KCNQ Modulators Reveal a Key Role for KCNQ Potassium Channels in Regulating the Tone of Rat Pulmonary Artery Smooth Muscle

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Received October 22, 2008; accepted January 15, 2009

ABSTRACT

Potassium channels are central to the regulation of pulmonary vascular tone. The smooth muscle cells of pulmonary artery display a background K^+ conductance with biophysical properties resembling those of KCNQ (K_v7) potassium channels. Therefore, we investigated the expression and functional role of KCNQ channels in pulmonary artery. The effects of selective KCNQ channel modulators were investigated on K^+ current and membrane potential in isolated pulmonary artery smooth muscle cells (PASMCs), on the tension developed by intact pulmonary arteries, and on pulmonary arterial pressure in isolated perfused lungs and in vivo. The KCNQ channel blockers, linopirdine and XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone], inhibited the noninactivating background K^+ conductance in PASMCs and caused depolarization, vasocon-

striction, and raised pulmonary arterial pressure without constricting several systemic arteries or raising systemic pressure. The KCNQ channel openers, retigabine and flupirtine, had the opposite effects. PASMCs were found to express KCNQ4 mRNA, at higher levels than mesenteric artery, along with smaller amounts of KCNQ1 and 5. It is concluded that KCNQ channels, most probably KCNQ4, make an important contribution to the regulation of pulmonary vascular tone, with a greater contribution in pulmonary compared with systemic vessels. The pulmonary vasoconstrictor effect of KCNQ blockers is a potentially serious side effect, but the pulmonary vasodilator effect of the openers may be useful in the treatment of pulmonary hypertension.

The pulmonary circulation delivers deoxygenated blood to the lungs at less than 20% of systemic pressure. This low pressure is maintained, at least in part, by the activity of K^+ channels in pulmonary artery smooth muscle cells (PASMCs), which mediate a background efflux of K^+ , driving the membrane potential to a negative value and preventing the opening of voltage-gated Ca^{2+} channels. The vasoconstrictor effect of agents causing membrane depolarization (Hara et al., 1980; Hasunuma et al., 1991) and vasodilation by drugs causing hyperpolarization (Clapp et al., 1993) illustrate the importance of membrane potential for pulmonary artery (PA) function.

The resting potential of PASMCs generally is agreed to

depend on a noninactivating K^+ conductance, but the molecular nature of the underlying K^+ channels is disputed. The voltage-gated channels, $K_v1.5$ (Archer et al., 1998; Moudgil et al., 2006; Remillard et al., 2007) and $K_v2.1/K_v9.3$ (Patel et al., 1997), have been widely studied as mediators of resting potential. Recent work suggests roles for the voltage-independent, two-pore domain channels, TASK-1 (Gurney et al., 2003; Olschewski et al., 2006) and TASK-2 (Gönczi et al., 2006). We showed that the resting potential depends on a background K^+ current (I_{KN}) comprising voltage-dependent and -independent components (Osipenko et al., 1997; Joshi et al., 2006) and suggested that TASK channels mediate the latter (Gurney and Joshi, 2006; Gurney et al., 2003). The biophysical and pharmacological properties of the voltage-dependent component differ, however, from those of $K_v1.5$ and $K_v2.1$ channels (Evans et al., 1996). Distinguishing properties of I_{KN} include a low voltage threshold for activation (below -60 mV), slow activation (time constant, ~ 1 s), absence of inactivation (Evans et al., 1996), and low sensitivity to 4-aminopyridine (Osipenko et al., 1997, 1998), which de-

This work was supported by the Biotechnology and Biological Sciences Research Council [Grant BBS/B/11761/2]; Tenovus Scotland; Leonardo da Vinci Programme; the Grant Agency of Charles University [Grant 2419/2007]; the Centre for Cardiovascular Research [Grant MSMT 1M 0510]; and the Czech Science Foundation [Grant 305/08/0108].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.147785.

ABBREVIATIONS: PASMC, pulmonary artery smooth muscle cell; PA, pulmonary artery; I_{KN} , noninactivating K^+ current; PSS, physiological salt solution; PG, prostaglandin; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AT, angiotensin II; TASK, TWIK-related acid-sensitive potassium.

polarizes PASMCs at concentrations above those required to inhibit $K_{V1.5}$ channels (Osipenko et al., 1997; Coetzee et al., 1999). Thus, although $K_{V1.5}$ may be important in preventing membrane depolarization, and it has been implicated in the development of pulmonary hypertension (Moudgil et al., 2006; Remillard et al., 2007), voltage-dependent channels with a lower activation threshold than $K_{V1.5}$ or $K_{V2.1}$ are the key determinants of I_{KN} and resting potential in PASMCs.

In early studies of I_{KN} , its kinetic properties were noted to bear a striking resemblance to the neuronal M-current (Evans et al., 1996). The channels responsible for the M-current are encoded by genes of the KCNQ (K_{V7}) family (Wang et al., 1998; Robbins, 2001). Together with the finding that KCNQ channel blockers are potent pulmonary vasoconstrictors (Joshi et al., 2006), the similarity of I_{KN} to the M-current led us to consider the possibility that KCNQ channels mediate the voltage-dependent component of I_{KN} . The KCNQ gene family has five members, KCNQ1 to 5, which carry out distinct functions when expressed in different tissues (Robbins, 2001). KCNQ1 is expressed in the heart, where it contributes to action potential repolarization, whereas KCNQ2, KCNQ3, and KCNQ5 contribute to the M-current and help to set the resting potential in neurons (Robbins, 2001; Wladyka and Kunze, 2006). KCNQ4 sets the resting potential and regulates submembrane Ca^{2+} concentration in inner ear hair cells (Oliver et al., 2003). KCNQ channel subunits originally were thought to be largely confined to these tissues, but it is becoming clear that they are also expressed in smooth muscle organs, including the vasculature where systemic arteries express KCNQ4 and smaller amounts of KCNQ1 and 5 (Ohya et al., 2003; Yeung et al., 2007).

This study investigated the expression and functional role of KCNQ channels in PA. Functional studies exploited a class of drugs that specifically inhibit or activate KCNQ channels at concentrations having little effect on other ion channels (Dalby-Brown et al., 2006). Linopirdine and XE991 (Dalby-Brown et al., 2006), originally developed as cognition enhancers, block KCNQ channels, with EC_{50} values in the low or submicromolar range. Both drugs are at least 20-fold less potent at inhibiting neuronal delayed rectifier, A-type, and BK_{Ca} currents and members of the EAG gene family (K_{V10-12}) and 100-fold less potent at inhibiting $K_{V1.2}$, $K_{V2.1}$, and $K_{V4.3}$ (Lamas et al., 1997; Schnee and Brown, 1998; Wang et al., 1998; Wladyka and Kunze, 2006). Retigabine and flupirtine are antiepileptic and analgesic drugs that act by selectively opening KCNQ2–5, but not homomeric KCNQ1 channels, at micromolar concentrations (Tatulian et al., 2001). The effects of these drugs were investigated on PASMC membrane potential and I_{KN} in rat isolated PASMCs and on pulmonary vascular function in rat isolated arteries, isolated perfused lungs, and in vivo. The results suggest a key role for KCNQ channels, particularly KCNQ4, in the control of membrane potential and tone in pulmonary arteries.

Materials and Methods

The functional effects of KCNQ channel modulators were investigated on preparations of rat intrapulmonary artery (200–300- μ m external diameter), isolated saline-perfused lungs, and in vivo hemodynamics, as described previously (Herget and McMurtry, 1987; Joshi et al., 2006). Experiments on isolated lungs and in vivo hemo-

dynamics were carried out in Prague and used adult male Wistar rats (age, 7–8 weeks; 240 ± 15 g), treated in accordance with the Declaration of Helsinki and the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). All procedures were approved by the Animal Studies Committee at Charles University, Prague. For experiments on isolated vessels and cells (in Manchester), male Sprague-Dawley rats (250–300 g) were killed by cervical dislocation in accordance with the UK Animals (Scientific Procedures) Act 1986, and the lungs were excised into physiological salt solution (PSS) containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4 , 0.5 mM KH_2PO_4 , 10 mM HEPES, 10 mM glucose, and 1 mM $CaCl_2$, pH 7.4.

Myography. PA rings were mounted in a wire myograph (DMT A/S, Aarhus, Denmark) in PSS at 37°C, under 5 mN basal tension. After 30 min equilibration, vessels were challenged three times with 50 mM KCl, and subsequent responses to drug application were measured as a percentage of the final KCl constriction. Where indicated, the endothelium was removed by rubbing the vessel lumen with a human hair. Linopirdine and XE991 (hydrochloride salts from Tocris Bioscience, Bristol, UK) were applied to vessels at resting tone, and the increase in force was measured relative to the response to 50 mM KCl. The effects of retigabine and flupirtine were tested on vessels precontracted with phenylephrine (1 μ M), prostaglandin (PG) $F_{2\alpha}$ (3 μ M), or 50 mM K^+ . The K^+ in the PSS was raised by equimolar replacement of Na^+ .

Isolated Saline-Perfused Lungs. Rats were anesthetized with sodium thiopental (50 mg/kg i.p.) and ventilated (peak inspiratory pressure, 12 cm of H_2O ; positive end expiratory pressure, 2 cm of H_2O) through a tracheal cannula with normoxic gas mixture (21% O_2 , 5% CO_2 , 74% N_2). The main PA and left ventricle were cannulated, and the lungs were perfused at 4 ml/min/100 g via the PA with bicarbonate-buffered salt solution containing albumin (4 g/100 ml) and meclofenamate (17 μ M) in 119 mM NaCl, 4.7 mM KCl, 1.16 mM $MgSO_4$, 17 mM $NaHCO_3$, 1.18 mM KH_2PO_4 , 3.2 mM $CaCl_2$, and 5.5 mM D-glucose. The pulmonary perfusion pressure was measured via a transducer connected to the inflow cannula (PowerLab; ADInstruments Ltd., Chalgrove, Oxfordshire, UK), and after equilibration for 15 min, two cycles of angiotensin II (0.2 μ g) followed by hypoxia (0% O_2 , 5% CO_2 , 95% N_2) were delivered to check viability. When pressure returned to baseline, linopirdine (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (0.5–10 μ M) was infused, and pressure was measured after reaching a stable level. Constrictor responses were measured as the peak increase in pulmonary perfusion pressure.

In Vivo Hemodynamics. Hemodynamic measurements were made in spontaneously breathing rats anesthetized with sodium thiopental (50 mg/kg i.p.). Mean systemic arterial pressure was measured via a cannula in the left carotid artery, and pulmonary arterial pressure was measured by a transducer introduced into the pulmonary artery via the jugular vein and right ventricle. Linopirdine dihydrochloride (Tocris Bioscience), dissolved in PSS, was injected via a venous catheter at 10-min intervals in cumulative bolus doses of 1, 5, and 5 mg/kg. Because 2.5 mg/kg i.v. linopirdine given to rats provided an immediate plasma concentration of 5.9 μ M (Rakestraw et al., 1994), these doses are expected to produce plasma concentrations covering the range over which linopirdine causes pulmonary vasoconstriction (Joshi et al., 2006). With a plasma half-life of 36 min (Rakestraw et al., 1994) the plasma concentration of linopirdine would decline by around 17% between doses and remain elevated at the end of each experiment.

Electrophysiology. PASMCs were isolated, and resting membrane potential and K^+ currents were studied using the whole-cell patch-clamp technique as described previously (Osipenko et al., 1998). Pipette solution contained 130 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 20 mM HEPES, and 0.5 mM Na_2GTP , pH 7.2. To isolate I_{KN} from other K^+ currents in the cell, 10 mM tetraethylammonium chloride and 10 μ M glibenclamide were added to the PSS, and the membrane was clamped at 0 mV for ≥ 5 min (Evans et al., 1996).

KCNQ Subunit Expression. RNA was extracted from rat arteries, brain, heart or isolated PASMCs and mRNA expression identified using reverse transcription (RT)-polymerase chain reaction (PCR), as described previously (Gurney et al., 2003). Primer sequences for amplifying KCNQ transcripts are listed in Table 1. Cycle parameters were typically 95°C for 10 min, followed by 20 to 35 cycles at 95°C for 1 min, 52°C to 58°C for 30 s, and 68°C for 1 min. Reverse transcriptase was omitted from control cDNA reactions. Products were resolved by agarose gel electrophoresis, purified, and verified by sequencing. Experiments were repeated on separate samples of RNA isolated from at least three rats.

Real-time quantitative RT-PCR with SYBR Green detection was used to quantify expression levels of KCNQ mRNAs, using the primers listed in Table 1. Reactions were carried out in 250 µl volumes containing 1 µl of cDNA, 12.5 µl of SYBR Green master mix, and 7.5 pmol each primer, using an Applied Biosystems 7500 PCR system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Cycling parameters were 95°C for 15 min followed by 40 cycles at 95°C for 1 min, 58°C for 40 s, and 68°C for 40 s. A dissociation step was performed for melting curve analysis, a single peak representing specificity. An absolute quantification method was used in which the input copy number was determined by relating the PCR signal to a standard curve. Expression levels were then normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), measured simultaneously in the same samples. Experiments were carried out in triplicate from the pooled RNA of three rats and repeated on separate extractions from two sets of animals.

To determine whether KCNQ4 protein was expressed in PASMCs, immunofluorescence experiments were carried out using three different KCNQ4 antibodies (G14, S18, and N10, 1:100 or 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously (Gurney et al., 2003). Staining was detected using the Alexa fluor 594-conjugated secondary antibody (Molecular Probes, Carlsbad, CA). Control cells were processed without primary antibody or with primary antibody preincubated with excess antigen.

TABLE 1

Primers for RT-PCR and quantitative RT-PCR

Forward (For) and reverse (Rev) primers for genes with the indicated accession numbers amplify transcripts of the size indicated. Internal (Int) primers amplified a region inside that was spanned by outer (Out) primer pairs and were used for real-time PCR.

Gene	Primer	Primer Pair Sequences (5'–3')	Predicted Size <i>bp</i>	Accession Number
KCNQ1	Out.For 1	AGC AGA GGC AGA AGC ACT TCA ACC	447	NM_0320773
	Rev1	GCA GCT GTG ACA CAT GGG TGA TG		
	Out.For 2	GGCATACTTGGCTCTGGGTTTG	194	
	Rev2	CTGGGGGAGAGCAAAGTGTG		
KCNQ2	Int. For1	GGCTCTGGGTTTGCACGTG		NM_133322
	Rev1	CATAGCACCTCCATGCAGTC	106	
	Out.For 1	ACG CCT TCT ACC GCA AGC TGC	460	
	Rev1	TAC CGA TCC CAA GAG CTT CCA GG		
KCNQ3	Out.For 2	CCGCAAGCTGCAGAATTTC	354	NM_031597
	Rev2	AAGACATTGCCCTGGGAGCC		
	Out.For1	AAG ACC AAA GCA TGA TGG GGA AGT T	807 (765)	
	Rev1	TGG AAG GGG TCC ATA TGG AAT CTG		
KCNQ4	Out.For2	AAG ACC AAA GCA TGA TGG GGA AGT T	731	XM_233477
	Rev2	CCC AGT GGA TGA CAT AGG CAT G		
	Out.For 1	CGCTTCCGGGCTCTCTAAGAC	561	
	Rev1	GTCTCTGGTCTACAGGGCTGTG		
KCNQ5	Int. For1	CCCCGCTGCTCTACTGAG	86	XM_237012
	Rev1	ATGACATCATCCACCGTGAG		
	Out.For1	GAT GCC AGT GTG ACG TCT CTG TGG	386	
	Rev1	CCT TTC CGG AGG ACC TGT TGA TAG		
GAPDH	Out.For2	GATGCCAGTGTGACGTGTCCGTGG	394	NC_005103.2
	Rev2	CCTTCCGGGAGGACCTGCTGGTAG		
	Int. For1	CGAGACAACGACAGATGACC	77	
	Rev1	TGGATTCAATGGATTGTACCTG		
GAPDH	For	CACCAGCATCACCCCATTT	157	
	Rev	CCATCAAGGACCCCTTCATT		

bp, base pair.

Results

Pulmonary-Selective Effects of KCNQ Channel

Blockers. Linopirdine and XE991 were shown previously to constrict PA while having little effect on mesenteric arteries (Joshi et al., 2006). XE991 has since been shown to constrict systemic arteries, especially if tone is already raised (Yeung et al., 2007). To directly assess the selectivity of the KCNQ blockers for PA, we compared the effects of linopirdine and XE991 on rat pulmonary, renal, mesenteric, femoral, coronary, carotid, cerebral, and tail arteries under identical conditions, using drug concentrations previously shown to maximally constrict PA (Joshi et al., 2006). The original traces in Fig. 1A (inset) confirm that 10 µM linopirdine and 1 µM XE991 constricted PA almost as effectively as 50 mM K⁺ but had a small effect on the renal artery, which responded to the drugs with constrictions of only 15 ± 5% (*n* = 4) and 17 ± 10% (*n* = 4), respectively, of the response to K⁺. The mesenteric artery was even less sensitive, with linopirdine and XE991 inducing only 5 ± 2% (*n* = 5) and 4 ± 1% (*n* = 6), respectively, of the response to K⁺. All other vessels failed to respond to either drug, despite robust responses to K⁺ (Fig. 1A).

The vasoconstrictor action of linopirdine was apparent in isolated, saline-perfused lungs (Fig. 1Bi). The effect was slow to develop compared with the response to angiotensin II (AT), taking ~10 min to reach maximum. This is consistent with the slow time course of constriction seen in isolated arteries (Joshi et al., 2006; see Fig. 1A, inset). In the absence of linopirdine, pressure remained stable over the same time course in at least five preparations, and reproducible responses to bolus injection of AT before and after perfusion

Fig. 1. PA effects of KCNQ blockers. A, constriction evoked by linopirdine (10 μ M) and XE991 (1 μ M) in the arteries indicated. Measured as percentage of K^+ -induced constriction ($n = 4-6$). Inset, original traces showing responses to linopirdine, XE991, and 50 mM K^+ (K) in pulmonary (left) and renal (right) arteries. Calibration bars, 1 mN vertical and 10 min horizontal. Bi, PA perfusion pressure in isolated, salt-perfused lungs before and during bolus injection of AT and infusion of linopirdine (10 μ M). Bii, concentration dependence of linopirdine-induced PA pressure increase; control pressure (c) in the absence of drug plotted for comparison; $n = 4$. Ci, mean systemic and PA pressures recorded in vivo before and during intravenous linopirdine at doses shown. Cii, dose-dependent increase in PA but not systemic pressure by linopirdine ($n = 5$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; unpaired Student's t test versus PA (A); analysis of variance followed by Tukey's comparison versus control (Bii and Cii).

with linopirdine indicated that the lungs remained viable. The increase in pulmonary perfusion pressure caused by linopirdine was concentration dependent, reaching significance above 0.5 μ M (Fig. 1Bii). Linopirdine also raised mean PA pressure in vivo, without raising mean systemic pressure (Fig. 1C). Figure 1Cii shows that the in vivo effect was dose dependent and significant above 1 mg/kg.

Vasodilator Effect of KCNQ Channel Activators.

When applied to rat PA, the KCNQ activator retigabine reduced the contractile response to phenylephrine in a concentration-dependent manner (Fig. 2A). Responses reached steady state within 10 to 15 min, but recovery took 40 min or so and required copious washing. To determine the site of action of retigabine, responses were compared in intact vessels and vessels with endothelium removed and nitric oxide and prostacyclin synthesis blocked by the addition of N^{ω} -nitro-L-arginine methyl ester (100 μ M) and indomethacin (10 μ M). Figure 2B shows that the vasodilator action of retigabine occurred independently of a functional endothelium. Figure 2B also shows that flupirtine, an analog of retigabine,

produced concentration-dependent dilation of pulmonary arteries, but with an approximately 5-fold lower potency. The phenylephrine-induced precontraction was 50% reversed by retigabine at 13 ± 4 μ M ($n = 5$) and by flupirtine at 62 ± 12 μ M ($n = 5$, $p < 0.05$). The effect of flupirtine was also endothelium-independent (data not shown).

If vasodilation were due to the opening of KCNQ channels, it should be inhibited by reducing the transmembrane K^+ gradient. As shown in Fig. 2C, the effect of retigabine was greatly reduced when the artery was precontracted with raised extracellular K^+ (50 mM). Figure 2D compares the effects of retigabine and flupirtine on vessels precontracted with either 50 mM K^+ or the receptor agonists phenylephrine (10 μ M) or PGF_{2 α} (3 μ M). At 10 and 100 μ M, retigabine and flupirtine were both significantly less effective ($p < 0.01$) at relaxing vessels constricted with K^+ than those constricted with phenylephrine or PGF_{2 α} . Retigabine reduced PGF_{2 α} constrictions by 50% at 14 ± 5 μ M ($n = 4$), not significantly different from the concentration causing 50% inhibition of phenylephrine constriction.

Fig. 2. Effect of KCNQ channel openers on rat PA. **A**, sustained constriction induced by 10 μ M phenylephrine followed by relaxation upon cumulative application of retigabine at concentrations indicated. Calibration bars, 1 mN vertical and 10 min horizontal. **B**, concentration-response curves for retigabine-induced relaxation in the presence (+E) or absence (-E) of endothelium and flupirtine ($n = 5$). Relaxation measured as percentage of constrictor-induced pretone. **C**, constriction induced by 50 mM K^+ followed by relaxation upon cumulative application of retigabine at concentrations indicated. Calibration as in **A**. **D**, histogram comparing mean relaxation amplitudes for flupirtine and retigabine (10 and 100 μ M) when the preconstrictor agent was phenylephrine (10 μ M), $PGF_{2\alpha}$ (3 μ M), or 50 mM K^+ ; $n = 4-6$. **E**, histogram comparing relaxation responses to KCNQ activators and levromakalim (levcrom), all at 10 μ M, in the absence and presence of 10 μ M glibenclamide ($n = 3$). **F**, histogram comparing constriction effects of linopirdine (10 μ M) and XE991 (1 μ M) in the presence and absence of retigabine at the concentrations indicated ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, paired Student's t test versus control.

Activation of K_{ATP} channels relaxes PA (Clapp and Gurney, 1992). To test whether the vasodilation caused by KCNQ activators could be explained by K_{ATP} channel opening, the ability of the K_{ATP} blocker glibenclamide (10 μ M) to inhibit their effects was investigated. Figure 2E shows that glibenclamide did not inhibit vasodilation evoked by retigabine (10 μ M) or flupirtine (10 μ M), but it did abolish vasodilation caused by the K_{ATP} channel opener levromakalim (10 μ M).

If the KCNQ blockers and activators act on the same channels, their effects might be expected to be antagonistic. Figure 2F shows that retigabine reduced the vasoconstrictor effects of linopirdine (10 μ M) and XE991 (1 μ M) in a concentration-dependent manner, with 100 μ M almost abolishing vasoconstriction. Flupirtine (10 μ M) had a similar effect (data not shown), increasing the linopirdine EC_{50} from 0.5 ± 0.1 to 2 ± 0.4 μ M ($n = 5$, $p < 0.05$) and the XE991 EC_{50} from 0.1 ± 0.03 to 1.8 ± 0.2 μ M ($n = 5$, $p < 0.05$).

Electrophysiological Effects of KCNQ Channel Blockers. The resting potential of rat PASMCs was -44 ± 2 mV ($n = 71$). Recordings were allowed to stabilize for at least 1 min before measuring or applying drugs. Both linopirdine and XE991 evoked depolarization (Fig. 3, A and B), which could take several minutes to reach steady state and often showed only partial recovery over the time course of the experiments (≤ 30 min), as found previously with recombinant KCNQ channels (Wickenden et al., 2001). Linopirdine depolarized cells by 9 mV (from -45 ± 3 to -36 ± 3 mV; $n = 8$, $p < 0.05$) at 1 μ M and by 15 mV (from -48 ± 3 to -33 ± 7 mV; $n = 5$, $p < 0.05$) at 10 μ M. Similar responses were seen with XE991 (Fig. 3B), and because the depolarization evoked by 5 μ M XE991 was not significantly larger than that evoked by 1 μ M, these seem to be maximal concentrations, consistent with XE991-induced vasoconstriction (Joshi et al., 2006). Note that these measurements include six of 30 cells that showed no response.

Fig. 3. Effects of KCNQ channel blockers on membrane potential and I_{KN} . **A**, membrane potential traces show depolarization upon application of linopirdine (10 μ M) or XE991 (5 μ M). **B**, mean depolarizations induced by linopirdine and XE991 at the concentrations shown (n indicated above bars). **C**, I_{KN} under control conditions, in the presence of 10 μ M linopirdine (left) or 5 μ M XE991 (right) and after 15 to 20 min of washing. Inset, voltage protocol. **D**, histogram comparing linopirdine and XE991 effects on I_{KN} measured at 0 mV (paired data, n indicated above bars). P, control PSS application in place of drug. *, $p < 0.05$. **E**, mean amplitude of delayed rectifier K^+ current, under control conditions, after 5 μ M XE991 application, then washout ($n = 5$). Inset, voltage protocol and typical traces before (con) and during (XE) XE991 application and after drug washout, recorded with a step to +40 mV.

Figure 3C illustrates reversible effects of linopirdine and XE991 on the noninactivating, background K^+ current, I_{KN} , which mirror their effects on membrane potential. The application of linopirdine or XE991 reduced I_{KN} amplitude at potentials above -60 mV. As shown in Fig. 3D, at 0 mV, linopirdine reduced current amplitude by 29% at $1 \mu\text{M}$ and 38% at $10 \mu\text{M}$, whereas XE991 reduced it by 36% at $5 \mu\text{M}$. As found with their effects on membrane potential and vessel tone, the inhibition took several minutes to develop, and recovery required washing for 15 min or more.

Linopirdine and XE991 can inhibit non-KCNQ channels at higher concentrations than used here (Lamas et al., 1997; Wang et al., 1998). Therefore, we tested the effects of the blockers on the delayed rectifier current of PSMCs, under the same conditions used to study I_{KN} . PSMCs were clamped at -80 mV, and a family of delayed rectifier currents, evoked by 200-ms steps to increasingly depolarized potentials, was applied at 5-s intervals. Figure 3E shows that $5 \mu\text{M}$ XE991 had no significant effect on the delayed rectifier current.

Electrophysiological Effects of KCNQ Channel Activators. The KCNQ activators, retigabine and flupirtine, both hyperpolarized PSMCs (Fig. 4A). Retigabine ($10 \mu\text{M}$) hyperpolarized five of seven cells with an average response of -7 ± 3 mV ($n = 7$). When applied to cells clamped at 0 mV, neither retigabine ($10 \mu\text{M}$) nor flupirtine ($10 \mu\text{M}$) had a measurable effect on I_{KN} . Because KCNQ channel activation may be saturated at 0 mV, precluding further activation by drugs (Tatulian et al., 2001), we investigated the effects of KCNQ activators at negative potentials where I_{KN} is too small to measure under physiological conditions. To facilitate measurements, the extracellular K^+ concentration was raised to 130 mM by equimolar substitution of Na^+ , shifting

Fig. 4. Effects of KCNQ channel openers on membrane potential and I_{KN} . A, membrane potential traces upon application of retigabine ($10 \mu\text{M}$) or flupirtine ($10 \mu\text{M}$). B, mean I_{KN} amplitude over a range of potentials, under control conditions (130 mM K^+) and after application of $10 \mu\text{M}$ retigabine ($n = 4$). Inset, voltage protocol. *, $p < 0.05$, paired Student's t test versus control.

Fig. 5. KCNQ transcripts in pulmonary artery. RT-PCR detection of KCNQ transcripts in whole-rat PA, heart, and brain (A) and isolated PSMCs (B). Each column represents a separate reaction with (+RT) or without (-RT) reverse transcriptase. M, size marker; B, water blank.

the K^+ equilibrium potential to 0 mV and amplifying I_{KN} (now inward) at negative potentials. Figure 4B shows that under these conditions, retigabine increased I_{KN} amplitude between -20 and -70 mV. At -60 mV, retigabine ($10 \mu\text{M}$) increased I_{KN} by 34% from a control amplitude of -70 ± 32 to -94 ± 37 pA ($n = 5$, $p < 0.05$).

KCNQ Channel Expression in PSMCs. The expression of KCNQ subunit mRNAs was first investigated in whole arteries, using heart and brain as positive controls (Fig. 5A). RT-PCR with KCNQ1 primers amplified the expected transcripts in heart but not brain. They also amplified a transcript in PA. Specific KCNQ2 primers detected transcripts in brain, but not PA. KCNQ3, 4, and 5 transcripts were amplified in brain and heart, as found before, and also in PA. RT-PCR performed on freshly dispersed PSMCs detected transcripts for KCNQ1, 4, and 5, but not KCNQ3 (Fig. 5B), which may originate from a different cell type. At least two sets of primer pairs were used to test the expression of each transcript, with the same result each time.

Figure 6A compares the expression profile of KCNQ subunit mRNAs in whole pulmonary and mesenteric artery measured relative to the housekeeping gene GAPDH. KCNQ4 was the most highly expressed subunit in both vessels, but its expression in PA was significantly higher (>10 -fold) than in the mesenteric artery. The highest expression of KCNQ1 was also in PA, but KCNQ5 expression was low in both vessels. Normalizing PA expression relative to the control tissues of brain and heart revealed that PA expresses KCNQ1 almost as well as heart, KCNQ4 expression is almost 20-fold higher in PA than brain, but KCNQ5 expression is small in PA compared with brain (Fig. 6B). Figure 6C shows that KCNQ4 protein is expressed in PSMCs. A similar staining pattern was seen with three antibodies directed against different parts of the KCNQ4 protein. In each case, staining had a

Fig. 6. Expression of KCNQ. A, expression profile of KCNQ1, 4, and 5 channels in whole-rat PA and mesenteric artery (MA) measured with qRT-PCR and normalized to GAPDH ($n = 3$). *, $p < 0.05$, analysis of variance with Tukey's pair-wise comparison versus KCNQ4 in PA. B, KCNQ subunit expression in PA expressed relative to brain and heart ($n = 3$). C, fluorescence images of PSMCs labeled separately with three different anti-KCNQ4 antibodies, G14, S18, and N10. Staining was absent in control cells treated identically but without primary antibody (fluorescence and bright-field images of the same cell shown). Calibration bar, 20 μ m.

punctate distribution and was present at the membrane. Labeling was consistently observed in PSMCs from three separate preparations, but not when the primary antibody was omitted from the incubation or was preincubated with excess antigen.

Discussion

This study demonstrates a key role for KCNQ channels, notably KCNQ4, in setting the resting membrane potential of PSMCs and regulating the intrinsic tone of PAs. We previously demonstrated a potent pulmonary vasoconstrictor action of the KCNQ channel blockers, linopirdine and XE991, on isolated vessels (Joshi et al., 2006). This study shows that the KCNQ blocking drugs preferentially constrict PAs over systemic arteries from different anatomical locations and that the pulmonary vasoconstriction results in elevated PA pressure, both in isolated lungs and in vivo. It is important that the rise in PA pressure occurred without a rise in systemic pressure, confirming a preferential action of KCNQ blockers on the pulmonary circulation.

The vasodilation produced by the KCNQ activators, retigabine and flupirtine, and their ability to antagonize the constrictor effects of KCNQ blockers, provide further evidence of a role for KCNQ channels in the pulmonary circulation. Vasodilation was endothelium-independent and inhibited by reducing the K^+ gradient across the plasmalemma, consistent with an action involving the activation of smooth muscle K^+ channels. The

vasodilation was not mediated by K_{ATP} channels because it was not prevented by glibenclamide. Retigabine and flupirtine relaxed agonist-constricted vessels with potencies comparable with their effects on KCNQ channels in other tissues. For example, flupirtine activated M-current in rat visceral sensory neurons at 10 to 20 μ M (Wladyka and Kunze, 2006), and retigabine activated the M-current in sympathetic neurones, with an EC_{50} of ~ 1 μ M (Tatulian et al., 2001). Retigabine is generally more potent than flupirtine (Blackburn-Munro et al., 2005), as found with their effects on PA.

The simplest explanation for the effects of KCNQ blockers and activators on PA is that by modulating KCNQ channels, they change the membrane potential and influence voltage-gated Ca^{2+} entry. The effects of the KCNQ modulators on isolated PSMCs confirm that they affect the background K^+ conductance and membrane potential. Both linopirdine and XE991 reduced I_{KN} and evoked membrane depolarization, whereas retigabine and flupirtine enhanced I_{KN} at negative potentials and evoked membrane hyperpolarization. The KCNQ blockers inhibited I_{KN} at potentials as low as -50 to -60 mV, implying an action on voltage-gated K^+ channels with a low activation threshold and similar to the effects of linopirdine on the neuronal M-current (Lamas et al., 1997). The ability of retigabine to enhance I_{KN} between -70 and -20 mV is also consistent with activation of a low threshold channel and is similar to its effects on the M-current in sympathetic neurons and several recombinant KCNQ channels (Tatulian et al., 2001). It is important that I_{KN} inhibition by linopirdine occurred without an effect on the delayed rectifier current, activated with short depolarizing steps from -80 mV, indicating a selective action on the slower activating, background K^+ conductance that regulates resting potential in PSMCs.

Linopirdine and XE991 evoked maximal depolarization at 10 and 1 μ M, respectively, which mirrors the concentration dependence of their effects on vessel tone. These concentrations are comparable with those inhibiting the M-current in rat hippocampal neurons and sympathetic ganglia (Lamas et al., 1997; Schnee and Brown, 1998) and background K^+ current in the node of Ranvier (Schwarz et al., 2006) and visceral sensory neurons (Wladyka and Kunze, 2006) but lower than required to inhibit a range of other K^+ channels (Lamas et al., 1997; Schnee and Brown, 1998; Wang et al., 1998; Wladyka and Kunze, 2006). Nevertheless, even at maximal concentrations, linopirdine and XE991 reduced I_{KN} (at 0 mV) and membrane potential by only 40%. That is probably because more than one channel mediates I_{KN} , reflected in voltage-dependent and -independent components (Gurney and Joshi, 2006; Gurney et al., 2003; Joshi et al., 2006). We previously reported that TASK channel blockers inhibited I_{KN} and membrane potential by 50% at most, and some PSMCs failed to respond (Gurney et al., 2003), in the same way that some PSMCs failed to respond to KCNQ modulators. Thus, KCNQ and TASK channels may contribute variably to I_{KN} and resting potential in different PSMCs.

PSMCs expressed KCNQ1, KCNQ4, and KCNQ5 subunits, with KCNQ4 mRNA the most abundant. The higher expression of KCNQ4 compared with the other subunits suggests that it is most probably responsible for the pharmacological effects of KCNQ modulators on pulmonary arterial smooth muscle. The higher expression of KCNQ4 in PA compared with mesenteric artery is also consistent with KCNQ4

mediating the larger pulmonary vasoconstrictor responses to linopirdine and XE991. Therefore, KCNQ4 subunits are likely to be mediators of I_{KN} and resting potential in PASMCs. Because the pharmacology of recombinant KCNQ channels is incompletely understood (Robbins, 2001), the sensitivity of the PA channels to KCNQ modulators is of limited help in identifying the specific subunits involved. We can rule out a major contribution from homomeric KCNQ1 channels because they are insensitive to retigabine (Tatulian et al., 2001). Homomeric KCNQ4 channels are less sensitive to block by linopirdine and XE991 (Robbins, 2001) than I_{KN} and the resting potential of PASMCs. On the other hand, KCNQ channel pharmacology can be influenced by interactions with KCNE proteins and other ancillary subunits (Strutz-Seeböhm et al., 2006), and we have yet to determine which, if any, of these are present in PA. It is also possible that KCNQ4 expressed in PASMCs is a specialized splice variant (Beisel et al., 2005), or it is expressed with KCNQ5 in a heteromeric channel assembly (Xu et al., 2007).

Expression of KCNQ4, with lower levels of KCNQ1 and 5, was recently reported in systemic arteries (Yeung et al., 2007; Mackie et al., 2008), and these studies did find effects of KCNQ modulators on systemic vessels. In agreement with the present work, however, XE991 was ineffective on mouse mesenteric artery and had little effect on femoral and carotid arteries, unless they were precontracted with phenylephrine (Yeung et al., 2007). Although the aorta was more responsive, constriction to 10 μ M XE991 was only 26% of the response to KCl (Yeung et al., 2007), compared with 75% at 1 μ M in PA. Perhaps lower KCNQ4 expression in systemic vessels gives rise to a smaller contribution to the resting potential, so that KCNQ channel inhibition alone provides insufficient depolarization to activate substantial voltage-gated Ca^{2+} entry. In contrast to our results and those of Yeung et al. (2007), however, linopirdine constricted mesenteric arteries mounted in a pressure myograph, albeit by only 35% of maximum (Mackie et al., 2008). Perhaps in these conditions, vessels experienced some pretone, but because vessel diameter was >300 μ m, myogenic tone was unlikely to be present (Sun et al., 1992). Harder to reconcile is the report that intravenous linopirdine raised mean arterial pressure (Mackie et al., 2008). In our study, systemic pressure was unchanged or even decreased at the highest dose of linopirdine. We cannot explain the discrepancy, but our findings concur with reports that linopirdine does not affect blood pressure in humans (Saletu et al., 1989; Pieniaszek et al., 1995).

Overall, this study implicates KCNQ channels, especially KCNQ4, as determinants of resting K^+ conductance and membrane potential in PASMCs. The results challenge the widely held view that $K_{v1.5}$ and $K_{v2.1}$ channels are the most important K^+ channels for regulating and modulating PA tone (Patel et al., 1997; Archer et al., 1998; Moudgil et al., 2006; Remillard et al., 2007) and provide evidence that KCNQ channels contribute to the regulation of PA tone in vivo. The results additionally indicate that pulmonary vasoconstriction is a potentially serious side effect of KCNQ blockers, which should be considered as a possible limitation to their development for clinical use. On the other hand, the ability of KCNQ activator drugs to dilate pulmonary arteries suggests that these drugs could provide an effective treat-

ment for lowering pulmonary vascular resistance in pulmonary hypertension.

Acknowledgments

We thank Roth Tate and Robin Plevin (University of Strathclyde, Glasgow, UK) for help with RT-PCR and AstraZeneca Pharmaceuticals (Alderley Park, Cheshire, UK) for the gift of retigabine.

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