in response. Changes in arterial tension were recorded continuously on Chart (version 5, ADInstruments) in conjunction with PowerLab (ADInstruments, Oxford, UK).

KCNQ overexpression in Xenopus laevis *oocytes*

Human KCNQ1, KCNQ4 and KCNQ5 constructs were subcloned into the pTLN expression vector; capped RNA was transcribed using SP6 RNA polymerase in mMessage mMachine kit (Ambion, Austin, TX, USA) after linearization of the cDNA with H*paI*. Individual stage V–VI oocytes were injected with KCNQ1, KCNQ4 and KCNQ5 encoding cRNA. Water-injected oocytes acted as controls. Oocytes were then kept at 17°C in ND96 solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH was adjusted to 7.4). The oocytes were purchased from EcoCyte Bioscience (Castrop-Rauxel, Germany).

Two-electrode voltage clamp electrophysiology

Two to three days after oocyte injection, two-electrode voltage clamp measurements were performed using an npi Turbotec amplifier (npi electronics, Tamm, Germany) and pClamp9 software (Axon Instruments, Sunnyvale, CA, USA). Oocytes were held at –80 mV and stepped to potentials between –80 mV and +40 mV for 2 s followed by repolarization to –40 mV. The effects of L-768,673, HMR1556 and JNJ282 (at 0.1, 1, 10 μM or DMSO equivalent in ND96) and R-L3 (at 1, 5 and 10 μM) were examined. All experiments were conducted at room temperature (approximately 22°C).

Drugs and solutions

All chemicals and drugs were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) and L-364 373 (R-L3) were obtained from Tocris (Bristol, UK). L-768,673, HMR1556, and JNJ39490282 were supplied by Janssen Pharmaceuticals. All drugs were prepared in either DMSO or distilled water.

Statistics

All responses are expressed as mean ± SEM, where *n* equals the number of rats from which arteries were taken. Where appropriate, multiple comparisons were performed by one-way ANOVA followed by Dunnett's *post* test. Changes in current of KCNQ overexpressed oocytes were determined by Student's paired *t*-test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Expression of K_v7.1 channels

While KCNQ1 transcripts have been identified in rat aorta, mesenteric artery and pulmonary artery (Mackie *et al.*, 2008; Joshi *et al.*, 2009; Jepps *et al.*, 2011) the only protein studies on K_v7.1 have been performed on rat cerebral artery (Zhong *et al.*, 2010) or murine aorta and portal vein (Yeung *et al.*, 2007; 2008). Single cell immunocytochemistry on smooth muscle cells isolated from rat mesenteric artery and thoracic aorta showed significant expression of K_v7.1 protein. In addition, co-staining with the membrane marker sodium potassium ATPase suggested that considerable K_v7.1 expression was localized to the plasma membrane (Figure 1).

Effects of K_v7 channel blockers on vascular tone

Previous studies have demonstrated that linopirdine and XE991, which block all types of K_v7 channel, evoke contractile responses in various rodent and human arterial preparations (Joshi *et al.*, 2006; Yeung *et al.*, 2007; Mackie *et al.*, 2008; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Ng *et al.*, 2011). In the present study, using tension myography, 10 μM linopirdine did not significantly contract mesenteric artery under basal conditions but in the presence of minimal methoxamine-induced pre-tone (<10% KCl response), linopirdine caused a contraction similar to KCl (60 mM) responses (mean contraction was 107.1 ± 24.0%, $n = 3$; $P < 0.01$). In contrast, none of the selective K_v7.1 blockers, at concentrations that produce considerable block of K^+ currents produced by the overexpression of KCNQ1 (10 μM L-768,673, 10 μM HMR1556, 1 μM JNJ282) (Towart *et al.*, 2009 and present study), produced any contraction of mesenteric arteries significantly different from the vehicle control (Figure 2A and B; Table 1). In addition, 10 μM L-768,673 failed to contract mesenteric vessels following pretreatment with 20 mM KCl (0.5 ± 0.8% relative to 60 mM KCl contraction; $n = 3$). It is worth highlighting that L-768,673, HMR1556 and JNJ282 at the concentrations used did not alter contractions evoked by 60 mM KCl (data not shown) indicating that the lack of effect of these compounds was not due to a direct effect on voltage-dependent calcium channels.

Application of linopirdine (10 μM) to segments of thoracic aorta caused robust contractions (approximately 130% of the KCl contraction; $P < 0.01$) but none of the K_v7.1 blockers had any effect on basal tone (Figure 2C and D). Intrapulmonary arteries have been described previously as preferential targets for K_v7 blockade compared with systemic arteries (Joshi *et al.*, 2009). However in this study, 10 μM linopirdine had minimal contractile effects in the absence of any pre-tone but, like the mesenteric arteries, linopirdine evoked a substantial contraction (~45% of KCl response; $P < 0.01$) when a low concentration of U46619 was applied. Similar to the aorta and mesenteric artery the K_v7.1 blockers did not contract intrapulmonary vessels under the same conditions where linopirdine produced a robust response (Figure 2E and F; Table 1).

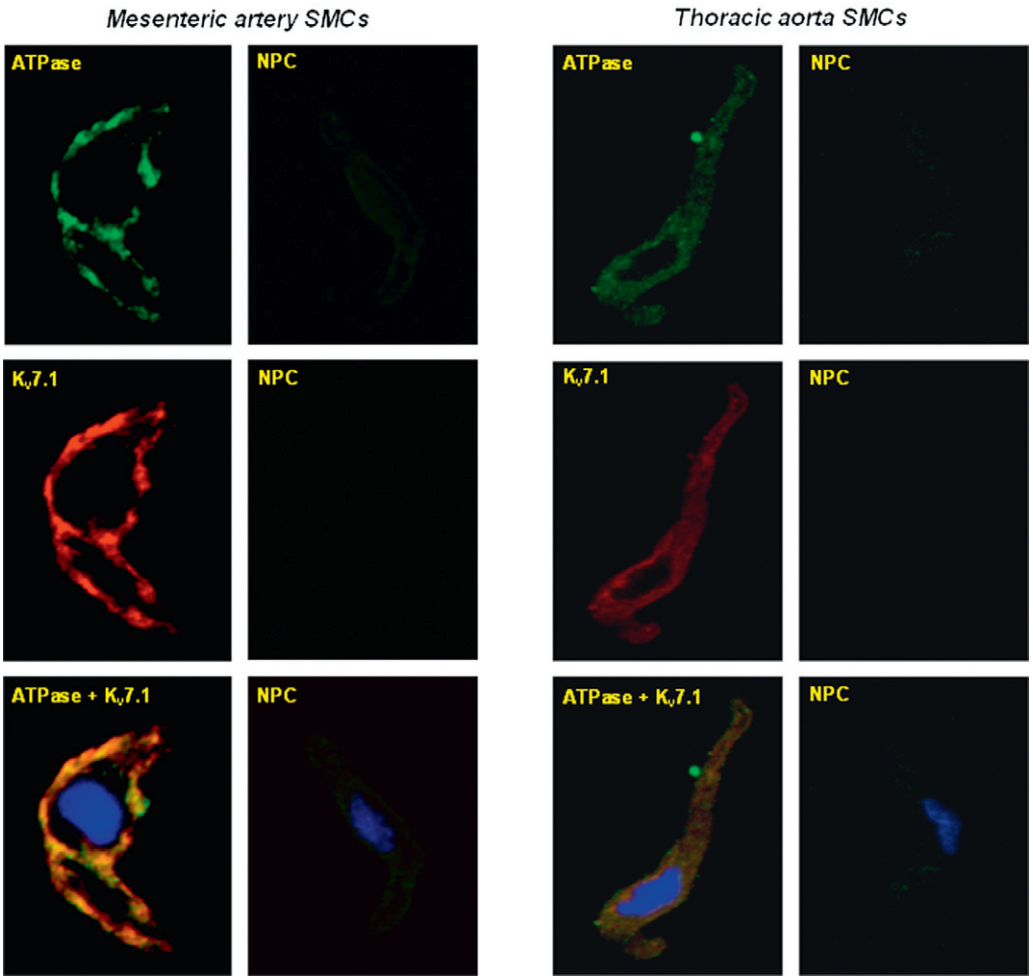

Figure 1

Identification of $K_v7.1$ protein in arterial myocytes. Representative fluorescence images from enzymatically isolated rat mesenteric artery and thoracic aorta SMCs co-incubated with the membrane marker anti sodium potassium ATPase (1:100; Abcam AB7671) and anti $K_v7.1$ (1:50 Millipore AB5932) or secondary antibodies only. Overlayed fluorescence image of sodium potassium ATPase, $K_v7.1$ and nucleus (DAPI) staining (bottom panels) indicates co-expression of $K_v7.1$ protein and sodium potassium ATPase. Fluorescence immunocytochemistry reveals expression of $K_v7.1$ protein in the plasma membrane of all tissues tested compared with minimal fluorescence in control cells. All images were time and gain matched. ATPase refers to sodium potassium ATPase. SMC, smooth muscle cell; NPC, no primary control.

Vasorelaxant effects of R-L3 and mefenamic acid

Having established that compounds that block either $K_v7.1$ or $K_v7.1$/KCNE1-encoded protein complexes (IKs) do not alter basal tension in mesenteric arteries, we investigated whether R-L3 and mefenamic acid, which augment $K_v7.1$ activity (Busch *et al.*, 1997; Seebohm *et al.*, 2003), had any functional impact. Application of the benzodiazepine R-L3 (0.1–3 μM) to segments of preconstricted mesenteric arteries produced concentration-dependent relaxations (pEC_{50}: 6.3 ± 0.4; E_{max}: 83.4 ± 3.3%, $n = 7$) (Figure 3A and B). This relaxant effect was fully reversed by the $K_v7.1$ selective blockers L-768,673 and HMR1556 but not JNJ282 (Figure 3C) and was not affected by the removal of the endothelium (confirmed by lack of response to carbachol) with a mean relaxation of 88 ± 1% ($n = 3$) to 3 μM RL-3 under these conditions. It is worth highlighting that the responses to S-1, an agent that activates $K_v7.2$–7.5 but not $K_v7.1$ (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005) and which has been shown previously to relax rat aorta and mesenteric artery (Jepps *et al.*, 2011) were not effected by the $K_v7.1$ inhibitors. L-768,673 did not reverse relaxations (3.5 ± 4.5%; $n = 3$) evoked by 10 μM S-1 (Figure 3D), while tone generated in the presence of the $K_v7.1$ blockers was fully relaxed by 10 μM S-1 ($n = 4$, data not shown). Similar to the mesenteric artery, R-L3 (0.1–3 μM) caused concentration-dependent relaxation in the aorta (pEC_{50}: 5.5 ± 0.2; E_{max}: 53.9 ± 7.5, $n = 8$; Figure 4A and B) and intrapulmonary arteries (pEC_{50}: 5.5 ± 0.2; E_{max}: 41.5 ± 3.5%, $n = 10$; Figure 4C and D; Table 1), yet these responses were not as potent as those observed in the mesenteric arteries.

In order to confirm the functional effect of activating $K_v7.1$ channels, an alternative $K_v7.1$ activator mefenamic acid was applied to preconstricted mesenteric arteries. Similar to RL-3, mefenamic acid (1–30 μM) relaxed these arteries in a concentration-dependent manner (E_{max}: 96.1 ± 0.8%, $n = 3$),

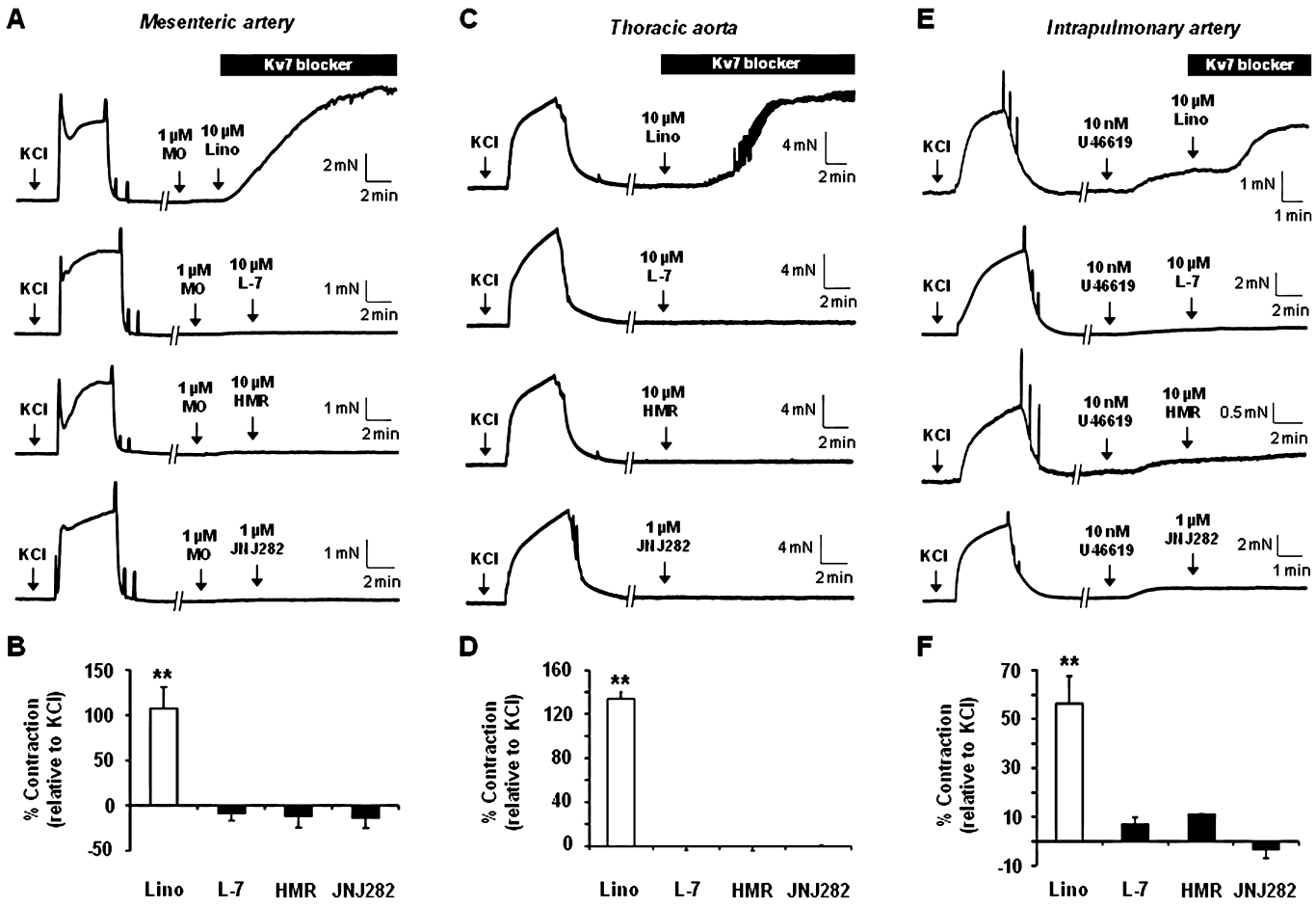

Figure 2

Effect of K_v7 blockers on arterial tone. Representative and mean smooth muscle contractions in response to 10 μM linopirdine or K_v7.1 blockers, 10 μM L-768,673 (L-7), 10 μM HMR1556 (HMR) and 1 μM JNJ282, in mesenteric arteries (A and B), thoracic aorta (C and D) and intrapulmonary arteries (E and F). It should be noted that mesenteric and intrapulmonary arteries were minimally preconstricted using methoxamine (MO) or U46619, respectively, before exposure to K_v7 blockers. $^{**}P < 0.01$ compared with tone in the absence of any K_v7 blockers using Dunnet's post hoc test following ANOVA ($P = 0.001$).

Table 1

Effect of K_v7 modulators in rat arterial preparations

	Mesenteric artery		Thoracic aorta		Intrapulmonary artery	
	Contraction (% of 60 mM KCl response)	% Reversal of RL-3 relaxation	Contraction (% of 60 mM KCl response)	% Reversal of RL-3 relaxation	Contraction (% of 60 mM KCl response)	% Reversal of RL-3 relaxation
Linopirdine	107.1 ± 24.0	–	133.6 ± 6.1	–	56.3 ± 11.3	–
L-768,673	–8.8 ± 7.6	106.8 ± 2.6	0.0 ± 0.0	71.2 ± 28.8	7.1 ± 2.8	52.6 ± 11.2
HMR1556	–12.1 ± 12.1	104.2 ± 5.9	0.0 ± 0.002	47.3 ± 20.6	11.1 ± 0.0	66.7 ± 33.3
JNJ282	–13.7 ± 10.3	–7.6 ± 5.5	0.2 ± 0.5	–	–3.3 ± 3.3	–
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
RL-3	6.3 ± 0.4	83.4 ± 3.3	5.4 ± 0.2	53.9 ± 7.5	5.5 ± 0.2	41.5 ± 3.5

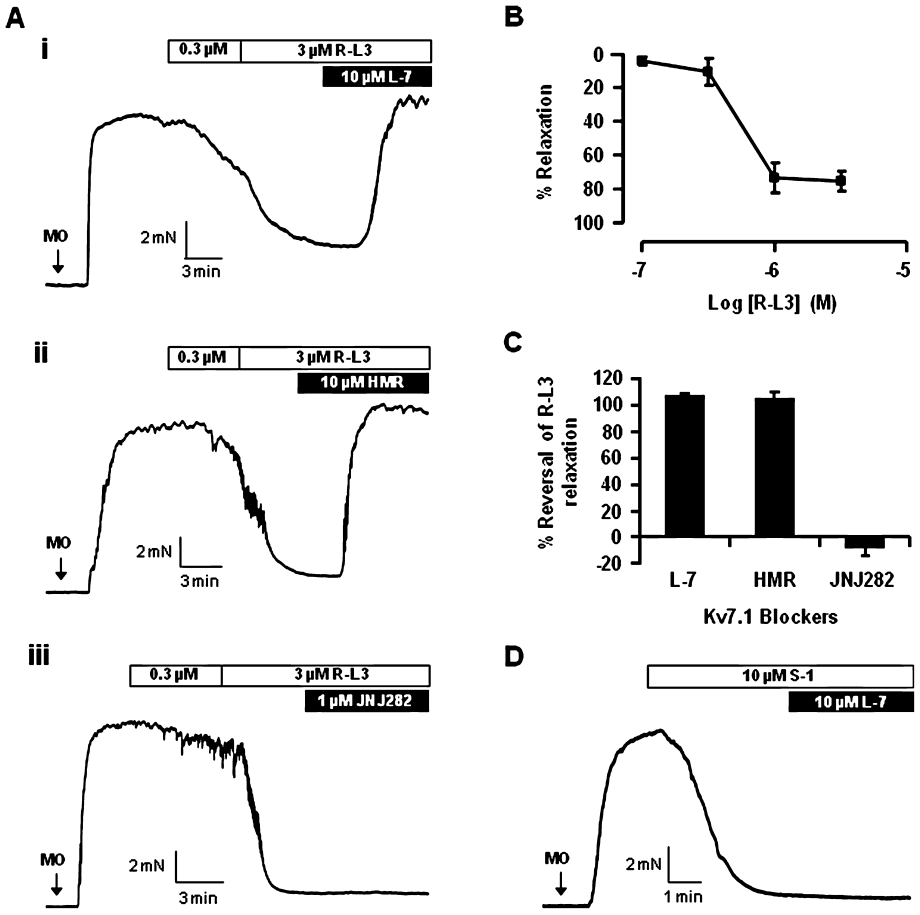

Figure 3

R-L3 evoked relaxation in mesenteric artery. Representative responses to R-L3 and subsequent exposure to $K_v7.1$ blockers, 10 μM L-768,673 (L-7; i), 10 μM HMR1556 (HMR; ii) or 1 μM JNJ282 (iii), in mesenteric arteries preconstricted with 10 μM methoxamine (A). Mean R-L3 concentration-dependent relaxation (B) and percentage reversal by $K_v7.1$ blockers (C). Representative relaxation to 10 μM S-1 in mesenteric arteries preconstricted with 10 μM methoxamine (MO) followed by subsequent exposure to 10 μM L-768,673 (L-7) is shown in (D).

which was reversed by 10 μM L-768,673 (Figure 5). These data are the first to show vascular effects of $K_v7.1$ stimulants and illustrates that both L-768,673 and HMR1556 were functionally active. Moreover, it provides strong evidence that $K_v7.1$-containing channel complexes are functionally relevant in the vasculature.

Effect of $K_v7.1$ channel blockers on heterologously expressed KCNQ channels

Previous studies have shown L-768,673, HMR1556 and JNJ282 to be effective blockers of the current formed by the association of $K_v7.1$ with KCNE1-encoded proteins (termed IKs); the prominent repolarizing current in the heart (Seebohm *et al.*, 2003; Towart *et al.*, 2009). However, there are few data on the impact of these agents on $K_v7.1$ channels alone or on the other K_v7 channels expressed in the vasculature. In addition, the present functional data indicate that while L-768,673 and HMR1556 can reverse the effects of R-L3, JNJ282 cannot, therefore the differential effects of these compounds on $K_v7.1$ channel activity was investigated. Overexpression of KCNQ1 in *Xenopus* oocytes resulted in robust K^+ currents in response to membrane depolarization (Figures 6 and 7). Application of 0.1 μM L-768,673 produced considerable inhibition of the KCNQ1-generated current at all test potentials ($n = 8$ oocytes). Application of 1 μM L-768,673 had no further effect on the evoked current. HMR1556 had a similar effect on KCNQ1-evoked K^+ currents (data not shown). In contrast, JNJ282 only produced minimal inhibition at 1 and 10 μM, concentrations which are far higher than the IC_{50} for blockade of IKs by this compound (Towart *et al.*, 2009). These experiments show that L-768,673 and HMR1556 were viable as $K_v7.1$ probes whereas JNJ282 was only effective against $K_v7.1$/KCNE1-encoded protein complexes. To provide more information about these agents the effect of L-768,673, HMR1556 and JNJ282 on currents produced by the overexpression of KCNQ4 and KCNQ5 was investigated. Figure 6 shows that none of the agents had an effect on the currents produced by the overexpression of KCNQ5 at concentrations that were functional in the isometric tension studies. However, L-768,673 but not HMR1556 nor JNJ282 had a pronounced inhibitory effect on KCNQ4

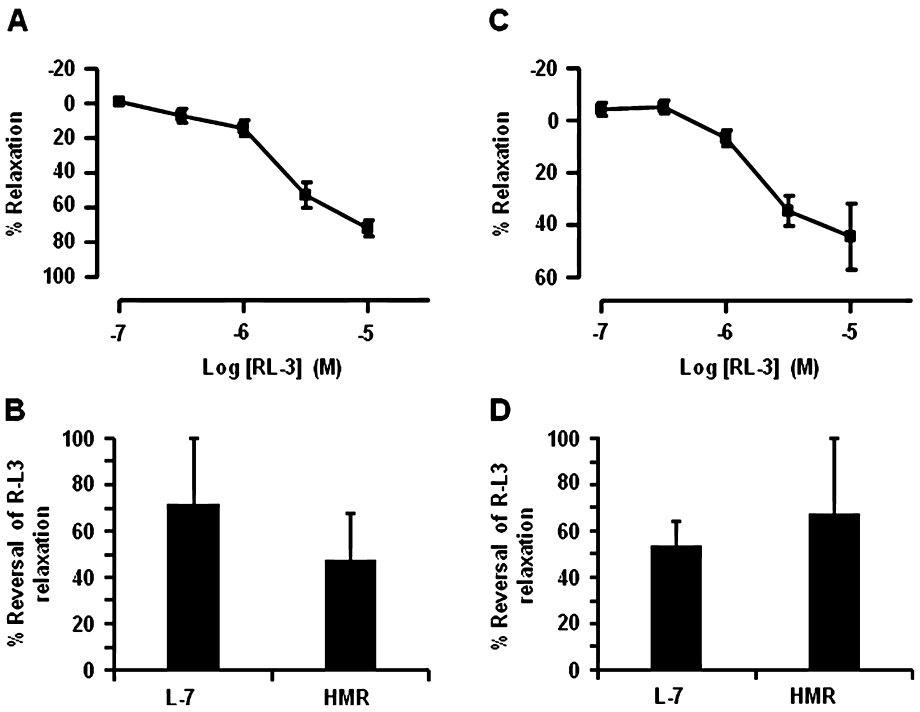

Figure 4

R-L3 evoked relaxation in thoracic aorta and intrapulmonary arteries. Mean R-L3 concentration-dependent relaxation and percentage reversal by K_v7.1 blockers, 10 μM L-768,673 and 10 μM HMR1556, in thoracic aorta (A and B) and intrapulmonary arteries (C and D) preconstricted with 3 μM methoxamine and 0.3 μM U46619, respectively.

generated currents at potentials positive to 0 mV (Figure 6B, D and F). These studies provide a thorough assay of the selectivity of these agents for the different K_V7 channels expressed in the vasculature.

Effect of R-L3 on heterologously expressed KCNQ channels

The effectiveness of RL-3 as a vasorelaxant in all blood vessels tested provides considerable evidence that K_v7.1-containing channels are functionally relevant. However, the effects of this agent could be mediated by stimulation of the K_v7.4 and K_v7.5 channels that are present in the vasculature. Figure 7 demonstrates that at concentrations that had a pronounced effect on currents produced by the overexpression of KCNQ1 (Figure 7A and B) RL-3 had no effect on KCNQ4 or KCNQ5-generated currents (Figure 7C). These data support the functional studies with L-768,673 and HMR1556 that suggest the relaxant effect of RL-3 is due to enhanced K_v7.1 activity.

Discussion

The current study shows that K_v7.1 channels are expressed in the plasmalemmal membrane of smooth muscle cells from rat mesenteric arteries and thoracic aortae but three structurally different K_v7.1 channel or IKs blockers (L-768,673, HMR1556 and JNJ282) at concentrations in excess of their IC_{50} for these channels had no effect on resting vascular tone, in contrast to the effect of the pan-K_v7 blocker linopirdine. However, this study provides unique evidence that direct activation of K_v7.1 channels is a highly effective dilator mechanism in both systemic and pulmonary arteries.

KCNQ1-encoded (K_v7.1) channels in co-assembly with the translated protein of KCNE1 (kcne1 or MinK) comprise the slow component of the delayed rectifier K^+ current (IKs) in cardiac myocytes (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996). Mutations to KCNQ1 or KCNE1 underlie a number of hereditary arrhythmias leading to long QT syndrome (Herbert *et al.*, 2002; Jespersen *et al.*, 2005). Consequently, several IKs blockers including chromanol 293B and its more potent analogue HMR1556 (Gogelein *et al.*, 2000), as well as the structurally different compound L-768,673, have been tested for use as potential anti-arrhythmic therapies. These drugs, as well as the highly potent compound JNJ282, reduce the IKs current and thus prolong repolarization and refractoriness leading to long QT (Antzelevitch, 2007). The effects of L-768,673, HMR1556 and JNJ282 on other cardiac ion channels (I_{Na}, I_{Ca}, I_{Kr}, I_{to}, I_{K1}) reveal very high selectivity for IKs (Towart *et al.*, 2009). The present study confirms that L-768,673 and HMR 1556 were good inhibitors of homomeric KCNQ1 channels, whereas JNJ282 was totally ineffective supporting the findings of Towart *et al.* (2009) that this compound is an inhibitor of the channel formed by the association of KCNQ1 + KCNE1 expression products that underlie the cardiac IKs. The results from the present study also show that neither HMR1556 nor JNJ282 affects currents generated by the expression of KCNQ4 or KCNQ5. L-768,673

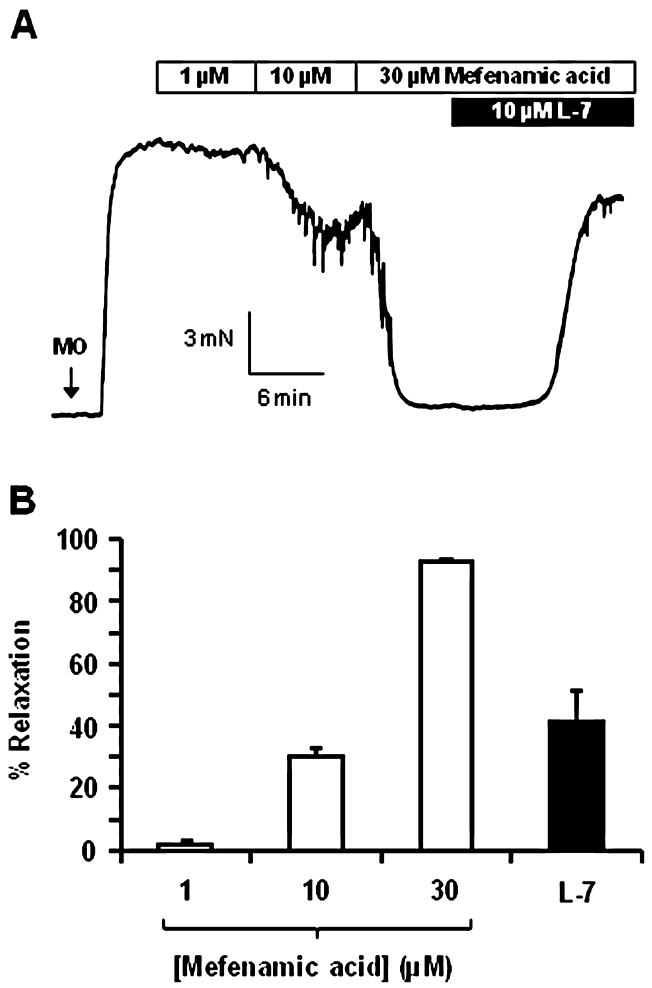

Figure 5

Mefenamic acid evoked relaxation in mesenteric artery. Representative trace (A) and mean data (B) of mefenamic acid concentration-responses in mesenteric arteries preconstricted with 10 µM methoxamine (MO) and subsequent blockade of $K_v7.1$ channels with 10 µM L-768,673 (L-7; $n = 3$).

also had no effect on KCNQ5-generated currents but reduced currents produced by KCNQ4 expression significantly. These studies provide an extensive addition to the pharmacological palette for studying KCNQ-encoded channels.

In previous studies it was observed that the selective $K_v7.1$ channel inhibitor chromanol 293B does not affect vascular tone, whereas under the same conditions the pan-K_v7 blockers linopiridne and XE991 contract arteries (Yeung and Greenwood, 2005; Yeung *et al.*, 2007; Ng *et al.*, 2011). These findings are now corroborated by the present study where linopirdine was shown to robustly contract systemic and pulmonary arteries, with the caveat that mesenteric and intrapulmonary vessels require a small degree of pre-tone, induced with a low concentration of methoxamine or U46619, respectively. This phenomenon has been observed previously in murine mesenteric arteries (Yeung *et al.*, 2007), but appears to be contradictory to previous results in intrapulmonary vessels (Joshi *et al.*, 2006) where contractions to XE991 and linopirdine did not require pre-tone and were of a similar magnitude to those elicited by 50 mM KCl. Although we cannot fully explain this discrepancy, it is worth noting that experimental conditions were not identical including differences in the normalized basal tension, which was ~1 mN (equivalent of 20 mmHg), almost 80% lower than in previous studies (Joshi *et al.*, 2006; 2009). Irrespective of these aspects, the simple message is that under the same conditions where linopiridne contracts blood vessels, compounds shown to selectively inhibit $K_v7.1$ channels have no effect. It is worth stressing that in the present study, none of the three different agents inhibited contractions elicited by 60 mM KCl proving that they did not block voltage-dependent calcium channels, which would preclude any contractile effect.

These data, in addition to previous observations with chromanol 293B, confirm that $K_v7.1$ channels are unlikely to contribute to resting vascular tone. However, due to the consistent expression of these channels in the vasculature, the persisting question of their function remains. A working scenario is that $K_v7.1$ channels under resting conditions are in a closed state and that direct activation would elicit a change in arterial tone. To test this hypothesis we used two IKs activators namely R-L3 and mefenamic acid (Busch *et al.*, 1994; 1997). The benzodiazepine R-L3 at concentrations <10 µM potentiates IKs (Salata *et al.*, 1998) by interacting with the fifth and sixth transmembrane domains of $K_v7.1$ subunits, binding sites that are distinct from where the benzodiazepine antagonist L-768,673 interacts (Seebohm *et al.*, 2003). In the present study, RL-3 enhanced currents produced by the overexpression of KCNQ1 but had no effect on KCNQ4 or KCNQ5-generated currents and relaxed preconstricted arteries. Interestingly, the potency of R-L3 in mesenteric vessels was greater than the dilator effects of the $K_v7.2$–7.5 channel activators retigabine, S-1, BMS-205352 and flupirtine (Mackie *et al.*, 2008; Jepps *et al.*, 2011). The finding that R-L3 relaxations were completely reversed by HMR1556 or L-768,673 confirmed that the dilator responses observed were due to modulation of $K_v7.1$ channels. Similarly, mesenteric arteries were relaxed by mefenamic acid, although with less potency than R-L3; however, these responses were only partially reversed by L-768,673, suggesting some non-selective effects of mefenamic acid such as blockade of Ca^{2+}-activated Cl^- channels (Greenwood and Large, 1995) at the higher concentrations used. Preconstricted thoracic aortae and intrapulmonary arteries were relaxed by ~70% and 45%, respectively, in response to 10 µM R-L3. These relaxations were reversed by 50–60% in the presence of HMR1556 or L-768,673 indicating that some effects of R-L3 at this higher concentration was not due to stimulation of $K_v7.1$ channels. Interestingly, R-L3 relaxations were not reversed by JNJ282 at concentrations that did not inhibit calcium channels. The lack of effect of JNJ282 cannot be fully explained, due to the lack of knowledge of the molecular mode of inhibition by the compound. However, in contrast to HMR1556 and L-768,673, which inhibit IKs as well as KCNQ1 alone (Seebohm *et al.*, 2003; present study), the poor interaction of JNJ282 with monomeric $K_v7.1$ isoforms (Figure 6) while being an effective IKs blocker (Towart *et al.*, 2009) may provide a possible explanation for the discrepency between the actions of these drugs. At present it is not possible to determine why $K_v7.1$-containing channels do not seem to contribute to the setting

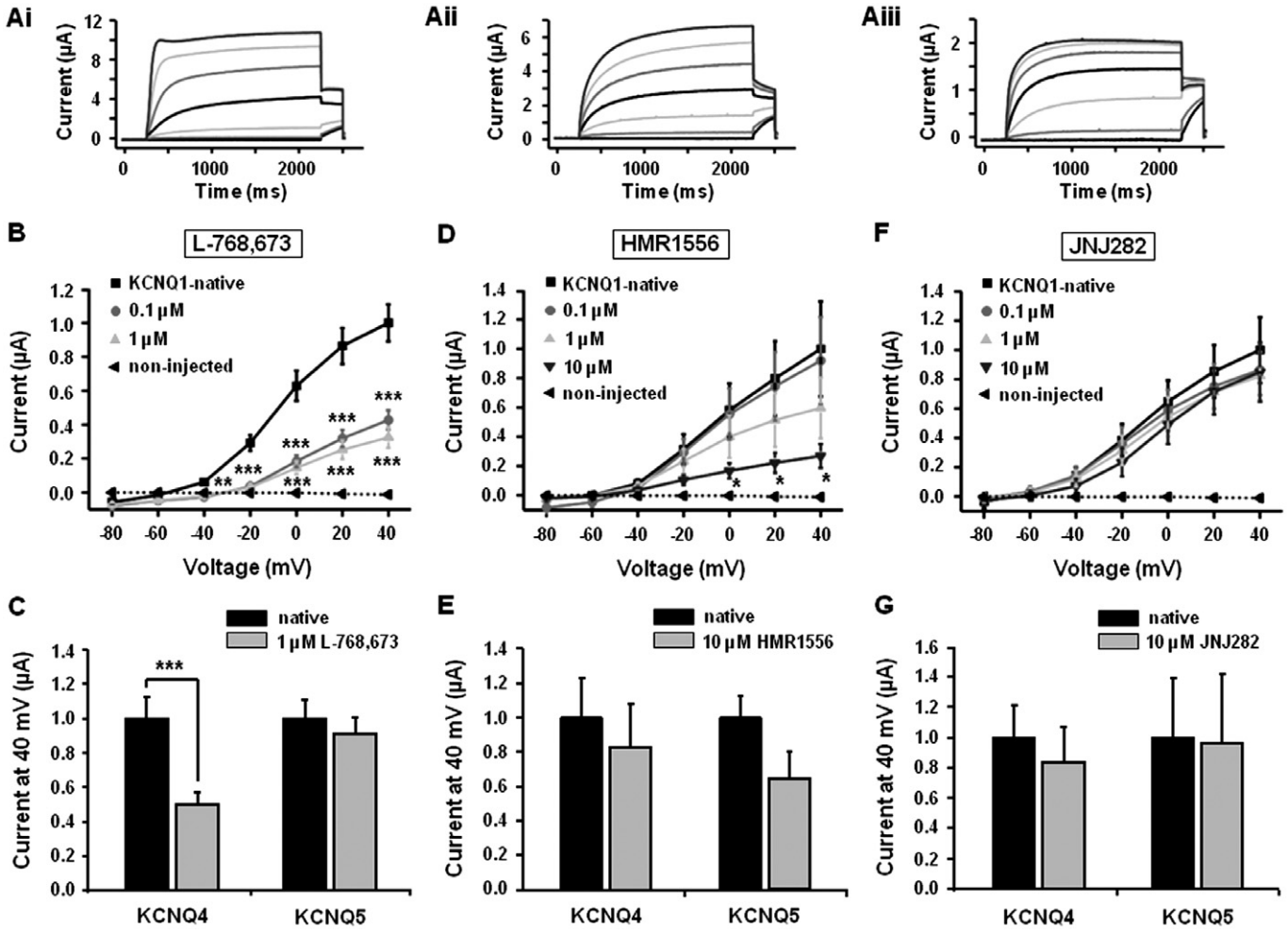

Figure 6

Effect of K_v7.1 blockers on currents produced by the overexpression of KCNQ1, 4 and 5 in Xenopus oocytes. Representative traces show currents produced by the overexpression of KCNQ1 (Ai), KCNQ4 (Aii) and KCNQ5 (Aiii) recorded at voltages between −80 mV and +40 mV. *I*/*V* curves of KCNQ1 as a summary of the current recordings obtained from oocytes in the presence of different concentrations of K_v7.1 channel blockers L-768,673 (n = 10; B), HMR 1556 (n = 9 to 10, D) and JNJ282 (n = 8; F). (C), (E) and (G) show the mean effects of L-768,673 (n = 8), HMR 1556 (n = 8 to 10) and JNJ282 (n = 2 to 5), respectively, on currents produced by the overexpression of KCNQ4 and KCNQ5 recorded at +40 mV. *P < 0.05, **P < 0.01 and ***P < 0.001 compared by Student's paired t-test.

of the membrane potential in these blood vessels. One possibility is that in the vascular smooth muscle, K_v7.1 associates with the product of KCNE4 genes expressed in blood vessels (Yeung *et al.*, 2007; Zhong *et al.*, 2010), which are known to effectively silence K_v7.1 activity (Grunnet *et al.*, 2003). Alternatively, a situation analogous to that seen in cardiac myocytes may exist where other potassium channels provide sufficient hyperpolarization to cover the lost control provided by K_V7.1 blockade (termed 'repolarization reserve'). Future work will concentrate on both of these aspects.

In conclusion, the results in this study dispel previous assumptions that K_v7.1 channels are functionally redundant in vascular smooth muscle. We have shown using a variety of potent K_v7.1 modulators that although these channels do not contribute to resting vascular tone they can evoke profound relaxation upon stimulation, particularly in resistance-like systemic arteries. Interestingly, it is unlikely to be the cardiac K_v7.1/MinK complex that exists in blood vessels. We speculate that vascular K_v7.1 channels may in part underlie vasodilatation pathways involved in rectifying vascular tone. It remains a challenge to determine the molecular architecture of vascular K_v7 channels and the respective contributions to physiological control of vascular tone.

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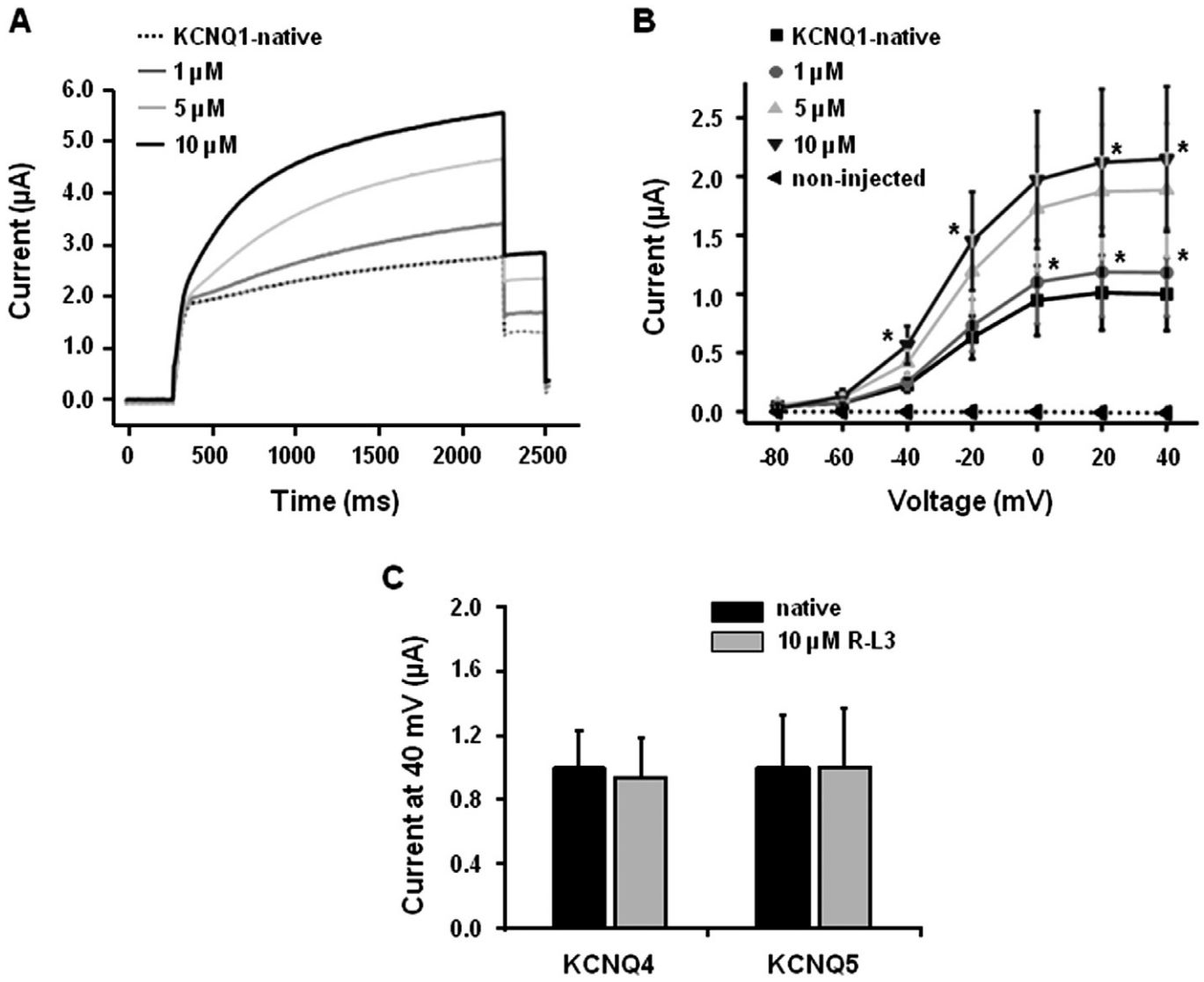

Figure 7

Effect of RL-3 on KCNQ1 expressed in Xenopus oocytes. (A) Representative effects of RL-3 (1–10 μM) on currents produced by the overexpression of KCNQ1. Oocytes were held at –80 mV stepped to +40 mV for 2 s and repolarized to –40 mV. Mean *I*/*V* data are shown in (B) ($n = 7$–9). (C) The lack of effect of 10 μM RL-3 on KCNQ4 and KCNQ5-generated currents ($n = 7$–9). *$P < 0.05$ compared by Student's paired *t*-test.

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Conflicts of interest

None.

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