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RESEARCH PAPER

Treatment with the K_v7 potassium channel activator flupirtine is beneficial in two independent mouse models of pulmonary hypertension

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Background and purpose: Voltage-gated potassium (K_v) channels contribute to resting membrane potential in pulmonary artery smooth muscle cells and are down regulated in patients with pulmonary arterial hypertension (PAH) and a contribution from K_v7 channels has been recently proposed. We investigated the effect of the K_v7 channel activator, flupirtine, on PAH in two independent mouse models: PAH induced by hypoxia and spontaneous PAH in mice over-expressing the 5-HT transporter ($SERT^+$ mice).

Experimental approach: Right ventricular pressure was assessed *in vivo* in mice chronically treated with flupirtine (30 $mg \cdot kg^{-1} \cdot day^{-1}$). In separate *in vitro* experiments, pulmonary arteries from untreated mice were mounted in a wire myograph. Relaxations to acute administration of flupirtine and contractions to K_v channel blocking drugs, including the K_v7 channel blocker linopirdine, were measured.

Key results: In wild-type (WT) mice, hypoxia increased right ventricular pressure, pulmonary vascular remodelling and right ventricular hypertrophy. These effects were attenuated by flupirtine, which also attenuated these indices of PAH in $SERT^+$ mice. In the *in vitro* experiments, flupirtine induced a potent relaxant response in arteries from untreated WT and $SERT^+$ mice. The relaxation was fully reversed by linopirdine, which potently contracted mouse pulmonary arteries while other K_v channel blockers did not.

Conclusions and implications: Flupirtine significantly attenuated development of chronic hypoxia-induced PAH in mice and reversed established PAH in $SERT^+$ mice, apparently via K_v7 channel activation. These results provide the first direct evidence that drugs activating K_v7 channels may be of benefit in the treatment of PAH with different aetiologies.

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Abbreviations: BMP, bone morphogenetic protein; BMPR-II, bone morphogenetic protein receptor type II; fPAH, familial pulmonary arterial hypertension; K_v, voltage-gated potassium; LV + S, left ventricle plus septum; mRVP, mean right ventricular pressure; PAH, pulmonary arterial hypertension; PASMCs, pulmonary artery smooth muscle cells; RV, right ventricle; RVH, right ventricular hypertrophy; SERT, 5-HT (serotonin) transporter

Introduction

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary arterial pressure, concomitant with increased pulmonary vascular resistance, pulmonary vascular remodelling, right ventricle (RV) failure and death (Chin and Rubin, 2008). About 70% of patients with the familial form of the disease (fPAH) have a mutation in the gene encoding bone morphogenetic protein (BMP) receptor type II (BMPR-II), a member of the transforming growth factor-β superfamily, as the primary genetic defect (Lane *et al.*, 2000; Machado *et al.*, 2001). Voltage-gated K^+ (K_v) channels, several families of which are expressed in pulmonary artery smooth muscle cells (PASMCs), contribute to resting membrane potential and have been implicated in the regulation of vascular smooth muscle function (Archer *et al.*, 1998; Yuan *et al.*, 1998). BMP can regulate K_v channel expression in PASMCs (Young *et al.*, 2006). Moreover, K^+ channel down-regulation is observed in PASMCs from patients with PAH (Yuan *et al.*, 1998; Remillard *et al.*, 2007) and in PASMCs from rats with chronic hypoxia-induced PAH (Smirnov *et al.*, 1994; Evans *et al.*, 1996; Platoshyn *et al.*, 2001). Considerable effort

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has focused on the importance of the $K_v1.5$ and $K_v2.1$ channels, where downregulation or dysfunction may be a predisposing factor in the development of pulmonary arterial vasoconstriction and pulmonary vascular remodelling in patients with fPAH (Yuan *et al*., 1998; Remillard *et al*., 2007). However, these particular K_v channels may not play a major role in maintaining PASMC resting potential as they have a very low probability of opening at the resting membrane potential (Evans *et al*., 1996; Gurney *et al*., 2003).

KCNQ (*KCNQ1–5*) genes encode a subfamily of voltage-gated K^+ channels, denoted $K_v7.1$-$K_v7.5$. These channels have been studied primarily in the heart, CNS and auditory pathway (Jentsch, 2000; Robbins, 2001; Jespersen *et al*., 2005; Jin *et al*., 2008), but were recently identified in vascular (Ohya *et al*., 2003; Yeung *et al*., 2007; Mackie *et al*., 2008) and uterine (McCallum *et al*., 2009) smooth muscle and may regulate Na^+ flux across pulmonary epithelial cells (Greenwood *et al*., 2009). *KCNQ* genes express the well described non-inactivating, outwardly rectifying K^+ currents ('M' currents) that play a pivotal role in controlling membrane excitability in neurones (Marrion, 1997; Robbins, 2001). The background K^+ current giving rise to the resting potential of PASMCs has biophysical properties similar to those of recombinant and native K_v7 channels (Evans *et al*., 1996). Combined with the finding that K_v7 channel blocking drugs are potent constrictors of rodent pulmonary arteries (Joshi *et al*., 2006), this suggests the possible involvement of K_v7 channels in the resting potential of PASMCs.

The K_v7 channel activator, flupirtine, is used in Europe as a non-opioid analgesic and in several human trials has proven to be essentially free of cardiovascular side effects (Herrmann *et al*., 1987; Hummel *et al*., 1991; Friedel and Fitton, 1993). Flupirtine was recently shown to activate recombinant $K_v7.4$ or $K_v7.5$ channels (Yeung *et al*., 2008). Its more potent analogue, retigabine, which is not yet available for clinical use, selectively activates $K_v7.2$–$K_v7.5$ channels while having little effect on the cardiac $K_v7.1$ channel (Main *et al*., 2000; Tatulian *et al*., 2001; Schenzer *et al*., 2005; Wuttke *et al*., 2005). Flupirtine and retigabine have been reported to relax vascular smooth muscle (Joshi *et al*., 2006; Yeung *et al*., 2007; Mackie *et al*., 2008). Here we investigated the effects of flupirtine (Wladyka and Kunze, 2006) on pulmonary vascular haemodynamics and indices of PAH in two mouse models of the disease. Mice with pulmonary hypertension, induced by chronic hypoxia, were used to investigate the effect of flupirtine on the development of PAH. Mice that over-express the 5-HT transporter (SERT) have established PAH and were therefore studied to determine the effects of flupirtine in an alternative model of PAH (MacLean *et al*., 2004). Importantly, we demonstrated that flupirtine treatment attenuated the indices of PAH in these two independent murine models of the disease. Flupirtine prevented the development of hypoxia-induced PAH and reversed PAH in $SERT^+$ mice, where the disease was already established.

Methods

In vivo *experiments*

All animal procedures and experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conformed to institutional regulations at the University of Glasgow. The generation and characterization of the $SERT^+$ mice have been described previously (MacLean *et al*., 2004; Mair *et al*., 2008). All mice were bred in the University of Glasgow and the genotype of each mouse was confirmed by PCR.

Exposure to hypoxia

Female wild-type (WT) control mice (C57BL/6XCBA strain, 5–6 months old; n = 8–10 mice per group) were maintained in normoxic or hypobaric/hypoxic conditions for 2 weeks as previously described (Keegan *et al*., 2001; MacLean *et al*., 2004). The hypobaric chamber was depressurized over the course of 2 days to 550 mbar (equivalent to 10% O_2). Temperature was maintained at 21°C to 22°C, and the chamber was ventilated with air at 45 $L{\cdot}min^{-1}$. As male $SERT^+$ mice do not develop PAH (MacLean *et al*., 2004), female mice were used in this study.

Drug administration protocol

Subsets of the WT and $SERT^+$ mice were given orally, either flupirtine (30 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$; n = 8–10 mice per group) or vehicle (1% carboxymethylcellulose, n = 8–10 mice per group) alone on each of the 14 experimental days. In those WT mice also exposed to hypoxia, flupirtine (or vehicle) was administered during the 14 days of hypoxic exposure. The effectiveness of the chosen dose of flupirtine has been demonstrated in models testing for anti-nociceptive and anti-Parkinson activity in rodents (Nickel, 1987; Szelenyi *et al*., 1989; Schmidt *et al*., 1997). Age-matched flupirtine and vehicle-treated control groups were maintained in room air. All animals were maintained in cages with a 12 h light/dark cycle and free access to food and water.

Under isoflurane (1.5% in O_2) anaesthesia, mean right ventricular pressure (mRVP) was measured via a 25-gauge needle advanced into the RV trans-diaphragmatically (MacLean *et al*., 2004; Morecroft *et al*., 2007). RVP and heart rate (HR; derived from the RVP trace) were recorded on a data acquisition system (MP 100, Biopac Systems). Depth of anaesthesia was confirmed by lack of a pinch withdrawal reflex applied to the hind paw.

Indices of PAH

The ratio of right ventricular weight to left ventricular weight plus septum [RV/(LV + S)] was used as an index of right ventricular hypertrophy (RVH) (MacLean *et al*., 2004; Morecroft *et al*., 2007) and the ratio of (LV + S) to body weight (bw) was also calculated. Sagittal sections were obtained from left lungs, stained with Elastica van Giessen stain and microscopically assessed for muscularization of pulmonary arteries (<80 μm external diameter) as described previously (MacLean *et al*., 2004; Morecroft *et al*., 2007). Lung sections from four to six mice from each group were studied.

Myography

Arteries from $SERT^+$ mice and control WT mice were studied. The animals were killed by an overdose of sodium pentobar-

bitone (200 mg·kg^{-1}, i.p.) and the lungs removed. Small pulmonary arteries (third order, first intralobar) of ~350 μm internal diameter and mesenteric arteries (first order branches) were dissected, cut into 2 mm long segments, threaded onto 40 μm stainless steel wires and mounted on an isometric myograph (610 M; Danish Myo Technology, Aarhus, Denmark) as described previously (Keegan *et al.*, 2001; Morecroft *et al.*, 2007). The vessels were maintained at 37°C in Krebs buffer solution (pH7.4) of the following composition (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5, glucose 11.0 and EDTA 0.023. Pulmonary arteries were aerated with 16% O_2/5% CO_2 balance N_2 and set up at tensions equivalent to their mean *in vivo* RVP (12–15 mmHg). Mesenteric arteries were gassed with 95% O_2/5% CO_2 and mounted under their optimal resting tensions, previously calculated to be 170 mg (Daly *et al.*, 2002). Vessel responses were recorded onto a computer using a data acquisition and recording software system (Myodaq and Myodata, Danish Myo Technology). After a 45 min equilibration period, the response to 50 mM KCl, the concentration that produced maximal contraction in these vessels, was measured. On washout and re-equilibration, vessels were contracted with phenylephrine to ~75% of the maximal phenylephrine-induced tone using a concentration of 10–100 nM and cumulative concentration-response curves to flupirtine (1 nM–0.1 mM) or retigabine (1 nM–30 μM, pulmonary arteries only) constructed. In a separate series of experiments on WT pulmonary and mesenteric arteries, following phenylephrine pre-constriction, concentration-response curves to flupirtine (1 nM–0.1 mM) were constructed immediately followed by constructing cumulative concentration-response curves to linopirdine (1 nM–0.1 mM) to assess if K_v7 channel block could reverse the flupirtine-induced relaxation. In other separate experiments, after challenging mouse pulmonary arteries with 50 mM KCl and subsequent washout, cumulative concentration-response curves were constructed for the K_v7 channel blocking drug linopirdine and several other K^+ channel blocking drugs. The EC_{50} for linopirdine acting at K_v7 channels is well documented in native tissues (Lamas *et al.*, 1997; Schnee and Brown, 1998; Joshi *et al.*, 2006) and expression systems (Robbins, 2001). The other K^+ channel blocking drugs included 4-aminopyridine (4-AP) and capsaicin, used as broad spectrum Kv channel inhibitors (Grissmer *et al.*, 1994; Coetzee *et al.*, 1999), tetraethylammonium ions (TEA), which inhibit BK_{Ca}, Kv1.1, Kv1.2 and Kv3.1 at low millimolar concentrations (Grissmer *et al.*, 1994; Coetzee *et al.*, 1999), glibenclamide for selective K_{ATP} channel block (Clapp and Gurney, 1992) and $ZnCl_2$, which blocks the TASK-like K^+ current in rabbit PASMCs (Gurney *et al.*, 2003).

The drug/molecular target nomenclature conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Data analysis

Contractile responses to K^+ channel blockers were normalized by expressing them as a percentage of the 50 mM KCl response of each arterial segment. Vasodilator responses were measured as a percentage of the pre-constrictor tone induced by phenylephrine. The maximal vasoconstrictor/vasodilator effect (E_{max}) and the pEC_{50} or pIC_{50} were calculated from a sigmoidal nonlinear regression curve fit. Individual comparisons were made using Student's unpaired t-test, as appropriate. Multiple comparisons were made using one-way ANOVA, followed by Neuman Keuls *post hoc* test. GraphPad Prism (version 4) was used to perform all statistical analyses and values of $P < 0.05$ were considered to be significant.

Materials and solutions

Flupirtine maleate, linopirdine dihydrochloride, glibenclamide, capsaicin and 4-AP were purchased from Tocris Cookson (Bristol, UK). Retigabine was a gift from Astra Zeneca. Stock solutions were prepared in distilled water (linopirdine), dimethylsulphoxide (other drugs) or 100 mM HEPES buffer (4-AP) with the pH adjusted to 7.3. Stock solutions of flupirtine for oral administration were made up in 1% caboxymethylcellulose in water. Phenylephrine hydrochloride, $ZnCl_2$, TEA chloride and carboxymethylcellulose were from Sigma-Aldrich (Poole, UK) and stock solutions were prepared in distilled water.

Results

In vivo experiments

As previously reported (MacLean *et al.*, 2004), under normoxia $SERT^+$ mice demonstrated markedly elevated mRVP, compared with WT mice ($P < 0.001$; Figure 1A). Treatment with flupirtine markedly reversed this elevation to levels commensurate with those in WT mice ($P < 0.001$ vs. vehicle-treated $SERT^+$ mice) (Figure 1A). As in previous studies (MacLean *et al.*, 2004), the $SERT^+$ mice displayed a low proportion of remodelled pulmonary arteries and this was not significantly affected by flupirtine treatment (Figure 1B). RVH, as indicated by an increase in the ratio RV/(LV + S), was observed in the $SERT^+$ mice compared with WT mice (Figure 1C) and this was also attenuated by flupirtine [Figure 1C ($P < 0.05$)].

Wild-type mice maintained in a hypoxic environment developed pulmonary hypertension within 14 days, with mRVP increased by around 50% compared with normoxic WT animals (Figure 1A). Flupirtine treatment attenuated the development of chronic hypoxia-induced pulmonary hypertension, with mRVP reduced to near normal values (Figure 1A). As a consequence of the increased RVP, the hypoxic WT mice developed RVH, with RV/(LV + S) increasing in the normoxic animals after exposure to hypoxia (Figure 1C; $P < 0.001$) and treatment with flupirtine reduced the hypertrophy, (Figure 1C; $P < 0.05$ vs. vehicle-treated hypoxic animals). In hypoxia-induced PAH, the rise in mRVP is associated with remodelling of the pulmonary arteries of diameter <80 μm. In control WT mice, the majority of vessels of this diameter were not remodelled, only a very small percentage being fully muscularized (Figure 1B). In hypoxic WT mice, a significant increase in the percentage of remodelled pulmonary arteries was evident (Figure 1B). Treatment with flupirtine resulted in a significant reduction in the percentage of remodelled arteries, compared with the vehicle-treated hypoxia group (Figure 1B).

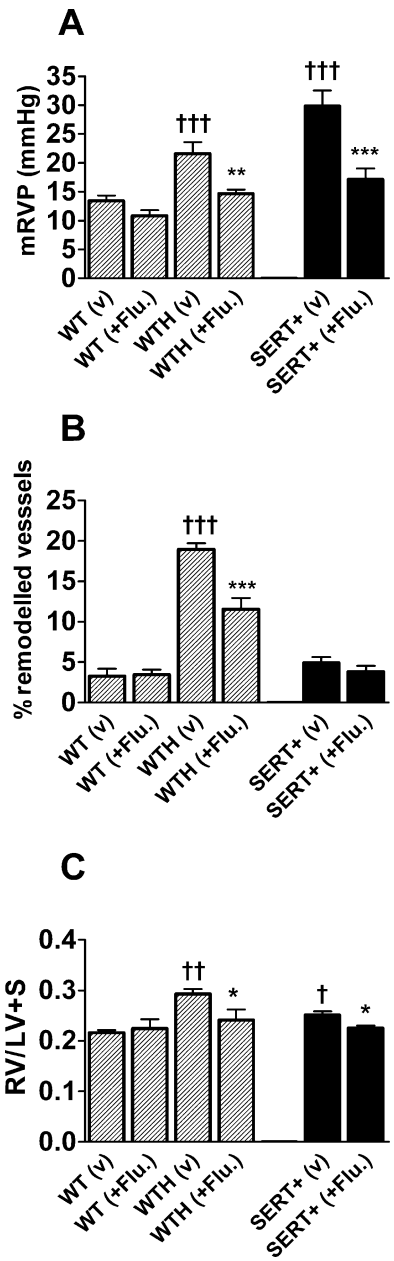

Figure 1 Effect of chronic flupirtine treatment on indices of PAH in anaesthetized mice. (A) Mean RVP (mRVP), (B) pulmonary vascular remodelling and (C) right ventricular hypertrophy in vehicle treated (v) and flupirtine (+Flu.) treated (30 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ by oral gavage) WT normoxic and hypoxic (WTH) mice and in normoxic SERT+ mice. *Value significantly less than corresponding value in vehicle treated mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). †Value significantly greater than corresponding value in normoxic WT mice (†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$). Data are expressed as mean ± SEM from six to eight experiments. LV + S, left ventricle plus septum; mRVP, mean right ventricular pressure; SERT+, mice that over-express the 5-HT transporter; WT, wild-type mice.

The effects of flupirtine were specific for the rise in mRVP seen in SERT+ mice and WT mice exposed to hypoxia for 14 days. The drug had no effect on the indices of PAH measured in normoxic WT mice (Figure 1). Flupirtine also had no significant effect on the HR, bw or (LV + S)/bw ratio in any of the treatment groups (Table 1).

Myography

Flupirtine relaxed pre-constricted pulmonary arteries from WT and SERT+ mice (Figure 2), although it was more potent in the WT vessels where it caused ~40% and ~60% relaxation at 10 and 30 μM respectively. Retigabine also relaxed pre-constricted pulmonary arteries from WT mice, with around 60% relaxation produced at 10 μM and 75% relaxation at 30 μM (Figure 3A). The effect of flupirtine was not restricted to the pulmonary circulation, because it also relaxed pre-constricted mesenteric arteries from WT mice (Figure 3A). Flupirtine appeared to be slightly more potent in relaxing mesenteric arteries and, unlike the response in pulmonary arteries, the relaxation response in mesenteric arteries reached a maximum at 30 μM (Figure 3A), allowing us to measure the pIC_{50} (5.6 ± 0.1) and E_{max} (94 ± 8%; $n = 6$ mice). To assess whether the relaxation effect of flupirtine was mediated by the opening of K_v7 channels, the ability of linopirdine to reverse the relaxation was investigated. In the continued presence of phenylephrine and 100 μM flupirtine, linopirdine was applied at increasing concentrations and the recovery of tension measured. Figure 3B shows that the response of pulmonary arteries to flupirtine was completely reversed by linopirdine with $pEC_{50} = 6.25 \pm 0.06$ ($n = 5$). In contrast, the response to flupirtine was only partially reversed by linopirdine in the mesenteric arteries (Figure 3B; $pEC_{50} = 6.47 \pm 0.13$, $n = 5$).

The effects of a range of different K^+ channel blockers were tested on the baseline tone of WT and SERT+ pulmonary arteries, in order to assess which of the K^+ channels might be responsible for maintaining a negative membrane potential in the PASMCs, thereby keeping the vessels relaxed. Only three drugs, linopirdine, 4-AP and TEA, produced sizeable constriction (Figure 4). In vessels from WT mice, linopirdine was around five orders of magnitude more potent than any other drug, producing constriction with $pEC_{50} = 6.37 \pm 0.18$ and $E_{max} = 56 \pm 4\%$ ($n = 7$) of the response to 50 mM KCl (Figure 4A). TEA and 4-AP were very weak vasoconstrictors, eliciting a significant response only at concentrations ≥10 mM (Figure 4A), while glibenclamide (data not shown), capsaicin (data not shown) and $ZnCl_2$ (Figure 4A) all failed to evoke constriction at concentrations up to 1 mM. A similar pattern of effects was observed in pulmonary arteries from the SERT+ mice (Figure 4B). Interestingly although, SERT+ vessels were significantly less responsive to linopirdine than WT vessels, with $pEC_{50} = 5.24 \pm 0.16$ ($P < 0.001$) and $E_{max} = 33 \pm 4$ % (Figure 4B; $n = 8$, $P < 0.001$). In contrast, SERT+ vessels appeared to have increased sensitivity to 4-AP, with 10 mM 4-AP evoking constriction amounting to 76 ± 8% ($n = 5$) of the response to 50 mM KCl, compared with 26 ± 7% of the KCl response in WT vessels (Figure 4B; $n = 6$, $P < 0.001$).

Discussion

This is the first report in which K_v7 channels have been directly implicated in PAH. The main finding is that treatment with the selective K_v7 channel activator flupirtine markedly attenuated the development of PAH induced by chronic hypoxia in mice and, moreover, completely reversed established PAH in mice over-expressing the SERT. In chronic

Table 1 Body weight (bw), heart rate and left heart weight to bw ratio in wild-type (WT) and SERT$^+$ mice after 14 days of chronic hypoxia and treatment with vehicle or flupirtine (30 $mg^{-1}\cdot kg^{-1}\cdot day^{-1}$)

Group	*Heart rate (beats·min^{-1})*	*bw (g)*	*LV + S/bw (mg·g^{-1})*
WT normoxic vehicle-dosed	365 ± 10	32.1 ± 1.3	3.22 ± 0.10
WT normoxic flupirtine-dosed	397 ± 32	28.0 ± 1.2	3.17 ± 0.22
WT hypoxic vehicle-dosed	379 ± 39	28.3 ± 1.1	3.04 ± 0.15
WT hypoxic flupirtine-dosed	418 ± 24	30.5 ± 1.6	3.13 ± 0.08
SERT$^+$ normoxic vehicle-dosed	469 ± 25	27.3 ± 4.8	3.43 ± 0.19
SERT$^+$ normoxic flupirtine-dosed	499 ± 29	22.8 ± 2.5	3.53 ± 0.11

Values shown are mean ± SEM; n = 6–8 animals per group.
LV + S, left ventricle plus septum; SERT$^+$, mice which over-express the 5-HT transporter.

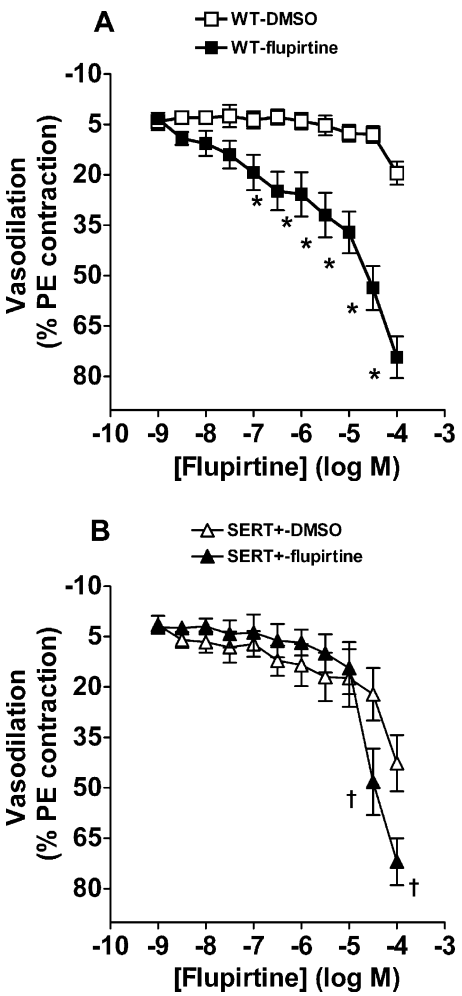

Figure 2 Vasodilator responses to the K_v7 channel activator flupirtine in precontracted pulmonary arteries. Cumulative concentration response curves to flupirtine in (A) WT and (B) SERT$^+$ mouse pulmonary arteries pre-constricted with phenylephrine (PE; 10–100 nM). Vehicle (DMSO) data are also shown and illustrate the fall in vascular tone in vessels set up in parallel with those to which flupirtine was added. *Value significantly greater than corresponding value in vehicle treated WT vessels (*$P < 0.001$). †Value significantly greater than corresponding value in vehicle treated SERT$^+$ vessels (†$P < 0.001$). Data are expressed as a percentage of the response to PE-induced preconstriction and shown as mean ± SEM from five to seven experiments. DMSO, dimethylsulphoxide; PE, phenylephrine; SERT$^+$, mice that over-express the 5-HT transporter; WT, wild-type mice.

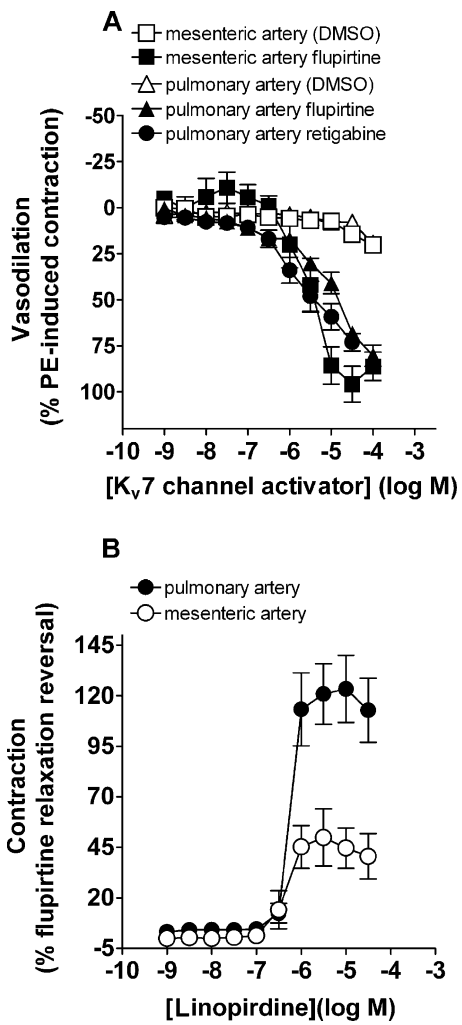

Figure 3 (A) Vasodilator responses to the K_v7 channel activators flupirtine and retigabine in WT mouse pulmonary arteries and flupirtine in mesenteric arteries pre-constricted with phenylephrine (PE; 10–100 nM). Vehicle (DMSO) data are also shown and illustrate the fall in vascular tone in vessels set up in parallel with those to which K_v7 channel activators were added. (B) Reversal by linopirdine of the flupirtine-induced vasodilator response shown in Figure 3A in the same WT mouse pulmonary and mesenteric arteries. Data are expressed as a percentage of the response to PE-induced preconstruction (A) and as a percentage reversal of flupirtine-induced vasodilation (B) and shown as mean ± SEM from five to eight experiments. DMSO, dimethylsulphoxide; PE, phenylephrine; WT, wild-type mice.

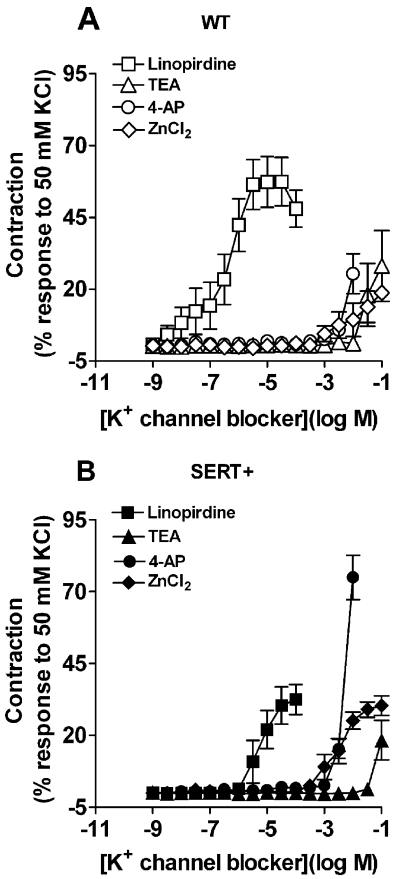

Figure 4 Vasoconstrictor responses to K^+ channel blockers in WT and $SERT^+$ mouse pulmonary arteries. Concentration response curves to linopirdine, TEA, 4-AP and $ZnCl_2$ in pulmonary arteries from (A) WT and (B) $SERT^+$ mice. Data are expressed as a percentage of the reference vasoconstrictor response to 50 mM KCl and shown as mean ± SEM from five to eight experiments. 4-AP, 4-aminopyridine; $SERT^+$, mice that over-express the 5-HT transporter; TEA, tetraethylammonium; WT, wild-type mice; $ZnCl_2$, zinc chloride.

hypoxic mice, flupirtine attenuated mRVP, pulmonary vascular remodelling and RVH, suggesting that K_v7 channels are present in mouse pulmonary vasculature and that their activation can prevent the development of hypoxic PAH. We propose that this is because flupirtine hyperpolarizes PASMCs, maintaining a negative membrane potential and preventing voltage-gated Ca^{2+} influx. This has recently been shown in rat pulmonary artery smooth muscle (Joshi *et al.*, 2009). The beneficial effect of flupirtine on PAH could be ascribed to several possible mechanisms, including its inhibitory effect on pulmonary vasoconstriction or, perhaps, the prevention of K_v channel down regulation, which has been proposed to mediate mitogenic effects on PASMCs (Burg *et al.*, 2008). Recent data from pharmacological studies strongly support the presence of functional K_v7 channels in mouse pulmonary arteries (Joshi *et al.*, 2006).

Several K^+ channel subfamilies have been implicated in the regulation of resting membrane potential in PASMCs, including a number of voltage-gated K^+ channels (Archer *et al.*, 1998; Remillard *et al.*, 2007) and two-pore domain channels, especially TASK-1 (Gurney *et al.*, 2003; Gardener *et al.*, 2004; Olschewski *et al.*, 2006). It was therefore important to identify which K^+ channels are likely to be open constitutively, contributing to PASMC resting membrane potential, and thus regulating resting tone in WT and $SERT^+$ mouse pulmonary arteries. The failure of glibenclamide to significantly affect baseline tone at concentrations known to selectively block K_{ATP} channels is consistent with findings on pulmonary arteries from other species (Clapp and Gurney, 1992) and implies that K_{ATP} channels contribute little to resting tone in WT or $SERT^+$ mice. Likewise, the lack of a significant contractile response to 4-AP or capsaicin, at concentrations inhibiting $K_v2.1$, and all K_v1 channels, and TEA at concentrations blocking $K_v1.1$, $K_v1.2$, $K_v3.1$ and BK_{Ca} channels (Grissmer *et al.*, 1994; Coetzee *et al.*, 1999), implies that BK_{Ca} and most Kv channels also make little contribution to resting tone in WT or $SERT^+$ mice. Selective blockers of TASK channels are not available, so we made use of Zn^{2+} ions, which at 100 μM depolarized rabbit PASMCs and inhibited a TASK-like K^+ current (Gurney *et al.*, 2003). Although $ZnCl_2$ failed to constrict mouse pulmonary arteries until the millimolar range, a role for TASK channels in regulating resting membrane potential in murine PASMCs cannot be ruled out, because Zn^{2+} can also block voltage-gated Ca^{2+} channels (Sun *et al.*, 2007), possibly counteracting potential constriction. The selective K_v7 channel blocker, linopirdine, was the only drug that induced potent vasoconstriction in pulmonary arteries from WT and $SERT^+$ mice, at concentrations shown to be selective for K_v7 channels in native tissues (Lamas *et al.*, 1997; Schnee and Brown, 1998) and expression systems (Robbins, 2001). Although interactions of linopirdine with other K^+ channels have been reported, these only occurred at higher concentrations (Wladyka and Kunze, 2006). The response to linopirdine is consistent with an earlier study on rat and mouse pulmonary arteries, which showed that it occurs independently of endothelium or nerve-derived mediators (Joshi *et al.*, 2006). Our results therefore strongly suggest that K_v7 channels may be the major K^+ channels regulating resting membrane potential and resting tone in mouse PASMCs.

Until further molecular and biophysical information is available, it is only possible to speculate which subtypes of the K_v7 channel are involved in regulating resting membrane potential and resting tone in mouse PASMCs as the β-subunit expression products of the *KCNE* genes can alter the biophysical and pharmacological properties of the K_v7 channels (Robbins, 2001). From *in vitro* studies, as flupirtine and retigabine are not active at $K_v7.1$ channels (Schenzer *et al.*, 2005; Yeung *et al.*, 2008), the effects of these drugs are likely to reflect actions on channels containing $K_v7.4$ and/or $K_v7.5$ channel subunits, although the weak effect of TEA on vessel tone argues against a major role for $K_v7.4$ homomers (Hadley *et al.*, 2000). The very weak effects of Zn^+ may rule out an involvement of a $K_v7.2$ + 7.3 heteromer; however, Zn^{2+} can potentiate $K_v7.5$ channels (Jensen *et al.*, 2005), causing vasodilation, which may counteract any vasoconstriction. Pulmonary arteries express *KCNQ4* mRNA abundantly, at higher levels than *KCNQ1*, and *KCNQ5* was also present, but not *KCNQ2* or *KCNQ3* (Joshi *et al.*, 2009). We, therefore, suggest that homomeric $K_v7.5$, or heteromeric channels containing $K_v7.4$ subunits, may be involved in the protective effects of flupirtine on the pulmonary circulation.

The lower potency of flupirtine in relaxing SERT$^+$ mouse arteries, compared with WT arteries, mirrors the loss of linopirdine constrictor potency and may reflect reduced *KCNQ* gene expression or possibly a change in the *KCNQ* subtype contributing to the channel. Although *KCNQ* expression has not been investigated in SERT$^+$ mice, the mice have been shown to express reduced levels of $K_v1.5$ and $K_v2.1$ mRNA (Guignabert *et al.*, 2006). Reduced *KCNQ* mRNA expression has also been shown in hypoxic rats (Joshi *et al.*, 2008). 5-HT is thought to modulate (inhibit) the activity of voltage-gated K^+ channels via signalling through the 5-HT_{2A} receptor (Cogolludo *et al.*, 2006) and human PASMCs treated with 5-HT show reduced $K_v1.5$ and $K_v2.1$ expression (Guignabert *et al.*, 2006). If K_v7 channels are the dominant regulators of resting potential, reduced *KCNQ* expression would be expected to cause depolarization, thereby promoting the opening of K^+ channels with a higher voltage threshold for activation, such as K_v1 or K_v2. An increased contribution of these channels to the resting potential may therefore explain why the loss of linopirdine potency was accompanied by an increase in the potency of 4-AP. A similar increase in 4-AP constrictor potency, due to loss of the background K^+ conductance and consequent membrane depolarization, was seen in hypoxic PAH despite concurrent loss of 4-AP sensitive current (Osipenko *et al.*, 1988).

The finding that flupirtine and its more potent analogue, retigabine, relaxed isolated, pre-constricted mouse pulmonary arteries indicates that flupirtine may mediate its beneficial effects on PAH at least in part by acting as a pulmonary arterial vasodilator to improve pulmonary haemodynamics. That the relaxation was completely reversed by linopridine is consistent with flupirtine acting via K_v7 channels. It is interesting that despite the reduced potency in isolated SERT$^+$ arteries, flupirtine *in vivo* reduced the indices of PAH in SERT$^+$ and chronic hypoxic mice while having no effect on WT mice. As the effect of flupirtine is only apparent in pre-constricted vessels, this probably reflects the minimal pulmonary vasoconstriction present in normoxic WT animals.

Although flupirtine also relaxed pre-constricted mouse mesenteric arteries, this effect may not be due entirely to activation of K_v7 channels, because it was only partly reversed by linopirdine. Our results are consistent with recent reports that flupirtine and retigabine dilated isolated systemic arteries from mice (Yeung *et al.*, 2007) and decreased mean arterial pressure in rats when given intravenously (Mackie *et al.*, 2008). This highlights a potential side effect of flupirtine treatment. It could be argued that the effect of flupirtine on RVP reflects a decrease in cardiac output rather than a change in pulmonary vascular resistance *per se*, as K_v7 channels are present in the heart (Sanguinetti *et al.*, 1996; Calloe *et al.*, 2007). This is unlikely, because the predominant K_v7 isoform in the heart is $K_v7.1$ (Sanguinetti *et al.*, 1996), which lacks the transmembrane tryptophan residue thought to be essential for the actions of flupirtine (Schenzer *et al.*, 2005; Wuttke *et al.*, 2005; Bentzen *et al.*, 2006). The lack of effect of flupirtine on HR is consistent with this and minimal cardiac effects are seen in humans undergoing flupirtine treatment (Hermann *et al.*, 1987). Although a central cardiovascular action of flupirtine cannot be ruled out, intracisternal administration of flupirtine does not alter mean arterial pressure in rabbits (Yoro *et al.*, 2008).

Pulmonary vascular remodelling occurs as a result of PASMC proliferation and attenuated PASMC apoptosis. Reduced K^+ channel activity in PAH patients is thought to promote PASMC proliferation through an increase in the concentration of free Ca^{2+} within the cytoplasm (Platoshyn *et al.*, 2000; Burg *et al.*, 2008), allowing for progression through the cell cycle. As flupirtine attenuated hypoxia-induced pulmonary vascular remodelling in WT mice, K_v7 channel dysfunction/down-regulation may be a feature of PAH, and K_v7 channel activation may inhibit PASMC proliferation. Several limitations to this study deserve comment. The study on chronic hypoxic mice concentrated on the preventive effects of flupirtine on the development of hypoxia-induced PAH, which is not easily extrapolated to patients with end-stage disease. Preventative therapy may, however, be useful, for example in carriers within families with a mutation in BMPR-II (Machado *et al.*, 2001). The SERT$^+$ mouse, with elevated pulmonary arterial pressure in the absence of a hypoxic stimulus (MacLean *et al.*, 2004), provided an example of established PAH, which may be a more clinically relevant endpoint. Although no single animal model precisely recapitulates the human disease, several drugs currently in use, including endothelin receptor antagonists (Rubin *et al.*, 2002; Galie *et al.*, 2008) and phosphodiesterase-5 inhibitors (Galie *et al.*, 2005), first demonstrated promising, beneficial results in experimental animal models (Zhao *et al.*, 2001). There is still a continual need to identify potential therapeutic targets and novel pathways that target vascular remodelling/vasoconstriction in order to halt or reverse the progression of this disease. The chronic hypoxic model of pulmonary hypertension still provides useful, as well as potentially relevant, data to the clinical setting, as structural remodelling through proliferation of PASMCs and fibroblasts and vasoconstriction of the pulmonary arteries both contribute to the progression of PAH, irrespective of different underlying causes (Humbert *et al.*, 2004; Simonneau *et al.*, 2004).

In this study we have shown that the K_v7 channel activator flupirtine is a potent pulmonary arterial vasodilator and markedly attenuates elevated RVP and RVH in two independent models of PAH. With the advent of new compounds that are more selective for the K_v7 channel subtypes, further investigation into the effects of these compounds on the pulmonary vasculature is warranted. Drugs that selectively activate $K_v7.2$–$K_v7.5$ potassium channels may prove a useful co-strategy to prevent and treat PAH in humans.

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Conflict of interest

None.

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Supporting Information

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

Supplemental paper KCNQ modulators reveal a key role for KCNQ potassium channels in regulating the tone of rat pulmonary artery smooth muscle.

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