BJP British Journal of Pharmacology

DOI:10.1111/bph.12805
www.brjpharmacol.org

RESEARCH PAPER

Vasorelaxant effects of novel $K_v7.4$ channel enhancers ML213 and NS15370

Correspondence
Thomas Jepps, Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. E-mail: tjepps@sund.ku.dk

Received
20 March 2014
Revised
25 April 2014
Accepted
14 May 2014

T A Jepps[1], B H Bentzen[1], J B Stott[2], O V Povstyan[2], K Sivaloganathan[2], W Dalby-Brown[3] and I A Greenwood[1,2]

[1]*Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark,* [2]*Vascular Biology Research Centre, Institute of Cardiovascular & Cell Sciences, St George's, London, UK, and* [3]*NeuroSearch A/S, Ballerup, Denmark*

BACKGROUND AND PURPOSE

The *KCNQ*-encoded voltage-gated potassium channel family ($K_v7.1$-$K_v7.5$) are established regulators of smooth muscle contractility, where $K_v7.4$ and $K_v7.5$ predominate. Various $K_v7.2$–7.5 channel enhancers have been developed that have been shown to cause a vasorelaxation in both rodent and human blood vessels. Recently, two novel K_v7 channel enhancers have been identified, ML213 and NS15370, that show increased potency, particularly on $K_v7.4$ channels. The aim of this study was to characterize the effects of these novel enhancers in different rat blood vessels and compare them with K_v7 enhancers (S-1, BMS204352, retigabine) described previously. We also sought to determine the binding sites of the new K_v7 enhancers.

KEY RESULTS

Both ML213 and NS15370 relaxed segments of rat thoracic aorta, renal artery and mesenteric artery in a concentration-dependent manner. In the mesenteric artery ML213 and NS15370 displayed EC_{50}s that were far lower than other K_v7 enhancers tested. Current-clamp experiments revealed that both novel enhancers, at low concentrations, caused significant hyperpolarization in mesenteric artery smooth muscle cells. In addition, we determined that the stimulatory effect of these enhancers relied on a tryptophan residue located in the S5 domain, which is the same binding site for the other K_v7 enhancers tested in this study.

CONCLUSIONS AND IMPLICATIONS

This study has identified and characterized ML213 and NS15370 as potent vasorelaxants in different blood vessels, thereby highlighting these new compounds as potential therapeutics for various smooth muscle disorders.

Abbreviations

BMS204352, (3S)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one; K_v, voltage-gated potassium channel; ML213, N-mesitylbicyclo[2.2.1]heptane-2-carboxamide; NS15370, (2-(3,5-difluorophenyl)-N-[6-[(4-fluorophenyl)methylamino]-2-morpholino-3-pyridyl]acetamide)hydrochloride; QPCR, quantitative PCR; S-1, (S)-N-[1-(3-morpholin-4-yl-phenyl)-ethyl]-3-phenyl-acrilamide; $V_{½}$, voltage at half-maximal activation

© 2014 The British Pharmacological Society

Table of Links

TARGETS	LIGANDS
K_v7.1 channels	Methoxamine
K_v7.2 channels	Linopirdine
K_v7.3 channels	Nicardipine
K_v7.4 channels	Paxilline
K_v7.5 channels	Retigabine
	BMS204352

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Five members of the *KCNQ* gene family have been identified (*KCNQ*1–5) that encode the voltage-gated potassium channels (K_v7.1–7.5). Expression of the different *KCNQ* isoforms varies among mammalian cell types (Soldovieri *et al.*, 2011), although the majority of research into these channels has focussed on their expression in the heart (*KCNQ*1) and nervous system (*KCNQ*2-5). *KCNQ*-encoded channels have also been identified in various mammalian smooth muscle tissues. In smooth muscles, the expression of *KCNQ*1, *KCNQ*4 and *KCNQ*5 dominates (Ohya *et al.*, 2003; Yeung *et al.*, 2007; Ng *et al.*, 2011; Chadha *et al.*, 2012a,b; Khanamiri *et al.*, 2013) with recent evidence that a K_v7.4/K_v7.5 heteromer is the main molecular species in vascular smooth muscle (Brueggemann *et al.*, 2014; Chadha *et al.*, 2014). In both rodent and human blood vessels, pharmacological modulators of *KCNQ*-encoded channels have been used to identify a crucial role for these channels in regulating smooth muscle contractility (Jepps *et al.*, 2013; Stott *et al.*, 2014). K_v7 channel enhancers, such as retigabine, S-1 and BMS204352, which enhance K_v7.2–K_v7.5, have been used to elucidate the importance of these channels in smooth muscle contractility and are effective vasorelaxants. Retigabine, S-1 and BMS204352 act on K_v7.2–7.5, but not K_v7.1 (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005), and shift both the activation threshold and voltage for half-activation in the hyperpolarizing direction, leading to an increase in current amplitude at more negative potentials. The mechanism of enhancement for these compounds occurs through direct binding to a tryptophan residue (Trp^{236} in K_v7.2; Trp^{265} in K_v7.3) in a hydrophobic pocket of the cytoplasmic part of S5, which stabilizes the channel in the open state (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005; Bentzen *et al.*, 2006; Lange *et al.*, 2009).

Recently two new activators have been described, ML213 and NS15370, which enhance the activity of K_v7.2 and K_v7.4, and K_v7.2–7.5 channels, respectively, with increased potency in overexpression systems compared with the K_v7 channel enhancers described previously. ML213 was identified by a high throughput fluorescent screen of the Molecular Libraries Small Molecule Repository and structure activity relationship studies, and has been shown to enhance K_v7.2 and K_v7.4 channels with EC_{50}s of 230 and 510 nM respectively (Yu *et al.*, 2011); whereas NS15370 was developed as a chemical analogue of retigabine and can enhance K_v7.2–7.5 channels with EC_{50}s ranging between 40 and 150 nM (Dalby-Brown *et al.*, 2013).

However, the role of K_v7 channels in controlling neuronal excitability has led to widespread interest in the development of novel pharmacological enhancers of these channels. Pharmacological enhancement of the 'neuronal' K_v7 channels suppresses neuronal activity and prevents the generation of seizures in various animal models of epilepsy (Dailey *et al.*, 1995; Rostock *et al.*, 1996; Tober *et al.*, 1996). Retigabine (Trobalt®) is effective in clinical trials as an anti-epileptic (Brodie *et al.*, 2010; French *et al.*, 2011) and is now on the market in both the United States and Europe. While the development of K_v7 channel enhancers is aimed predominantly at treating neurological disorders, the aim of this study was to determine whether the new K_v7 channel enhancers, ML213 and NS15370, were effective at suppressing contractile activity in vascular smooth muscle, thus highlighting these enhancers as possible therapeutics for various vascular disorders. Therefore, this study compared the effects of three established enhancers of K_v7.2–7.5 with those of the two novel agents in various rat blood vessels, and also determined the effects of the novel enhancers on the resting membrane potential of rat mesenteric artery smooth muscle cells. In addition, we determine whether these new activators rely on the same tryptophan residue essential for the binding of retigabine and the other K_v7 enhancers.

Methods

All experiments were performed on arteries from male, 12–16-week-old Wistar rats (Charles River UK, Ltd., Margate, UK) killed by cervical dislocation. A total of 30 animals were used in this study.

Quantitative PCR (QPCR)

QPCR analysis of KCNQ isoform expression in the rat thoracic aorta, renal artery and mesenteric artery was performed

Table 1

KCNQ 1–5 assays used for QPCR analysis of rat blood vessels

Gene	Primer sequence (+) sense, (–) antisense	GenBank accession number	Amplicon (bp)	Region spanned
KCNQ1	(+) 5′-CCATCTTTGTTCATCCCCATCT-3′	NM_032073	100	1797–1896
	(–) 5′- CCAGTTGTGTCACCTTGTCTT -3′			
KCNQ2	(+) 5′-GGTGTCTCATTCTTCGCTCTT-3′	NM_133322	100	1023–1122
	(–) 5′-TCCGCCGTTTCTCAAAGTG-3′			
KCNQ3	(+) 5′-ATACACATTTATCTGCTCTTCCTTTTA-3′	NM_031597	122	3299–3420
	(–) 5′-TGCTCTCAGTTTATCCGAATCAA-3′			
KCNQ4	(+) 5′-GCTCATCTTCGCCTCTTTCC-3′	XM_233477	112	861–972
	(–) 5′-GCCAATGGTCGTCAGTGTAAT-3′			
KCNQ5	(+) 5′-CCTGGCGTACACGAGAGTAT-3′	XM_001071249	80	2383–2462
	(–) 5′-TTTGACTGGGCGAACTGAAC-3′			

The analysis was designed and optimized by PrimerDesign, Ltd.

as described previously in Khanamiri *et al.* (2013). Briefly, RNA was extracted using the RNEasy Micro Extraction Kit (Qiagen, Manchester, UK) and reverse transcribed using Oligo$(dT)_{12–18}$ primers and Moloney murine leukemia virus (Life Technologies). Quantitative analysis of specific genes of interest within our cDNA samples was determined using Precision-iC SYBR green mastermix (PrimerDesign, Ltd., Southampton, UK) with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hertfordshire, UK). Duplicate reactions were performed in 10 μL volumes containing 5 μL Precision-iC SYBR green master mix (PrimerDesign, Ltd.), 300 nM primer (PrimerDesign, Ltd.), 5–10 ng cDNA and made up to 10 μL with nuclease-free water. The following cycling conditions were used: initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min, and data were collected during each cycling phase. Melt curve analysis, to ensure each primer set amplified a single, specific product, completed the protocol. Reverse transcription-negative samples and no-template controls were run alongside all reactions to assess contamination. Quantification cycle (Cq) values were determined using Bio-Rad CFX96 Manager 3.0 software and the single threshold mode.

The geNorm reference gene selection kit (PrimerDesign, Ltd.) was used to identify the most stable reference genes and to determine optimal number of reference genes required for reliable normalization of the genes of interest in our samples (Vandesompele *et al.*, 2002). All reference genes in the rat geNorm reference gene selection kit and the *KCNQ*1–5 assays (Table 1) were designed and optimized by PrimerDesign, Ltd.

Myography

Arteries were cleaned of adherent tissue and segments (~2 mm) were mounted in a myograph (Danish Myo Technology, Aarhus, Denmark) for isometric tension recording. The composition of the physiological salt solution (PSS) in the chambers was 125 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 25.4 mM $NaHCO_3$, 1 mM Na_2HPO_4, 0.6 mM $MgSO_4$ and 10 mM glucose, maintained at 37°C and aerated with 95% O_2/5% CO_2. The vessels were allowed to equilibrate for 30 min before undergoing a passive force normalization procedure (Mulvany & Halpern, 1977). Before the application of the K_v7 enhancers, the arterial segments were contracted with the α_1-adrenoceptor agonist methoxamine (3 μM in the thoracic aorta and renal artery, 10 μM in the mesenteric artery), which was determined previously to produce a sub-maximal contraction (80–90% of the maximal contraction) in the respective vessels (data not shown).

Mesenteric artery myocyte isolation and electrophysiology

Following dissection, mesenteric arteries were bathed for 10 min in nominally Ca^{2+}-free PSS before incubation at 37°C in Ca^{2+}-free PSS containing collagenase type IA (2 mg·mL^{-1}), protease type X (1 mg·mL^{-1}) and DL-dithiothreitol (1 mg·mL^{-1}) for 15 min followed by a 10 min wash in Ca^{2+}-free PSS solution at room temperature. Single cells were liberated by gentle mechanical agitation with a wide bore Pasteur pipette and the suspension was transferred to experimental chambers and kept for at least 30 min at room temperature to allow adherence. Cells were used in patch-clamp experiments within 6 h of isolation. Membrane potential recordings were made using standard amphotericin B (300 μg·mL^{-1}) perforated-patch technique in current clamp mode. Patch pipettes were fire-polished and had a resistance of 4–8 MΩ when filled with the pipette solution of the following composition: 126 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 0.5 mM EGTA, pH was adjusted to 7.2 with KOH and PSS was used as an external solution. All experiments were performed at room temperature. The electrical signals were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). Electrical signals were generated and digitized at 1 kHz using a Digidata 1322A hosted by a PC running pClamp 9.0 software (Axon Instruments, Sunnyvale, CA, USA). Data were analysed and plotted using pClamp and MicroCal Origin software. All data are presented as mean ± SEM. Comparative analysis of the data was performed using Student's *t*-test.

HEK cell transfection and electrophysiology

Monoclonal HEK293 cells stably expressing human $K_v7.4$ were grown in DMEM, supplemented with 10% FBS (Th Geyer, Renningen, Germany) supplemented with Glutamax (Substrate Department, the Panum Institute, Copenhagen, Denmark) and incubated at 37°C in 5% CO_2. In addition, HEK293 cells were transiently transfected with $hK_v7.4$-W242L mutant channels using siLentFect™ Lipid (Bio-Rad), according to manufacturer's instructions. The point mutation W242L of human $K_v7.4$ was constructed as described previously (Bentzen *et al.*, 2006).

Potassium currents were recorded from HEK cells stably expressing human $K_v7.4$ using a QPatch 16 HT automated patch-clamp system, (Sophion-Bioscience, Ballerup, Denmark), with disposable single-hole QPlates (Sophion-Bioscience). On the day of the experiments, HEK293 cells were rinsed with PBS, detached from T175 bottles with 5 mL Detachin (Th Geyer) and re-suspended in serum-free medium supplemented with 100 U·mL^{-1} penicillin/streptomycin, 0.04 mg·mL^{-1} Soy bean trypsin inhibitor and 25 mM HEPES (Sigma-Aldrich, Brondby, Denmark) at a density of 1–5 million cells mL^{-1}. Cells were transferred to a QPatch stirring station, and allowed to recover for 10 min before the experiment was initiated. Conventional whole-cell patch-clamp electrophysiology was used to record currents from HEK cells expressing $hK_v7.4$-W242L. In all experiments, the extracellular solution contained the following: 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 4 mM KCl and 10 mM glucose. The pH was adjusted to 7.4 with NaOH and the osmolarity to 305 mOsm with sucrose. The intracellular solution contained: 1.75 mM $MgCl_2$, 10 mM EGTA, 110 mM KCl, 5.4 mM $CaCl_2$ and 4 mM Na_2-ATP, pH adjusted to 7.4 with KOH and osmolarity to 295 mOsm. The intracellular free calcium concentration was 100 nM. $K_v7.4$ currents were elicited every 5 s by depolarizing the membrane potential to –10 mV for 500 ms from a holding potential of –80 mV. After 2 min of stabilization, 6 periods of increasing concentrations of ML213 or NS15370 were added for 1 min to determine the stimulatory effects of the enhancers on the $K_v7.4$ current at –10 mV. A double step protocol from –100 mV to +60 mV (20 mV increments, 500 ms duration) returning to –30 mV to measure the tail current was applied before addition of the K_v7 enhancer and after the highest concentration.

Drugs

Retigabine, S-1, BMS204352 and NS15370 were synthesized at NeuroSearch A/S (Ballerup, Denmark). Linopirdine was purchased from Sigma-Aldrich, and ML213 was purchased from Tocris (Bristol, U.K.).

Results

Relative expression of KCNQ *isoforms in rat blood vessels*

Figure 1 shows the relative abundance of *KCNQ* gene expression in the cDNA from the rat thoracic aorta (n = 4), renal artery (n = 6) and mesenteric artery (n = 4). In the thoracic aorta and renal artery the relative expression of *KCNQ*1 predominated with *KCNQ*4 and *KCNQ*5 also being present. In the mesenteric artery *KCNQ*4 expression dominated relative to *KCNQ*1 and *KCNQ*5, which were also present. *KCNQ*3 was observed in the renal artery, but its expression was variable among samples, whereas in the thoracic aorta and mesenteric artery, *KCNQ*3 expression was negligible. *KCNQ*2 was never detected in these arteries (Figure 1).

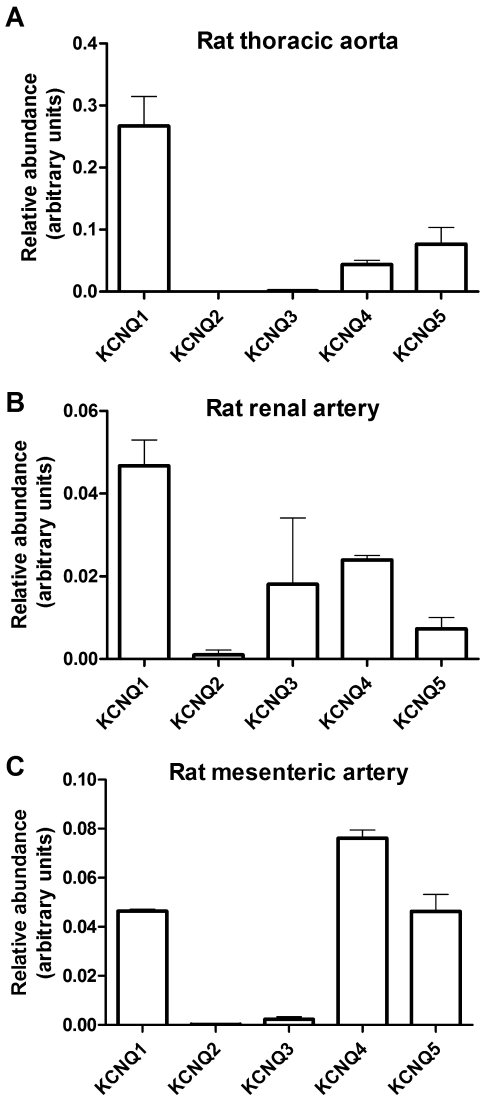

Figure 1

QPCR analysis of relative abundance of *KCNQ* genes in (A) the rat thoracic aorta (n = 4); (B) the rat renal artery (n = 6); (C) the rat mesenteric artery (n = 4), normalized to the reference genes. The relative abundance of each gene was calculated using the $2^{-\Delta Cq}$ method. Data represent the mean ± SEM.

ML213 and NS15370 are potent vasorelaxants of rat blood vessels

Following contraction with the α_1-adrenoceptor agonist methoxamine, application of increasing concentrations of ML213 and NS15370 to segments of rat thoracic aorta, renal artery and mesenteric artery resulted in concentration-dependent relaxations, which were considerably different

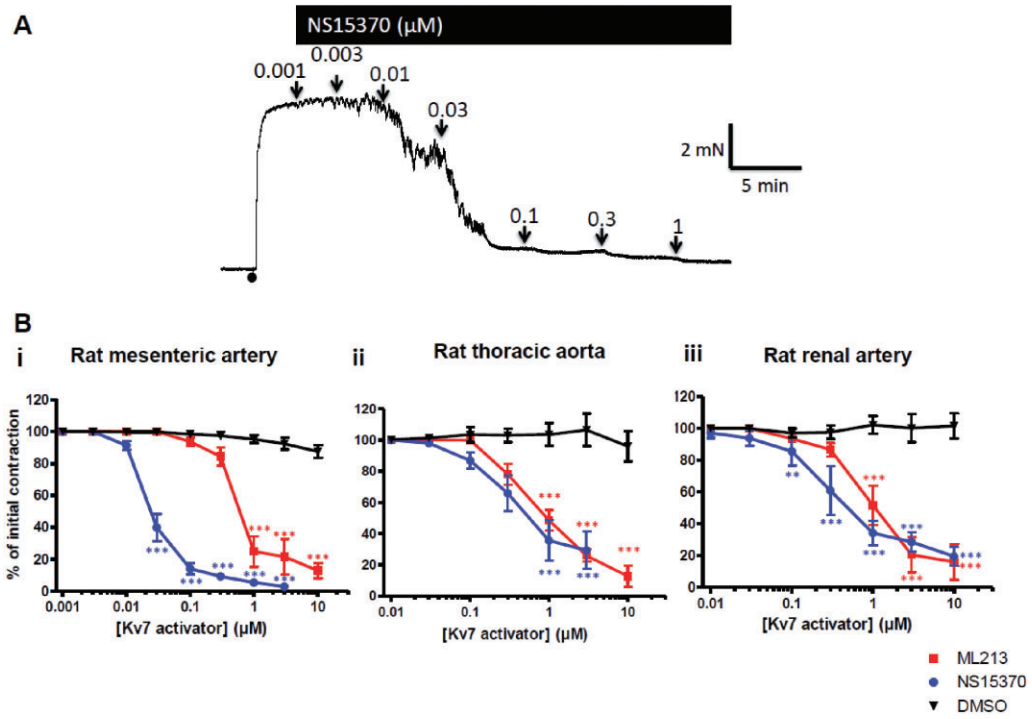

Figure 2

NS15370 and ML213 cause a vasorelaxation in different precontracted rat blood vessels. (A) Representative effect of increasing concentrations of NS15370 on a segment of small mesenteric artery mounted in a wire myograph precontracted with 10 μM methoxamine. (B) Concentration–effect curves for ML213 and NS15370 on (i) small mesenteric arteries, (ii) thoracic aorta and (iii) renal arteries. Each point is the mean of 7–10 animals ± SEM. A Bonferroni *post hoc* test was performed following a two-way ANOVA and *** $P < 0.001$.

from the minimal loss of tone seen when vehicle alone was applied (Figure 2A). When comparing the three arteries, ML213 and NS15370 were more potent at relaxing precontracted segments of mesenteric artery than the thoracic aorta and renal artery (n = 7–10; Table 2). In the mesenteric artery, pre-application of the K_v7 blocker linopirdine (10 μM) prevented ML213 from relaxing the artery (n = 6; Figure 3). The effect of NS15370 was also blunted significantly in the presence of 10 μM linopirdine (n = 6; Figure 3). Similar effects were observed in the aorta with 15 μM ML213 producing a 100 ± 2% and 10 ± 2% relaxation in the absence and presence of 10 μM linopirdine ($P < 0.001$; n = 5; data not shown). The lack of response to the K_v7 activators in the presence of linopirdine was not due to a reduced ability of the vessels to relax as application of 1 μM nicardipine caused a vasorelaxation in all vessels tested (Figure 3). Neither agent was able to relax arteries contracted with 60 mM KCl (e.g. tone with 15 μM ML213 was 100 ± 4% of initial, n = 6; data not shown). These data reveal ML213 and NS15370 to be highly potent relaxants of precontracted rat arteries.

ML213 and NS15370 are more potent in the vasculature than other K_v7 enhancers

Previously, other structurally different enhancers of $K_v7.2$–7.5 have been used to study the role of K_v7 channels in numerous blood vessels (Yeung *et al.*, 2007; 2008; Mackie *et al.*, 2008; Joshi *et al.*, 2009; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Mani *et al.*, 2011; Ng *et al.*, 2011; Chadha *et al.*, 2012a; 2014; Khanamiri *et al.*, 2013). We therefore undertook a series of experiments to compare the effects of S-1, BMS204352 and retigabine with the relaxant ability of the two novel compounds. Under isometric conditions, all the K_v7 channel enhancers tested relaxed precontracted segments of thoracic aorta, renal artery and mesenteric artery with differing potencies (see Table 2). All relaxations were prevented by prior application of linopirdine. Statistical analysis of the EC_{50} values in Table 2 (one-way ANOVA followed by a Bonferroni multiple comparisons test) showed that in the thoracic aorta NS15370 and ML213 were significantly more potent at causing a vasorelaxation compared with S-1, BMS204352 and retigabine. In the renal and mesenteric arteries, NS15370 and ML213 were significantly more potent compared with retigabine, and NS15370 showed significant differences compared with S-1 in the mesenteric artery (Figure 4 and Table 2).

ML213 and NS15370 hyperpolarize mesenteric artery smooth muscle cells

Current-clamp recordings were performed to determine the action of ML213 and NS15370 on the resting membrane potential of single smooth muscle cells freshly isolated from rat mesenteric arteries. As shown in Figure 5, the membrane potential in mesenteric artery smooth muscle cells was often superimposed by transient hyperpolarizations. These were sensitive to paxilline and, therefore, reflect the sporadic activation of calcium-activated potassium channels. We did not investigate this aspect further. Importantly, application of

Table 2

Comparison of the EC_{50} values of different K_v7 activators when applied to the rat thoracic aorta, renal artery and mesenteric artery under isometric conditions

	EC_{50}s (μM)		
K_v7 channel enhancer	**Thoracic aorta (*n*)**	**Renal artery (*n*)**	**Mesenteric artery (*n*)**
NS15370	0.55 ± 0.13 (7)	0.72 ± 0.21 (7)	0.03 ± 0.00 (8)
ML213	1.03 ± 0.09 (10)	1.03 ± 0.16 (7)	0.75 ± 0.09 (9)
S-1	8.96 ± 0.8 (11)	0.76 ± 0.16 (6)	2.75 ± 0.26 (11)
BMS204352	10.08 ± 1.15 (7)	3.31 ± 0.51 (6)	2.06 ± 0.21 (9)
Retigabine	8.75 ± 1.22 (8)	9.21 ± 2.76 (6)	10.59 ± 1.47 (7)
Bonferroni's multiple comparison test			
NS15370 vs. ML213	ns	ns	ns
NS15370 vs. S-1	***	ns	**
NS15370 vs. BMS204352	***	ns	ns
NS15370 vs. Retigabine	***	***	***
ML213 vs. S-1	***	ns	ns
ML213 vs. BMS204352	***	ns	ns
ML213 vs. Retigabine	***	***	***

The EC_{50} values were analysed statistically using a Bonferroni multiple comparison test, where $^{**}P < 0.01$ and $^{***}P < 0.005$; ns, not significant.

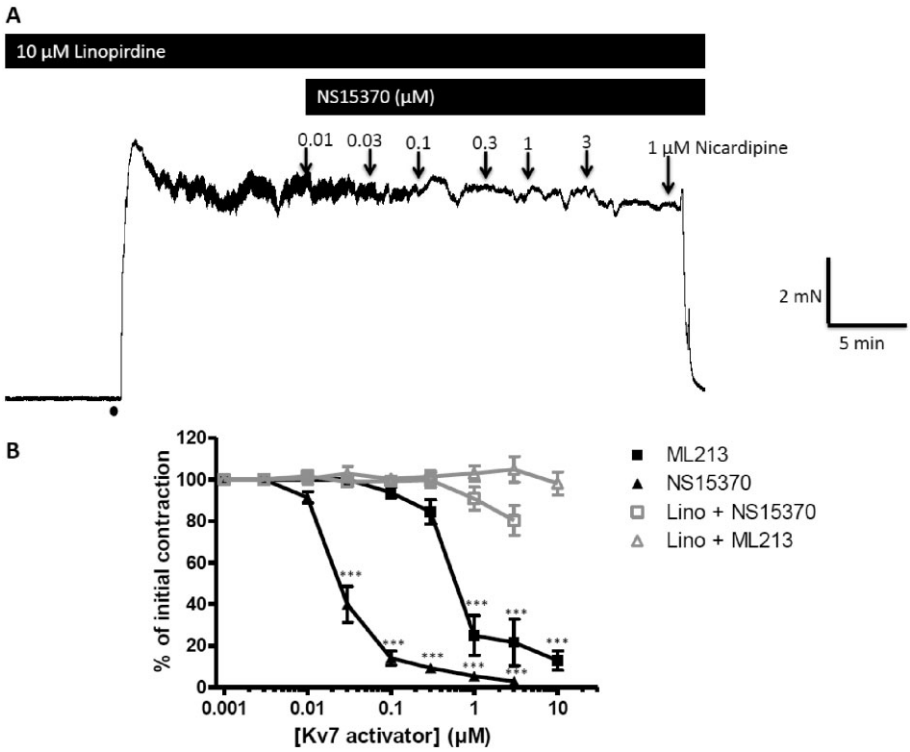

Figure 3

The effects of the K_v7 channel enhancers in the presence of a K_v7 channel blocker. (A) Representative isometric recording traces of the effect of NS15370 on precontracted (10 μM methoxamine) segments of mesenteric artery in the presence of the K_v7 blocker linopirdine (10 μM). At the end of the experiment 1 μM nicardipine was applied to show the vessel was still able to fully relax. (B) Mean data summerizing the effect of ML213 and NS15370 in the presence and absence of 10 μM linopirdine on segments of mesenteric artery ($n = 6$). Data represent the mean ± SEM. A Bonferroni *post hoc* test was performed following a two-way ANOVA, and $^{**}P < 0.01$ and $^{***}P < 0.001$.

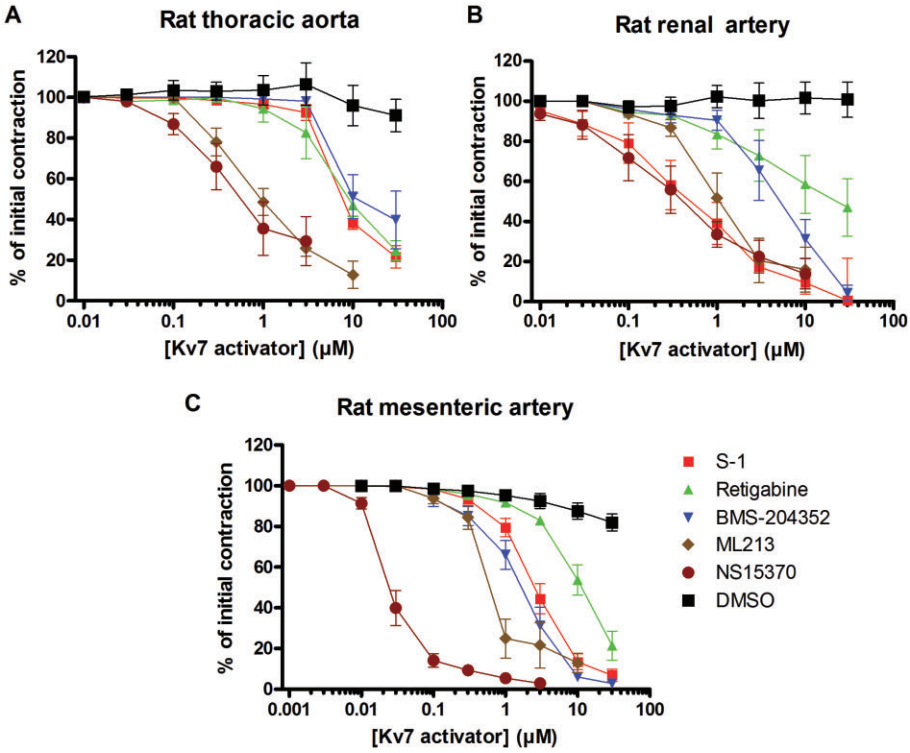

Figure 4

Comparison of the vasorelaxant effects of different K_v7 channel enhancers in the (A) thoracic aorta, (B) renal artery and (C) mesenteric artery. Data represent the mean ± SEM taken from 6 to 11 animals.

10 μM ML213 produced a significant hyperpolarization within a few minutes of application ($P < 0.001$; $n = 11$ cells). Application of NS15370 also hyperpolarized the membrane potential significantly at lower concentrations than ML213 (Figure 5B).

Effect of the novel enhancers relies on a tryptophan residue in the S5 transmembrane domain of $K_v7.4$

In QPatch experiments, application of increasing concentrations of ML213 and NS15370 stimulated $K_v7.4$ currents at –10 mV with EC_{50} values of 1.8 ± 0.3 μM and 42 ± 8 nM, respectively ($n = 6$ and 8 cells). As a tryptophan residue in the S5 pore domain is crucial for the stimulatory effects of retigabine, S-1 and BMS204352 (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005; Bentzen *et al.*, 2006), but is not involved with the enhancing effects of another $K_v7.2$–7.5 enhancer, ICA27243 (Padilla *et al.*, 2009; Blom *et al.*, 2010); we investigated whether NS15370 and ML213 activities relied on this tryptophan residue. In the HEK cells overexpressing *KCNQ*4, ML213 (10 μM) caused a significant increase in steady-state current (Figure 6A), which was associated with a significant leftward shift in the voltage-dependence of channel activation with the voltage at half-maximal activation ($V_{½}$) of activation changing by –33.0 ± 3.9 mV ($n = 7$ cells). In contrast, in cells overexpressing a *KCNQ*4 mutant with the tryptophan-242 residue changed to a leucine (*KCNQ*4-W242L), ML213 had no effect on the steady-state currents and produced a 1.6 ± 4.4 mV ($n = 7$ cells) change in the $V_{½}$ of activation, which was not significant according to a two-way ANOVA (Figure 6B). NS15370 (1 μM) had more complex effects on the currents produced by the stable expression of $hK_v7.4$, with enhancement (not significant according to a two-way ANOVA and Bonferroni's *post hoc* test) seen at potentials negative to 0 mV and current depression at more positive membrane potentials (Figure 7A). However, 1 μM NS15370 produced a leftward shift in the voltage-dependence of activation with a decrease in $V_{½}$ of –39.9 ± 1.7 mV ($n = 8$ cells). These effects were not observed in cells expressing the $K_v7.4$ mutant ($n = 7$ cells; Figure 7B).

Discussion

The results of this study show that two newly identified compounds, ML213 and NS15730, are highly potent vasorelaxants in a range of blood vessels. Moreover, these agents are more potent vasorelaxants than retigabine, S-1 and BMS204352, which have been used along with flupirtine to investigate vascular effects of K_v7 channels previously (Yeung *et al.*, 2007; 2008; Mackie *et al.*, 2008; Joshi *et al.*, 2009; Zhong *et al.*, 2010; Jepps *et al.*, 2011; Mani *et al.*, 2011; Chadha *et al.*, 2012a; 2014; Khanamiri *et al.*, 2013). In addition, ML213 and NS15730 produced marked hyperpolarization of the membrane in freshly isolated smooth muscle cells. These effects could be sufficient to counter any depolarizing

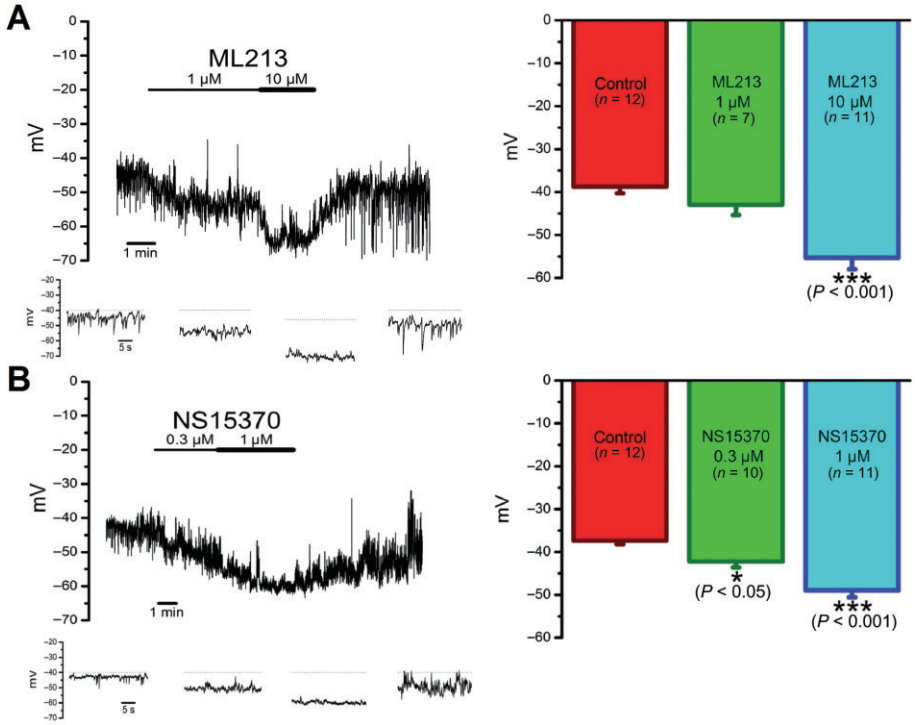

Figure 5

(A) ML213 and (B) NS15370 hyperpolarize the resting membrane potential of mesenteric artery smooth muscle cells. (Left panel) Representative traces of current-clamp recordings performed on smooth muscle cells isolated from rat mesenteric arteries to show the hyperpolarization caused by application of ML213 and NS15370. Expanded examples of the recordings are shown in four insets beneath each of the main current-clamp recordings. (Right panel) Mean effect on the membrane potential of different concentrations of the K_v7 channel enhancers (n = 7–12 cells) ± SEM. According to a one-way ANOVA followed by a Bonferroni *post hoc* test, *$P < 0.05$ and ***$P < 0.001$ significantly different from control.

surges that would be likely to lead to increased opening of voltage-dependent calcium channels and contraction. All effects on smooth muscle cells were attenuated by either high-potassium bathing solutions or specific K_v7 blockers, such as linopirdine, consistent with both agents producing arterial relaxation through specific enhancement of K_v7 channel activity and subsequent hyperpolarization.

This study also determined the relative expression of different *KCNQ* isoforms in the rat thoracic aorta, renal artery and mesenteric artery. In these vessels, *KCNQ*1, *KCNQ*4 and *KCNQ*5 expression was detected, *KCNQ*3 expression was variable and *KCNQ*2 message was never detected, suggesting that this gene is not expressed in these arteries or the copy number is considerably lower than detectable levels for this assay. These data support previous findings that *KCNQ*1, *KCNQ*4 and *KCNQ*5 dominate in vascular smooth muscle. Furthermore, the present study shows that the relative levels of *KCNQ* isoform expression vary considerably between different vessels, with *KCNQ*1 expression predominating in the aorta and renal artery, whereas in third-order mesenteric arteries, *KCNQ*4 expression predominated.

A particularly interesting observation in this study was the increased potency of ML213 and NS15730 in the mesenteric artery compared with the aorta or renal artery. ML213 and NS15730 affects $K_v7.2$ and 7.4 and $K_v7.2$–7.5, respectively, without affecting $K_v7.1$ (Yu *et al.*, 2011; Dalby-Brown *et al.*, 2013). The expression profiles of the *KCNQ* isoforms in these vessels suggest that the relative contributions of *KCNQ*4 and *KCNQ*5 are greater in the mesenteric artery than the other vessels tested. In the thoracic aorta and renal artery, *KCNQ*1 expression predominates, which if translated in these ratios to a protein level, might explain the reduced potency of the K_v7 enhancers in these vessels compared with the mesenteric artery. Considering the pharmacological profiles of the K_v7 enhancers and given that *KCNQ*3 expression is rarely detected in the vasculature and *KCNQ*2 is never detected (Ohya *et al.*, 2003; Yeung *et al.*, 2007; Ng *et al.*, 2011; Chadha *et al.*, 2012a,b; Chadha *et al.*, 2014; Brueggemann *et al.*, 2014; this study), the effect of ML213 at the concentrations reported in the present study are likely to be due to the enhancement of native $K_v7.4$ channels, whereas the effects of NS15730 are probably due the enhancement of both $K_v7.4$ and 7.5. Recently, proximity ligation assays in cerebral and mesenteric arteries (Brueggemann *et al.*, 2014; Chadha *et al.*, 2014) have shown that, in these vessels, $K_v7.4$ subunits are likely to exist as either homomeric $K_v7.4$ channels or a $K_v7.4$/$K_v7.5$ heteromultimer. Therefore, the increased potency of ML213 and NS15730 in the mesenteric artery might reflect a dominance of the $K_v7.4$ or $K_v7.4$/$K_v.7.5$ channels compared with in the aorta or renal artery. However, linopirdine and XE991, which block all K_v7 channels, rarely contract mesenteric arteries especially in the absence of any vasoconstrictor pre-tone, whereas aorta and renal arteries contract readily to these agents (Chadha *et al.*, 2012a,b). Future studies need to

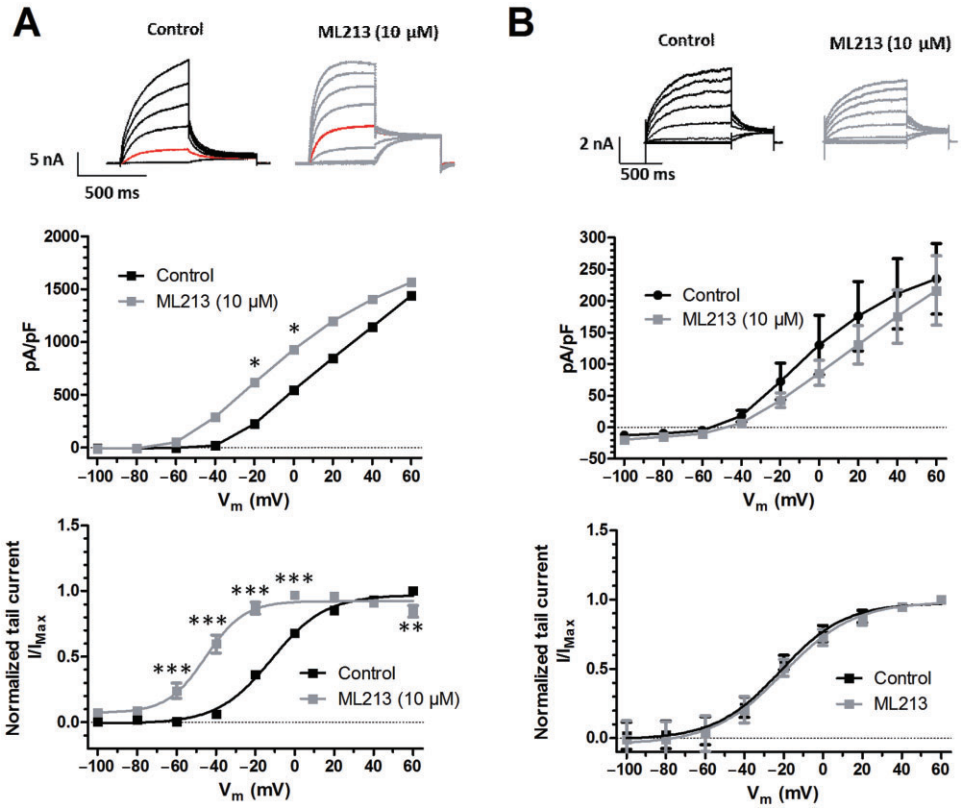

Figure 6

Effect of ML213 on (A) $hK_v7.4$ channels and (B) on mutated $hK_v7.4$-W242L channels. (Top panel) Representative current traces recorded from HEK293 cells either (A) stably expressing $hK_v7.4$ or (B) transiently transfected with $hK_v7.4$-W242L before and after application of ML213. Red traces represent the current elicited at −20 mV. (Middle panel) Current–voltage relationship comparing the effect of ML213 on $hK_v7.4$ and $hK_v7.4$-W242L. Current values are plotted against test potential before and after application of the drug. Currents were elicited by depolarizing steps from −100 to +60 mV (20 mV increments, 500 ms duration for the $hK_v7.4$ currents and 1 s for the $hK_v7.4$-W242L currents) from a holding potential of −80 mV. A Bonferroni *post hoc* test was performed following a two-way ANOVA and $^*P < 0.05$. (Bottom panel) Voltage-dependence of activation. Curves were obtained by normalizing the tail current at −30 mV against the maximal tail current measured in each experiment and plotted as a function of the preceding step potential ($n = 6$). Finally, a Boltzmann fit was performed to determine the $V_{½}$ values. A two-way ANOVA followed by a Bonferroni multiple comparisons test was performed, and $^{**}P < 0.01$ and $^{***}P < 0.001$.

focus on the various factors that are responsible for the regional effectiveness of K_v7 modulators.

In the mesenteric artery, this study found the relaxant EC_{50} values of the previously reported enhancers (S-1, BMS204352 and retigabine) to range between 2 and 11 μM, whereas ML213 displayed an EC_{50} of 0.74 μM and NS15370 was particularly potent with an EC_{50} of 0.026 μM. To date, no other K_v7 channel enhancers have been shown to relax blood vessels with such potency. ML213 and NS15370 also relaxed thoracic aorta segments with increased potency compared with the other enhancers tested, and in the renal artery, as well as NS15370 and ML213, S-1 was also equally potent at causing vasorelaxations. The low relaxant EC_{50}s presented in this study for NS15370 are in keeping with EC_{50} values estimated from concentration–response curves using a Fluorimetric Imaging Plate Reader-based Tl^+ influx assay (Dalby-Brown *et al.*, 2013), as well QPatch data presented in this study. Importantly, the vasorelaxant effects were inhibited by the K_v7 channel blocker linopirdine, suggesting the effects of these new enhancers can be attributed specifically to K_v7 channel enhancement.

We tested the effect of the two novel K_v7 channel enhancers on HEK293B cells stably expressing *KCNQ*4 and used a mutated *KCNQ*4 to determine the binding site of these novel enhancers. NS15370 and ML213 caused hyperpolarizing shifts in the $V_{½}$ of activation as previously reported (Dalby-Brown *et al.*, 2013). This is in keeping with the effects other pharmacological enhancers of K_v7 channels that have also been shown to cause a hyperpolarizing shift in the $V_{½}$ of activation, including BMS204352 (Schrøder *et al.*, 2001), S-1 (Bentzen *et al.*, 2006) and retigabine (Main *et al.*, 2000). In this study, we also observed that NS15370 inhibited currents at positive potentials and the threshold for a net inhibition became more negative with increasing concentration. These findings are in agreement with those of Dalby-Brown *et al.* (2013), who also observed a current depression at higher concentrations of this enhancer. This effect has also been reported for retigabine and S-1 in overexpression systems (Main *et al.*, 2000; Bentzen *et al.*, 2006). Moreover, remarkably similar bimodal effects were reported for retigabine on native K^+ currents in mouse portal vein smooth muscle cells

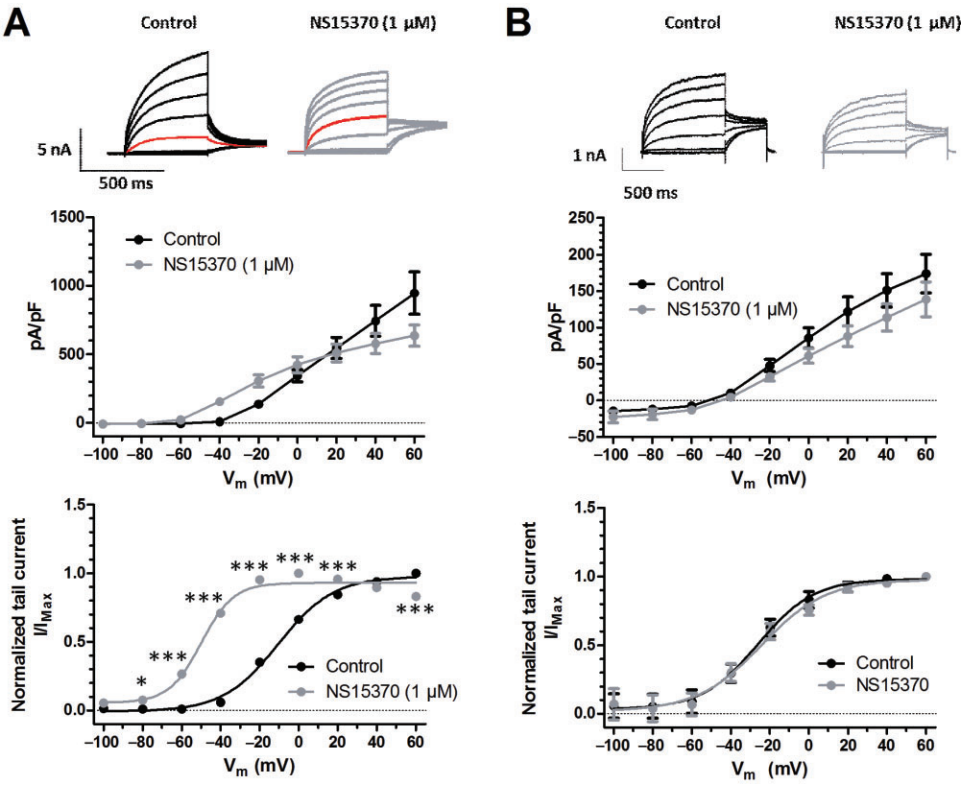

Figure 7

Effect of NS15370 on (A) $hK_v7.4$ channels and (B) on mutated $hK_v7.4$-W242L channels. (Top panel) Representative current traces recorded from HEK293 cells either (A) stably expressing $hK_v7.4$ or (B) transiently transfected with $hK_v7.4$-W242L before and after application of NS15370. (Middle panel) Current–voltage relationship comparing the effect of NS15370 on $hK_v7.4$ and $hK_v7.4$-W242L. Current values are plotted against test potential before and after application of drug. Currents were elicited by depolarizing steps from −100 to +60 mV (20 mV increments, 500 ms duration for the $hK_v7.4$ currents and 1 s for the $hK_v7.4$-W242L currents) from a holding potential of −80 mV. A Bonferroni *post hoc* test was performed following a two-way ANOVA and no points were found to be significantly different from their respective control. (Bottom panel) Voltage-dependence of activation. Tail current–voltage relationship was obtained by plotting the normalized tail current amplitude at −30 mV against the maximal tail current and plotted as a function of the preceding step potential (n = 6–8). Finally a Boltzmann fit was performed to determine the $V_{½}$ values. A Bonferroni *post hoc* test was performed following a two-way ANOVA, and $^*P < 0.05$ and $^{***}P < 0.001$.

(Yeung *et al.*, 2008), and were observed in voltage clamp studies on mesenteric arteries in the present study (data not shown). Interestingly, both the stimulatory and inhibitory effects of NS15370 were lost in HEK cells overexpressing a *KCNQ*4 mutant with the tryptophan-242 residue changed to a leucine that has been determined to be crucial for retigabine's action. This residue is found in a hydrophobic pocket of the S5 domain and only when the channel is in the open state is this residue available for the K_v7 channel enhancers to bind and stabilize the channel in the open configuration (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005; Lange *et al.*, 2009). The finding that ML213 and NS15730 produced marked membrane hyperpolarization and are reliant upon this essential tryptophan residue adds credence to the view that K_v7 channels contribute to the resting membrane conductance in smooth muscle cells.

Recently, the use of retigabine (Trobalt) to pharmacologically enhance K_v7 channels has been shown to prevent seizure generation by suppressing neuronal activity (Brodie *et al.*, 2010; French *et al.*, 2011). However, retigabine enhances $K_v7.2$–7.5 channels, which leads to various smooth muscle side effects (Jepps *et al.*, 2013); therefore, to treat epilepsy, it is desirable to produce K_v7 enhancers with increased specificity and potency for $K_v7.2$–7.3 channels. NS15370 and ML213 are two such activators that were identified in an attempt to develop such an anti-epileptic, or possibly to treat other neurological disorders such as mania and psychosis (Dalby-Brown *et al.*, 2013). However, the data presented in this study show that these activators are also potent vasorelaxants in a range of blood vessels and can hyperpolarize the resting membrane potential of mesenteric artery smooth muscle cells. Moreover, ML213 has been shown to inhibit contractions in pig detrusor muscle suggesting that the use of selective $K_v7.4$ channel modulators could be used in the treatment of detrusor overactivity (Svalø *et al.*, 2013). This study has only focused on vascular smooth muscle; however, it is also known that K_v7 channels are expressed and regulate non-vascular smooth muscle contractility, including the digestive system (Ohya *et al.*, 2002; Jepps *et al.*, 2009; Ipavec *et al.*, 2011), uterus (McCallum *et al.*, 2009; 2011), bladder (Streng *et al.*, 2004; Rode *et al.*, 2010; Svalø *et al.*, 2011; 2013) and airways (Brueggemann *et al.*, 2012), all of which should

be considered when trying to characterize a new K_v7 channel enhancer as an anti-epileptic. It is now widely recognized that the 'neuronal' K_v7 channels are not exclusively expressed in neurones, and this study highlights the possibility that these new compounds have potential as therapeutics for various smooth muscle disorders.

Acknowledgements

We would like to thank the staff at the Biological Research Facility at St. George's University of London. J. B. S. and S. P. were funded by the British Heart Foundation and by Medical Research Council grants (PG/12/63/29824 and MR/K019074/1) respectively.

Author contributions

T. A. J., B. H. B., J. B. S., O. V. P. and K. S. performed experiments and analysed data. T. A. J., B. H. B., J. B. S. and W. D. B. and I. A. G. were involved in the preparation and submission of the paper. I. A. G. also provided funding.

Conflict of interests

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Ion channels. Br J Pharmacol 170: 1607–1651.

Bentzen BH, Schmitt N, Calloe K, Dalby-Brown W, Grunnet M, Olesen SP (2006). The acrylamide (S)-1 differentially affects Kv7 (*KCNQ*) potassium channels. Neuropharmacology 51: 1068–1077.

Blom SM, Schmitt N, Jensen HS (2010). Differential effects of ICA-27243 on cloned Kv7 channels. Pharmacology 86: 174–181.

Brodie MJ, Lerche H, Gil-Nagel A, Elger C, Hall S, Shin P *et al.* (2010). Efficacy and safety of adjunctive ezogabine (retigabine) in refractory partial epilepsy. Neurology 75: 1817–1824.

Brueggemann LI, Kakad PP, Love RB, Solway J, Dowell ML, Cribbs LL *et al.* (2012). Kv7 potassium channels in airway smooth muscle cells: signal transduction intermediates and pharmacological targets for bronchodilator therapy. Am J Physiol Lung Cell Mol Physiol 302: L120–L132.

Brueggemann LI, Mackie AR, Cribbs LL, Freda J, Tripathi A, Majetschak M *et al.* (2014). Differential protein kinase C-dependent modulation of Kv7.4 and Kv7.5 subunits of vascular Kv7 channels. J Biol Chem 289: 2099–2111.

Chadha PS, Zunke F, Zhu HL, Davis AJ, Jepps TA, Olesen SP *et al.* (2012a). Reduced *KCNQ*4-encoded voltage-dependent potassium channel activity underlies impaired β-adrenoceptor-mediated relaxation of renal arteries in hypertension. Hypertension 59: 877–884.

Chadha PS, Zunke F, Davis AJ, Jepps TA, Linders JT, Schwake M *et al.* (2012b). Pharmacological dissection of Kv7.1 channels in systemic and pulmonary arteries. Br J Pharmacol 166: 1377–1387.

Chadha PS, Jepps TA, Carr G, Stott JB, Zhu HL, Cole WC *et al.* (2014). Contribution of Kv7.4/Kv7.5 heteromers to intrinsic and calcitonin gene-related peptide-induced cerebral reactivity. Arterioscler Thromb Vasc Biol 34: 887–893.

Dailey JW, Cheong JH, Ko KH, Adams-Curtis LE, Jobe PC (1995). Anticonvulsant properties of D-20443 in genetically epilepsy-prone rats: prediction of clinical response. Neurosci Lett 195: 77–80.

Dalby-Brown W, Jessen C, Hougaard C, Jensen ML, Jacobsen TA, Nielsen KS *et al.* (2013). Characterization of a novel high potency positive modulator of Kv7 channels. Eur J Pharmacol 709: 52–63.

French JA, Abou-Khalil BW, Leroy RF, Yacubian EM, Shin P, Hall S *et al.* (2011). Randomized, double-blind, placebo-controlled trial of ezogabine (retigabine) in partial epilepsy. Neurology 76: 1555–1563.

Ipavec V, Martire M, Barrese V, Taglialatela M, Currò D (2011). Kv7 channels regulate muscle tone and nonadrenergic noncholinergic relaxation of the rat gastric fundus. Pharmacol Res 64: 397–409.

Jepps TA, Greenwood IA, Moffatt JD, Sanders KM, Ohya S (2009). Molecular and functional characterization of Kv7 K^+ channel in murine gastrointestinal smooth muscles. Am J Physiol Gastrointest Liver Physiol 297: G107–G115.

Jepps TA, Chadha PS, Davis AJ, Harhun MI, Cockerill GW, Olesen SP *et al.* (2011). Downregulation of Kv7.4 channel activity in primary and secondary hypertension. Circulation 124: 602–611.

Jepps TA, Olesen SP, Greenwood IA (2013). One man's side effect is another man's therapeutic opportunity: targeting Kv7 channels in smooth muscle disorders. Br J Pharmacol 168: 19–27.

Joshi S, Sedivy V, Hodyc D, Herget J, Gurney AM (2009). *KCNQ* modulators reveal a key role for *KCNQ* potassium channels in regulating the tone of rat pulmonary artery smooth muscle. J Pharmacol Exp Ther 329: 368–376.

Khanamiri S, Soltysinska E, Jepps TA, Bentzen BH, Chadha PS, Schmitt N *et al.* (2013). Contribution of Kv7 channels to basal coronary flow and active response to ischemia. Hypertension 62: 1090–1097.

Lange W, Geissendörfer J, Schenzer A, Grötzinger J, Seebohm G, Friedrich T *et al.* (2009). Refinement of the binding site and mode of action of the anticonvulsant Retigabine on *KCNQ* K^+ channels. Mol Pharmacol 75: 272–280.

Mackie AR, Brueggemann LI, Henderson KK, Shiels AJ, Cribbs LL, Scrogin KE *et al.* (2008). Vascular *KCNQ* potassium channels as novel targets for the control of mesenteric artery constriction by vasopressin, based on studies in single cells, pressurized arteries, and *in vivo* measurements of mesenteric vascular resistance. J Pharmacol Exp Ther 325: 475–483.

Main MJ, Cryan JE, Dupere JR, Cox B, Clare JJ, Burbidge SA (2000). Modulation of *KCNQ*2/3 potassium channels by the novel anticonvulsant retigabine. Mol Pharmacol 58: 253–262.

Mani BK, Brueggemann LI, Cribbs LL, Byron KL (2011). Activation of vascular *KCNQ* (Kv7) potassium channels reverses spasmogen-induced constrictor responses in rat basilar artery. Br J Pharmacol 164: 237–249.

McCallum LA, Greenwood IA, Tribe RM (2009). Expression and function of Kv7 channels in murine myometrium throughout oestrous cycle. Pflugers Arch 457: 1111–1120.

McCallum LA, Pierce SL, England SK, Greenwood IA, Tribe RM (2011). The contribution of Kv7 channels to pregnant mouse and human myometrial contractility. J Cell Mol Med 15: 577–586.

Mulvany MJ, Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 41: 19–26.

Ng FL, Davis AJ, Jepps TA, Harhun MI, Yeung SY, Wan A *et al*. (2011). Expression and function of the K+ channel *KCNQ* genes in human arteries. Br J Pharmacol 162: 42–53.

Ohya S, Asakura K, Muraki K, Watanabe M, Imaizumi Y (2002). Molecular and functional characterization of ERG, *KCNQ*, and KCNE subtypes in rat stomach smooth muscle. Am J Physiol Gastrointest Liver Physiol 282: G277–G287.

Ohya S, Sergeant GP, Greenwood IA, Horowitz B (2003). Molecular variants of *KCNQ* channels expressed in murine portal vein myocytes: a role in delayed rectifier current. Circ Res 92: 1016–1023.

Padilla K, Wickenden AD, Gerlach AC, McCormack K (2009). The *KCNQ*2/3 selective channel opener ICA-27243 binds to a novel voltage-sensor domain site. Neurosci Lett 465: 138–142.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al*. (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–1106.

Rode F, Svalø J, Sheykhzade M, Rønn LC (2010). Functional effects of the *KCNQ* modulators retigabine and XE991 in the rat urinary bladder. Eur J Pharmacol 638: 121–127.

Rostock A, Tober C, Rundfeldt C, Bartsch R, Engel J, Polymeropoulos EE *et al*. (1996). D-23129: a new anticonvulsant with a broad spectrum activity in animal models of epileptic seizures. Epilepsy Res 23: 211–223.

Schenzer A, Friedrich T, Pusch M, Saftig P, Jentsch TJ, Grötzinger J *et al*. (2005). Molecular determinants of *KCNQ* (Kv7) K^+ channel sensitivity to the anticonvulsant retigabine. J Neurosci 25: 5051–5060.

Schrøder RL, Jespersen T, Christophersen P, Strøbaek D, Jensen BS, Olesen SP (2001). *KCNQ*4 channel activation by BMS-204352 and retigabine. Neuropharmacology 40: 888–898.

Soldovieri MV, Miceli F, Taglialatela M (2011). Driving with no brakes: molecular pathophysiology of Kv7 potassium channels. Physiology 26: 365–376.

Stott JB, Jepps TA, Greenwood IA (2014). Kv7 potassium channels: a new therapeutic target in smooth muscle disorders. Drug Discov Today 19: 413–424.

Streng T, Christoph T, Andersson KE (2004). Urodynamic effects of the K^+ channel (*KCNQ*) opener retigabine in freely moving, conscious rats. J Urol 172: 2054–2058.

Svalø J, Hansen HH, Rønn LC, Sheykhzade M, Munro G, Rode F (2011). Kv7 positive modulators reduce detrusor overactivity and increase bladder capacity in rats. Basic Clin Pharmacol Toxicol 110: 145–153.

Svalø J, Bille M, Parameswaran Theepakaran N, Sheykhzade M, Nordling J, Bouchelouche P (2013). Bladder contractility is modulated by Kv7 channels in pig detrusor. Eur J Pharmacol 715: 312–320.

Tober C, Rostock A, Rundfeldt C, Bartsch R (1996). D-23129: a potent anticonvulsant in the amygdala kindling model of complex partial seizures. Eur J Pharmacol 303: 163–169.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A *et al*. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: 34.

Wuttke TV, Seebohm G, Bail S, Maljevic S, Lerche H (2005). The new anticonvulsant retigabine favors voltage-dependent opening of the Kv7.2 (*KCNQ*2) channel by binding to its activation gate. Mol Pharmacol 67: 1009–1017.

Yeung S, Schwake M, Pucovský V, Greenwood IA (2008). Bimodal effects of the Kv7 channel activator retigabine on vascular K+ currents. Br J Pharmacol 155: 62–72.

Yeung SY, Pucovský V, Moffatt JD, Saldanha L, Schwake M, Ohya S *et al*. (2007). Molecular expression and pharmacological identification of a role for Kv7 channels in murine vascular reactivity. Br J Pharmacol 151: 758–770.

Yu H, Wu M, Townsend SD, Zou B, Long S, Daniels JS *et al*. (2011). Discovery, synthesis, and structure activity relationship of a series of N-Aryl-bicyclo[2.2.1]heptane-2-carboxamides: characterization of ML213 as a novel *KCNQ*2 and *KCNQ*4 potassium channel opener. ACS Chem Neurosci. 2: 572–577.

Zhong XZ, Harhun MI, Olesen SP, Ohya S, Moffatt JD, Cole WC *et al*. (2010). Participation of *KCNQ* (Kv7) potassium channels in myogenic control of cerebral arterial diameter. J Physiol 588: 3277–3293.