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

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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. We therefore 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, inhibited the non-inactivating background K^+ conductance in PASMCs, caused depolarisation, vasoconstriction 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 likely 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.

INTRODUCTION

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 is generally agreed to depend on a non-inactivating 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 (Gonczi et al, 2006). We showed that the resting potential depends on a background K^+ current (I_{KN}) comprising voltage-dependent and voltage-independent components (Joshi et al, 2006; Osipenko et al, 1997) and suggested that TASK channels mediate the latter (Gurney & Joshi, 2002; 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 -60mV), slow activation (time constant ~1s), absence of inactivation (Evans et al, 1996) and low sensitivity to 4-aminopyridine (Osipenko et al, 1997; Osipenko et al, 1998), which depolarises 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 depolarisation, 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 neuronal M-current (Evans et al, 1996). The channels responsible for M-current are encoded by genes of the KCNQ (K_V7) family (Robbins, 2001; Wang et al, 1998). Together with the finding that KCNQ channel blockers are potent pulmonary vasoconstrictors (Joshi et al, 2006), the similarity of I_{KN} to 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, KCNQ1-5, which carry out distinct functions when expressed in different tissues (Robbins, 2001). KCNQ1 is expressed in the heart where it contributes to action potential repolarisation, whereas KCNQ2, KCNQ3 and KCNQ5 contribute to M-current and help to set the resting potential in neurons (Robbins, 2001; Wladka et al, 2006). KCNQ4 sets resting potential and regulates sub-membrane Ca^{2+} concentration in inner ear hair cells (Oliver et al, 2003). KCNQ channel subunits were originally 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, originally developed as cognition enhancers, block KCNQ channels with EC_{50} values in the low or sub-micromolar range. Both drugs are at least 20-fold less potent at inhibiting neuronal delayed rectifier, A-type and BK_{Ca} currents, as well as members of the EAG gene family (K_V10-12), and 100-fold less potent at inhibiting $K_V1.2$, $K_V2.1$ and $K_V4.3$ (Wang et

al, 1998; Wladyka et al, 2006; Lamas et al, 1997; Schnee et al, 1998). Retigabine and flupirtine are antiepileptic and analgesic drugs that act by selectively opening KCNQ2-5, but not homomeric KCNQ1 channels, at micromolar concentrations (Tatulain 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.

METHODS

The functional effects of KCNQ channel modulators were investigated on preparations of rat intrapulmonary artery (200-300 μm external diameter), isolated saline-perfused lungs and *in vivo* haemodynamics, as described previously (Herget et al, 1987; Joshi et al, 2006). Experiments on isolated lungs and *in vivo* haemodynamics were carried out in Prague and used adult male Wistar rats (age 7-8 weeks, 240 ± 15g), treated in accordance with the Declaration of Helsinki and with the Guide for the Care and use of Laboratory Animals as adopted and promulgated by the U. S. National Institutes of Health. 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 (250-300g) were killed by cervical dislocation in accordance with the UK Animals (Scientific Procedures) Act 1986 and the lungs excised into physiological salt solution (PSS) containing (mmol/L): NaCl 120; KCl 5; $MgCl_2$ 1; NaH_2PO_4 0.5; KH_2PO_4 0.5; HEPES 10; glucose 10; $CaCl_2$ 1; pH 7.4.

Myography

PA rings were mounted in a wire myograph (Danish Myo Technology) 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 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) were applied to vessels at resting tone and the increase in force measured relative to the response to 50 mM KCl. The effects of retigabine and flupirtine were tested on vessels pre-constricted with phenylephrine (1 μM), prostaglandin (PG) $F_{2\alpha}$ (3 μM) or 50 mM K^+. The K^+ in the PSS was raised by equimolar replacement of Na^+.

Isolated saline-perfused lungs

Rats were anaesthetised with sodium thiopental (50 mg/kg i.p.) and ventilated (peak inspiratory pressure 12 cm H_2O, positive end expiratory pressure 2 cm 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 perfused at 4 ml/min/100g via the PA with bicarbonate-buffered salt solution (BSS) containing albumin (4 g/100ml) and meclofenamate (17 μM) in (in mM): NaCl 119, KC1 4.7, $MgSO_4$ 1.16, $NaHC0_3$ 17, KH_2PO_4 1.18, $CaCl_2$ 3.2, d-glucose 5.5. The pulmonary perfusion pressure was measured via a transducer connected to the inflow cannula (PowerLab, AD Instruments) and after equilibration for 15 min, two cycles of angiotensin II (0.2 μg) 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) dissolved in DMSO (0.5-10 μM) was infused and pressure measured after reaching a stable level. Constrictor responses were measured as the peak increase in pulmonary perfusion pressure.

In vivo haemodynamics

Haemodynamic measurements were made in spontaneously breathing rats anaesthetised with sodium thiopental (50 mg/kg ip). Mean systemic arterial pressure was measured via a cannula in the left carotid artery and pulmonary arterial pressure (PAP) measured by a transducer introduced into the pulmonary artery via the jugular vein and right ventricle. Linopirdine dihydrochloride (Tocris), dissolved in PSS, was injected via a venous catheter at 10 min intervals in cumulative bolus doses of 1, 5 and 5 mg/kg. Since 2.5 mg/kg linopirdine given i.v. to rats provided an immediate plasma concentration of 5.9 μM (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 studied using the whole-cell patch-clamp technique as previously described (Osipenko et al, 1998). Pipette solution contained (mM): KCl 130, $MgCl_2$ 1, ethyleneglycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 1, HEPES 20, Na_2GTP 0.5; pH 7.2. To isolate I_{KN} from other K^+ currents in the cell, 10 mM tetraethylammonium chloride (TEA) and 10 µM 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 polymerase chain reaction (RT-PCR) as previously described (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 3 rats.

Real time quantitative RT-PCR (qPCR) 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 25µl volumes containing 1µl cDNA, 12.5 µl SYBR Green master mix and 7.5 pmol of each primer, using an Applied BioSystems 7500 PCR system according to the manufacturer's instructions. Cycling parameters were 95^{o}C for 15min followed by 40 cycles at 95^{o}C for 1 min, 58^{o}C for 40 s and 68^{o}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 normalised against glyceraldehyde 3-phosphate

dehydrogenase (GAPDH), measured simultaneously in the same samples. Experiments were carried out in triplicate from the pooled RNA of 3 rats and repeated on separate extractions from two sets of animals.

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

RESULTS

Pulmonary selective effects of KCNQ channel blockers

Linopirdine and XE991 were previously shown 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 renal artery, which responded to the drugs with constrictions only 15 ± 5% (n=4) and 17 ± 10% (n=4) respectively, of the response to K^+. Mesenteric artery was even less sensitive, 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 5 preparations and reproducible responses to bolus injection of AT before and after perfusion 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 μM (Fig. 1Bii). Linopirdine also raised mean PA pressure (PAP) *in vivo*, without raising mean systemic pressure (Fig. 1C). Fig. 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 addition of N(G)-nitro-L-arginine methyl ester (L-NAME, 100μM) and indomethacin (10μM). Fig. 2B shows that the vasodilator action of retigabine occurred independently of a functional endothelium. Fig. 2B also shows that flupirtine, an analogue of retigabine, produced concentration-dependent dilation of pulmonary arteries, but with an approximately 5-fold lower potency. The phenyephrine-induced pre-constriction was 50% reversed by retigabine at 13 ± 4 μM (n=5) and by flupirtine at 62 ± 12 μM (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 pre-constricted with raised extracellular K^+ (50 mM). Fig. 2D compares the effects of retigabine and flupirtine on vessels pre-constricted with either 50 mM or the receptor agonists phenylephrine (10 μM) or $PGF_{2\alpha}$ (3 μM). At 10 and 100 μM, retigabine and flupirtine were both significantly less effective ($p<0.01$) at relaxing vessels constricted with K^+ than those constricted with phenylephrine or $PGF_{2\alpha}$. Retigabine reduced $PGF_{2\alpha}$ constrictions by 50% at 14±5 μM (n=4), not significantly different from the concentration causing 50% inhibition of phenylephrine constriction.

Activation of K_{ATP} channels relaxes PA (Clapp & 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 μM) to inhibit their effects was investigated. Fig 2E shows that glibenclamide did not inhibit vasodilation evoked by retigabine (10 μM) or flupirtine (10 μM), but it did abolish vasodilation caused by the K_{ATP} channel opener levcromakalim (10 μM).

If the KCNQ blockers and activators act on the same channels, their effects might be expected to be antagonistic. Fig. 2F shows that retigabine reduced the vasoconstrictor effects of linopirdine (10 μM) and XE991 (1 μM) in a concentration-dependent manner, with 100 μM almost abolishing vasoconstriction. Flupirtine (10 μM) had a similar effect (not shown), increasing the linopirdine EC_{50} from 0.5 ± 0.1 μM to 2 ± 0.4 μM (n=5, $P<0.05$) and the XE991 EC_{50} from 0.1 ± 0.03 μM to 1.8 ± 0.2 μM (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 stabilise for at least 1 min before measuring or applying drugs. Both linopirdine and XE991 evoked depolarization (Fig. 3A, 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 depolarised cells by 9 mV (from -45 ± 3 to -36 ± 3 mV; n=8, $p<0.05$) at 1 μM and by 15 mV (from –48 ± 3 to –33 ± 7 mV; n=5, $p<0.05$) at 10 μM. Similar responses were seen with XE991 (Fig. 3B) and as the depolarisation evoked by 5 μM XE991 was not significantly larger than that evoked by 1 μM, these appear to be maximal concentrations, consistent with XE991-induced vasoconstriction (Joshi et al, 2006). Note that these measurements include 6 out of 30 cells that showed no response.

Fig. 3C illustrates reversible effects of linopirdine and XE991 on the non-inactivating, 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 μM and 38% at 10 μM, while XE991 reduced it by 36% at 5 μM. 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 (Wang et al, 1998; Lamas et al, 1997). We therefore tested the effects of the blockers on the delayed rectifier current of PASMCs, under the same conditions used to study I_{KN}. PASMCs were clamped at -80 mV and a family of delayed rectifier currents evoked by 200 ms steps to increasingly depolarised potentials, applied at 5s intervals. Fig. 3E shows that 5 μM XE991 had no significant effect on the delayed rectifier current.

Electrophysiological effects of KCNQ channel activators

The KCNQ activators, retigabine and flupirtine, both hyperpolarised PASMCs (Fig. 4A). Retigabine (10 μM) hyperpolarised 5 out of 7 cells with an average response of -7 ± 3 mV (n=7). When applied to cells clamped at 0 mV, neither retigabine (10 μM) nor flupirtine (10 μM) had a measurable effect on I_{KN}. As KCNQ channel activation may be saturated at 0 mV, precluding further activation by drugs (Tatulain 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 the K^+ equilibrium potential to 0 mV and amplifying I_{KN} (now inward) at negative potentials. Fig. 4B shows that under these conditions, retigabine increased I_{KN} amplitude between -20 and -70 mV. At -60 mV retigabine (10 μM) increased I_{KN} by 34% from a control amplitude of -70 ± 32 pA to -94 ± 37 pA (n=5, $P<0.05$).

KCNQ channel expression in PASMCs

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 PASMCs 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.

Fig. 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 mesenteric artery. The highest expression of KCNQ1 was also in PA, but KCNQ5 expression was low in both vessels. Normalising 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). Fig 6C shows that KCNQ4 protein is expressed in PASMCs. A similar staining pattern was seen with three antibodies directed against different parts of the KCNQ4 protein. In each case, staining had a punctuate distribution and was present at the membrane. Labelling was consistently observed in PASMCs from 3 separate preparations, but not when the primary antibody was omitted from the incubation or was pre-incubated with excess antigen.

DISCUSSION

This study demonstrates a key role for KCNQ channels, notably KCNQ4, in setting the resting membrane potential of PASMCs 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*. Importantly, 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, as well as their ability to antagonise the constrictor effects of KCNQ blockers, provides 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 to their effects on KCNQ channels in other tissues. For example, flupirtine activated M-current in rat visceral sensory neurons at 10-20 μM (Wladyka et al, 2006) and retigabine activated M-current in sympathetic neurones with an EC_{50} of ~1 μM (Tatulain 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 PASMCs confirm that they affect the background K^+ conductance and membrane

potential. Both linopirdine and XE991 reduced I_{KN} and evoked membrane depolarisation, whereas retigabine and flupirtine enhanced I_{KN} at negative potentials and evoked membrane hyperpolarisation. 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 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 M-current in sympathetic neurons and several recombinant KCNQ channels (Tatulain et al, 2001). Importantly, I_{KN} inhibition by linopirdine occurred without an effect on the delayed rectifier current, activated with short depolarising steps from -80 mV, indicating a selective action on the slower activating, background K^+ conductance that regulates resting potential in PASMCs.

Linopirdine and XE991 evoked maximal depolarisation at 10 μM and 1 μM, respectively, which mirrors the concentration dependence of their effects on vessel tone. These concentrations are comparable to those inhibiting M-current in rat hippocampal neurons and sympathetic ganglia (Lamas et al, 1997; Schnee et al, 1998) and background K^+ current in the node of Ranvier (Wickenden et al, 2001) and visceral sensory neurons (Wladyka et al, 2006), but lower than required to inhibit a range of other K^+ channels (Wang et al, 1998; Wladyka et al, 2006; Lamas et al, 1997; Schnee et al, 1998). 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 voltage-independent components (Gurney & Joshi, 2002; 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 PASMCs failed to respond (Gurney et al, 2003), in the same way that some PASMCs failed to respond to KCNQ modulators. Thus KCNQ and TASK channels may contribute variably to I_{KN} and resting potential in different PASMCs.

PASMCs 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 likely 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. KCNQ4 subunits are therefore likely to be mediators of I_{KN} and resting potential in PASMCs. As 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 as 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-Seebohm 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 specialised 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, XE911 was ineffective on mouse mesenteric artery and had little effect on femoral and carotid arteries unless they were pre-constricted with phenylephrine (Yeung et al, 2007). Although aorta was more responsive, constriction to 10 μM XE991 was only 26% of the response to KCl (Yeung et al, 2007), compared with 75% at 1 μM 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 depolarisation 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 pre-tone, but as vessel diameter was >300μm, myogenic tone was unlikely to be present (Sun et al, 1992). Harder to reconcile is the report that i.v. 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 (Pieniaszek et al, 1995; Saletu et al, 1989).

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 treatment for lowering pulmonary vascular resistance in pulmonary hypertension.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: PA effects of KCNQ blockers. A, Constriction evoked by linopirdine (10μM) and XE991 (1 μM) in the arteries indicated. Measured as % K^+-induced constriction (N=4-6). Inset: original traces showing responses to linopirdine, XE991 and 50 mM K^+ (K) in pulmonary (left panel) and renal (right panel) arteries. Calibration bars: 1mN vertical; 10 min horizontal. Bi, PA perfusion pressure in isolated, salt-perfused lungs before and during bolus injection of angiotensin II (AT) and infusion of linopirdine (10 μM). Bii, Concentration dependence of linopirdine-induced PA pressure increase; control pressure (c) in absence of drug plotted for comparison; N=4. Ci, Mean systemic and PA pressures recorded *in vivo* before and during i.v. 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 t-test versus PA (A) or control (Biii); ANOVA followed by Tukey's comparison versus control (Bii, Cii).

Figure 2: Effect of KCNQ channel openers on rat PA. A, sustained constriction induced by 10μM phenylephrine followed by relaxation upon cumulative application of retigabine at concentrations indicated. Calibration bars: 1mN vertical; 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 % constrictor-induced pre-tone. 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 μM) when the preconstrictor agent was phenylephrine (10μM), $PGF_{2\alpha}$ (3 μM) or 50 mM K^+; N=4-6. E, Histogram comparing relaxation responses to KCNQ activators and levcromakalim (levcrom), all at 10 μM, in the absence and presence of 10 μM glibenclamide (n=3). F, Histogram comparing constrictor effects of linopirdine (10 μM) and XE991 (1 μM) in the presence and

absence of retigabine at the concentrations indicated (n=4). *P<0.05, **P<0.01, ***P<0.001, paired t-test versus control.

Figure 3: Effects of KCNQ channel blockers on membrane potential and I_{KN}. A, Membrane potential traces show depolarisation upon application of linopirdine (10 μM) or XE991 (5 μM). B, Mean depolarisations induced by linopirdine and XE991 at the concentrations shown (n indicated above bars). C, I_{KN} under control conditions, in the presence of 10 μM linopirdine (left panel) or 5 μM XE991 (right panel) and after 15-20 min of washing. Voltage protocol inset. D, Histogram comparing linopirdine and XE991 effects on I_{KN} measured at 0 mV (paired data, n indicated above bars). P indicates control PSS application in place of drug. *P<0.05. E, Mean amplitude of delayed rectifier K^+ current measured using the voltage protocol inset, under control conditions, after 5 μM XE991 application then washout (n=5). Inset shows typical traces before (con) and during XE991 application (XE) and after drug washout, recorded with a step to +40mV.

Figure 4: Effects of KCNQ channel openers on membrane potential and I_{KN}. A, Membrane potential traces upon application of retigabine (10 μM) or flupirtine (10 μM). B, Mean I_{KN} amplitude over a range of potentials, under control conditions (130 mM K^+) and following application of 10 μM retigabine (n=4). Voltage protocol inset. *p<0.05, paired t-test versus control.

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

Figure 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

normalised to GAPDH (n=3). *P<0.05 ANOVA with Tukey's pairwise comparison versus KCNQ5 in PA. B, KCNQ subunit expression in PA expressed relative to brain and heart (n=3). C, Fluorescence images of PASMCs labelled 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 20μm.

Table 1: Primers for RT-PCR and qPCR. 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 spanned by outer (Out) primer pairs and were used for real time PCR.

Gene	Primer	Primer pair sequences (5'-3')	Predicted size bp	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 1	GGCATACTTGGCTCTGGGTTTG	194	
	Rev1	CTGGGGGAGAGCAAAGTGTG		
	Int. For1	GGCTCTGGGTTTGCACTG		
	Rev1	CATAGCACCTCCATGCAGTC	106	
KCNQ2	Out.For 1	ACG CCT TCT ACC GCA AGC TGC	460	NM_133322
	Rev1	TAC CGA TCC CAA GAG CTT CCA GG		
	Out.For 1	CCGCAAGCTGCAGAATTTCC	354	
	Rev1	AAGACATTGCCCTGGGAGCC		
KCNQ3	Out.For1	AAG ACC AAA GCA TGA TGG GGA AGT T	807 (765)	NM_031597
	Rev1	TGG AAG GGG TCC ATA TGG AAT CTG		
	Out.For1	AAG ACC AAA GCA TGA TGG GGA AGT T	731	
	Rev1	CCC AGT GGA TGA CAT AGG CAT G		
KCNQ4	Out.For 1	CGCTTCCGGGCCTCTCTAAGAC	561	XM_233477
	Rev1	GTCCTCGTGGTCTACAGGGCTGTG		
	Int. For1	CCCCGCTGCTCTACTGAG	86	
	Rev1	ATGACATCATCCACCGTGAG		
KCNQ5	Out.For1	GAT GCC AGT GTG ACG TCT CTG TGG	386	XM_237012
	Rev1	CCT TTC CGG AGG ACC TGT TGA TAG		
	Out.For1	GATGCCAGTGTGACGTGTCCGTGG	394	
	Rev1	CCTTTCCGGAGGACCTGCTGGTAG		
	Int. For1	CGAGACAACGACAGATGACC	77	
	Rev1	TGGATTCAATGGATTGTACCTG		
GAPDH	For	CACCAGCATCACCCCATTT	157	NC_005103.2
	Rev	CCATCAAGGACCCCTTCATT		

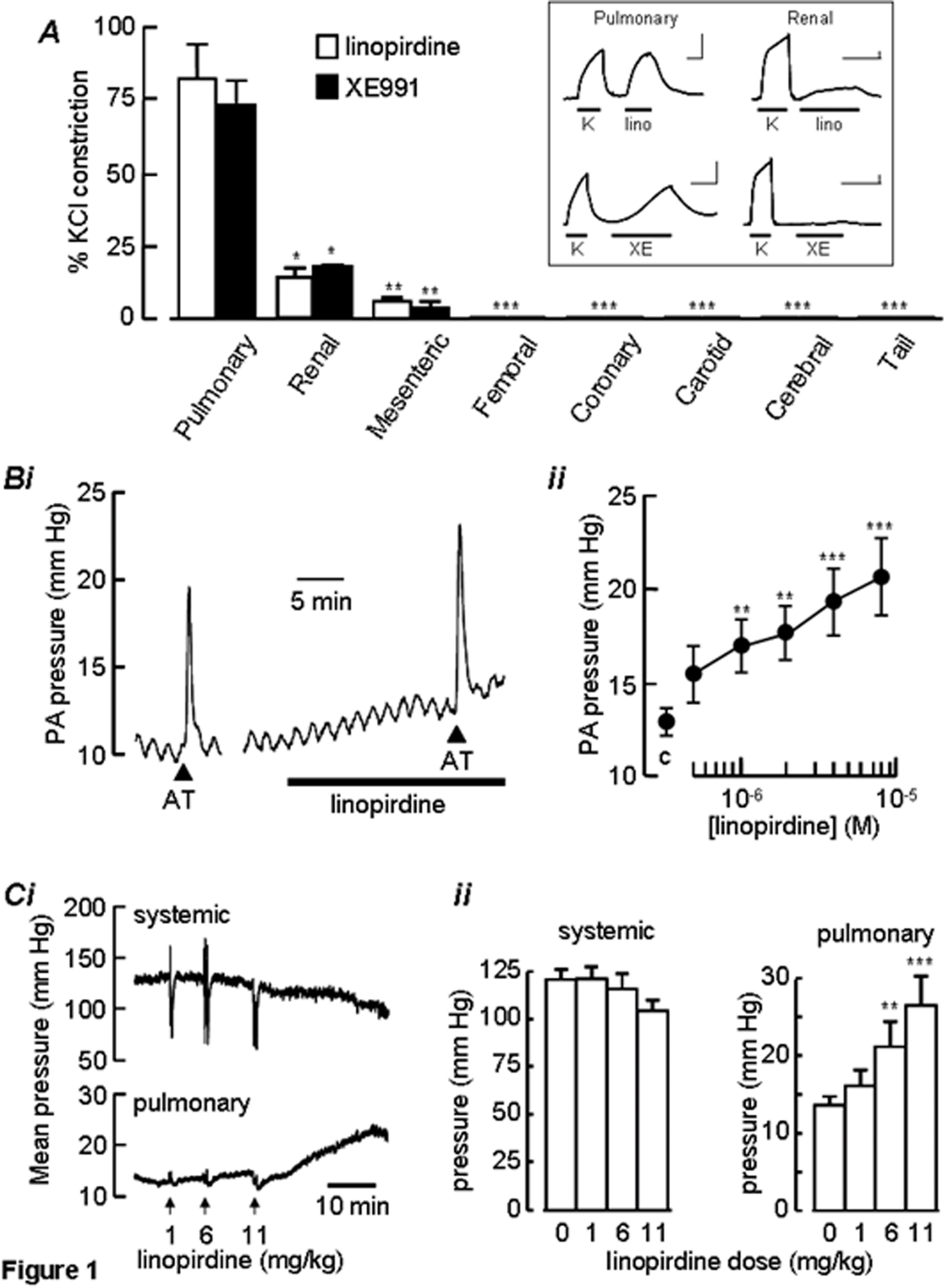

A
% KCl constriction
linopirdine
XE991
Pulmonary
Renal
K
lino
XE
Pulmonary
Renal
Mesenteric
Femoral
Coronary
Carotid
Cerebral
Tail
Bi
PA pressure (mm Hg)
5 min
AT
linopirdine
ii
PA pressure (mm Hg)
[linopirdine] (M)
Ci
Mean pressure (mm Hg)
systemic
pulmonary
10 min
linopirdine (mg/kg)
ii
systemic
pulmonary
pressure (mm Hg)
linopirdine dose (mg/kg)

Figure 1

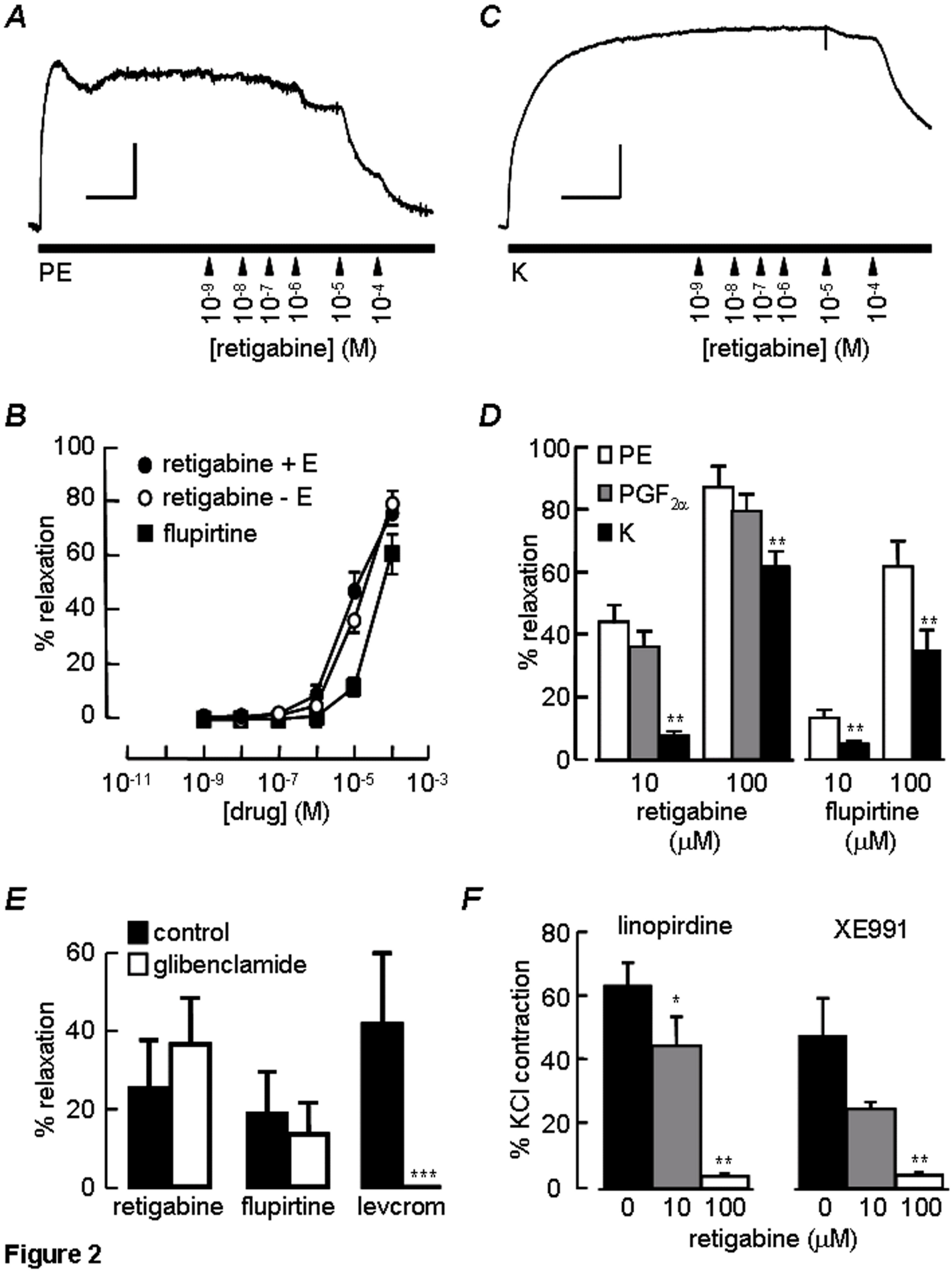
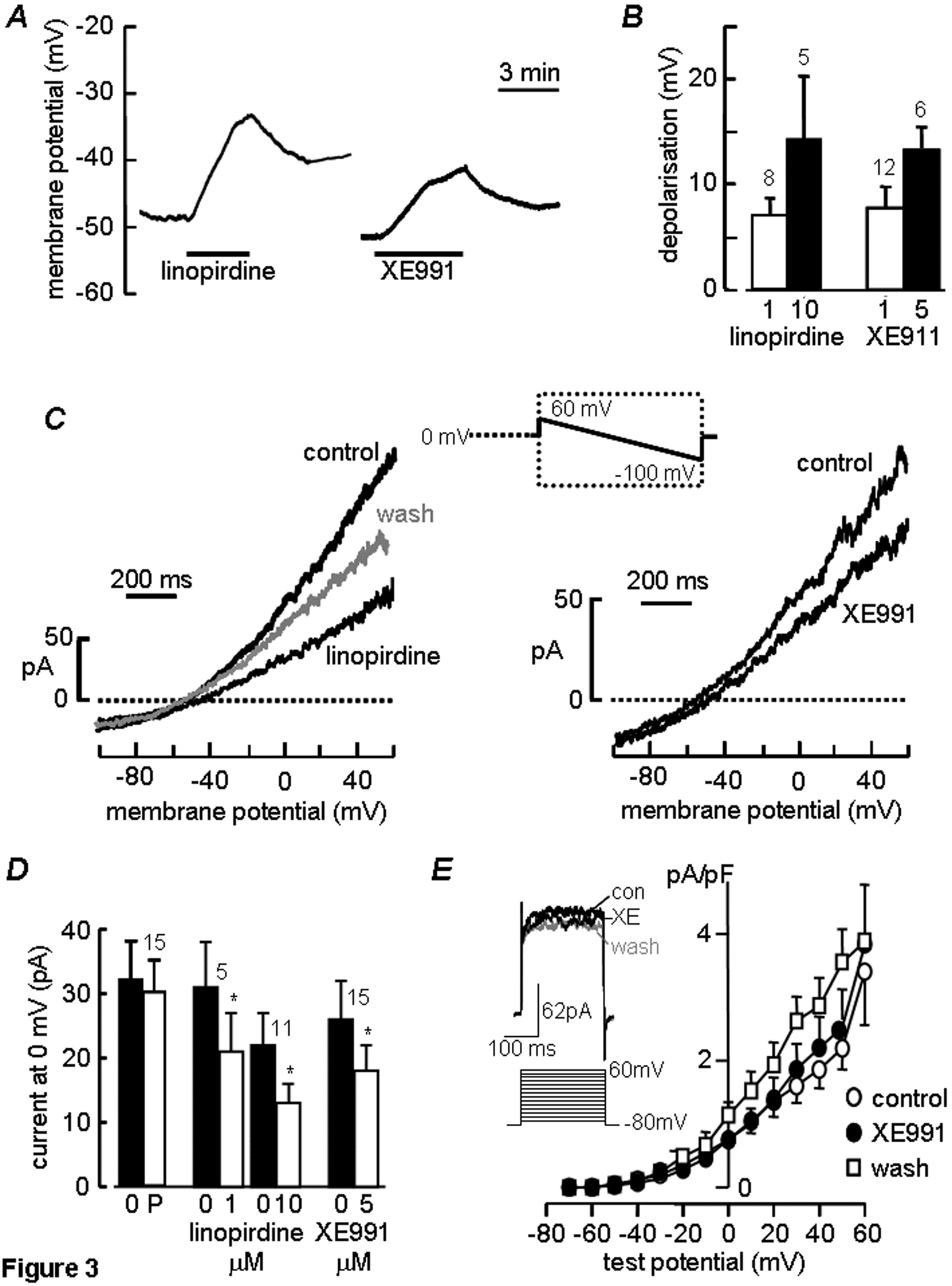

A
PE
10-9
10-8
10-7
10-6
10-5
10-4
[retigabine] (M)
C
K
10-9
10-8
10-7
10-6
10-5
10-4
[retigabine] (M)
B
retigabine + E
retigabine - E
flupirtine
% relaxation
100
80
60
40
20
0
10-11
10-9
10-7
10-5
10-3
[drug] (M)
D
PE
PGF2α
K
% relaxation
100
80
60
40
20
0
**
**
**
**
10
100
retigabine
(μM)
10
100
flupirtine
(μM)
E
control
glibenclamide
% relaxation
60
40
20
0
***
retigabine
flupirtine
levcrom
F
linopirdine
XE991
% KCl contraction
80
60
40
20
0
*
**
**
0
10
100
0
10
100
retigabine (μM)

Figure 2


A
membrane potential (mV)
-20
-30
-40
-50
-60
3 min
linopirdine
XE991
B
depolarisation (mV)
20
10
0
8
5
12
6
1 10
linopirdine
1 5
XE911
C
control
wash
linopirdine
200 ms
50
pA
0
-80 -40 0 40
membrane potential (mV)
60 mV
0 mV
-100 mV
control
XE991
200 ms
50
pA
0
-80 -40 0 40
membrane potential (mV)
D
current at 0 mV (pA)
40
30
20
10
0
15
5
11
15
0 P
0 1
0 10
0 5
linopirdine
μM
XE991
μM
E
pA/pF
con
XE
wash
62pA
100 ms
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2
0
control
XE991
wash
-80 -60 -40 -20 0 20 40 60
test potential (mV)

Figure 3

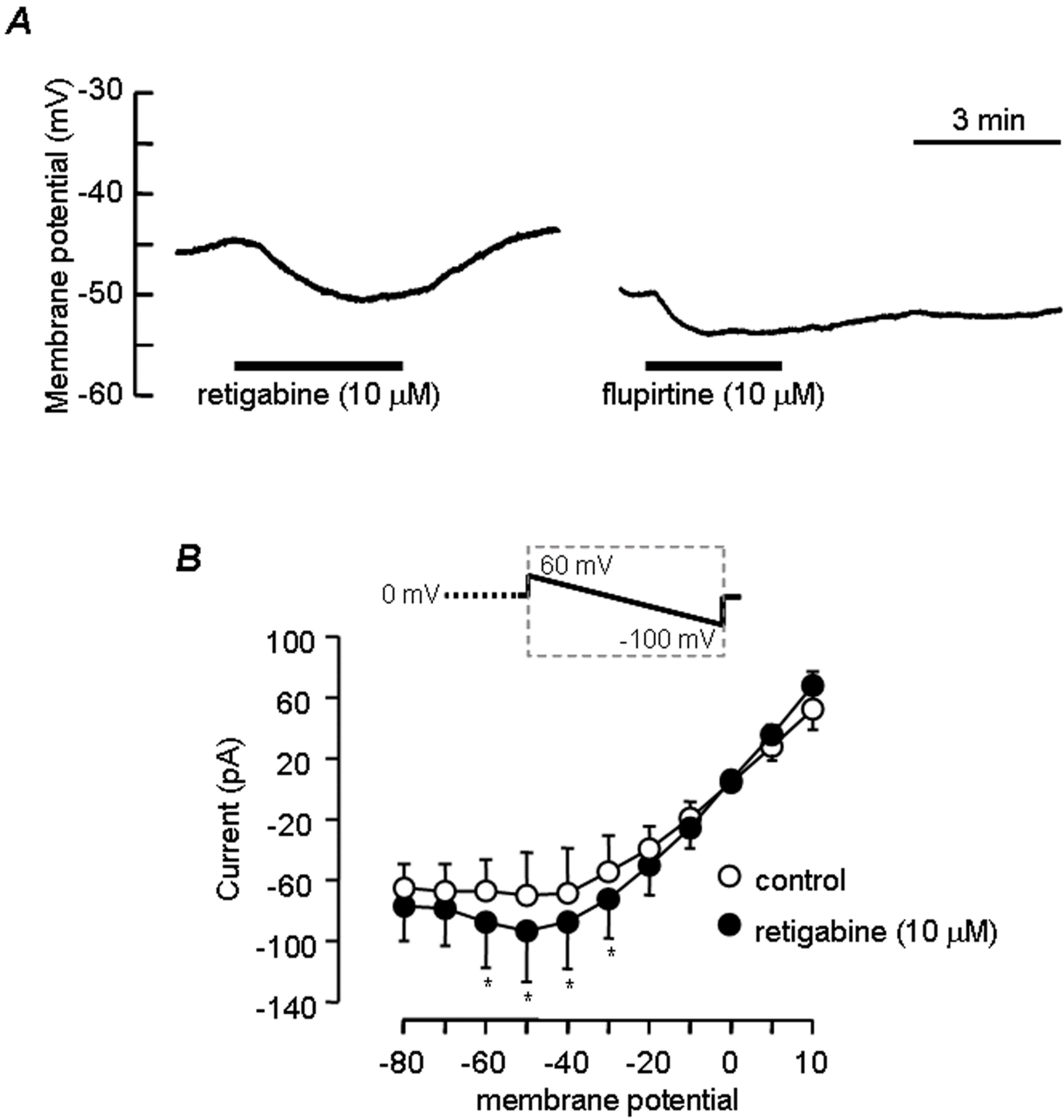
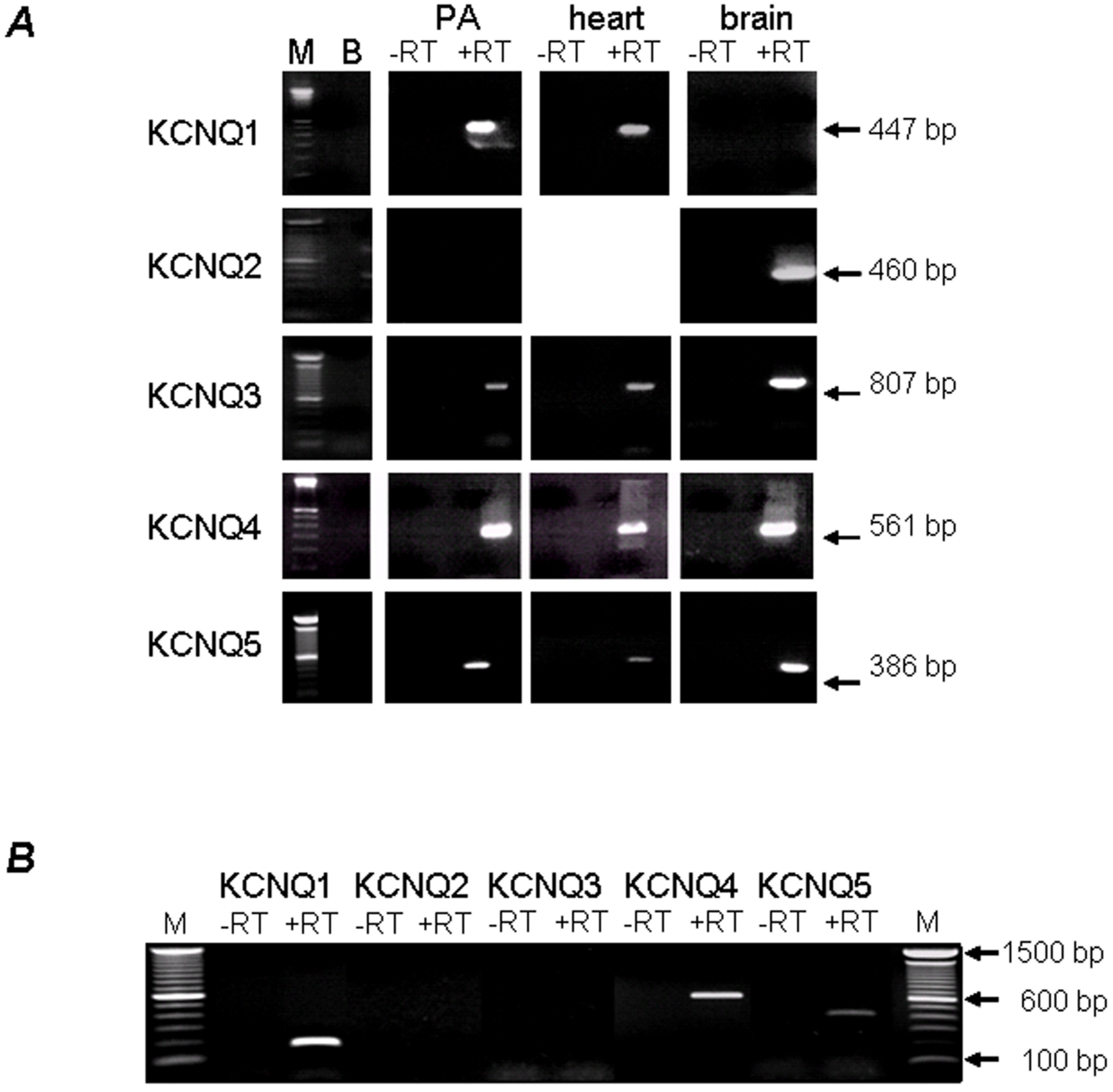

A
Membrane potential (mV)
-30
-40
-50
-60
3 min
retigabine (10 μM)
flupirtine (10 μM)
B
0 mV
60 mV
-100 mV
100
60
20
-20
-60
-100
-140
Current (pA)
control
retigabine (10 μM)
-80
-60
-40
-20
0
10
membrane potential

Figure 4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PA
heart
brain
M
B
-RT
+RT
-RT
+RT
-RT
+RT
KCNQ1
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KCNQ2
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KCNQ3
807 bp
KCNQ4
561 bp
KCNQ5
386 bp
B
KCNQ1
KCNQ2
KCNQ3
KCNQ4
KCNQ5
M
-RT
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+RT
-RT
+RT
-RT
+RT
-RT
+RT
M
1500 bp
600 bp
100 bp

Figure 5

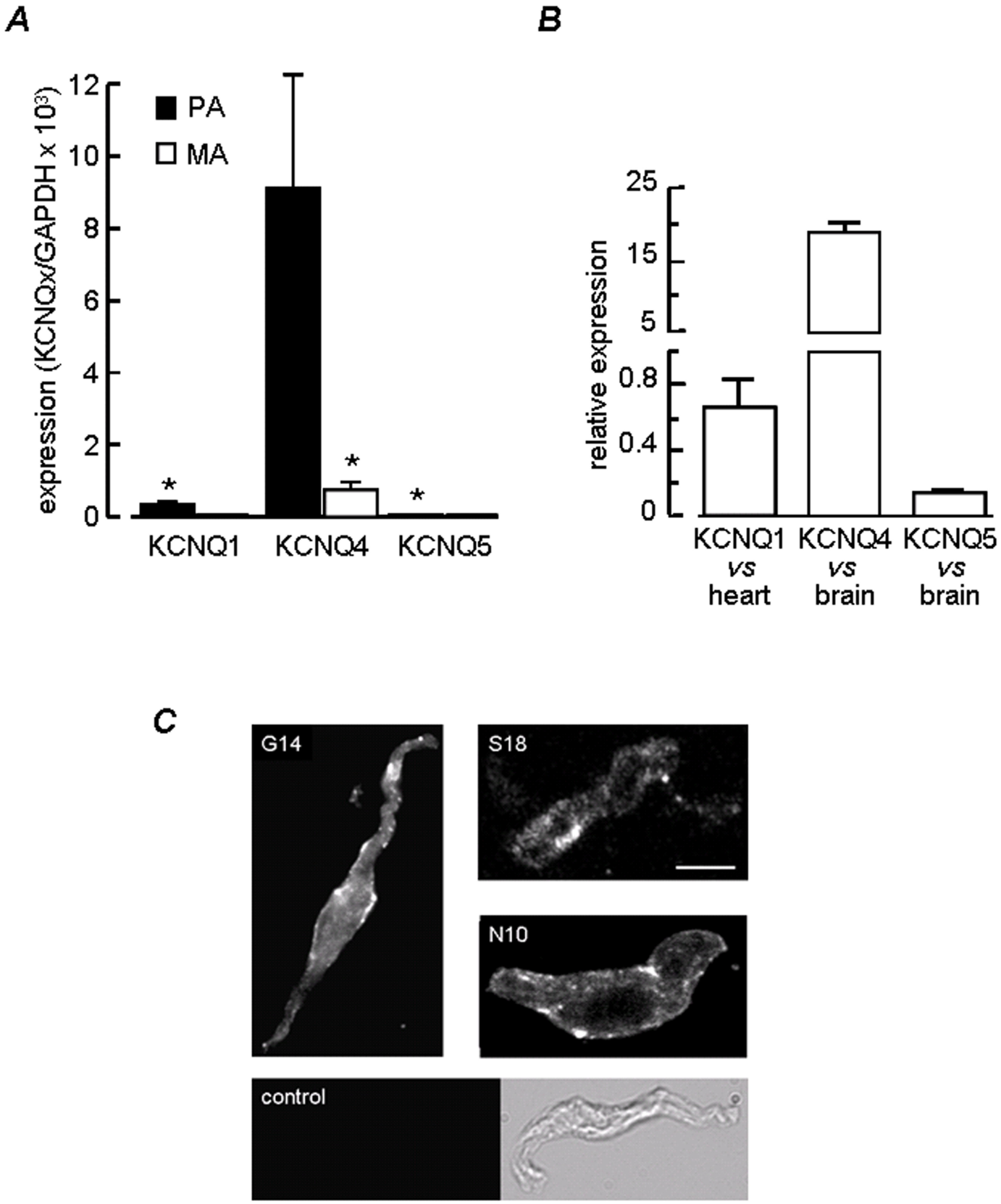
A
B
C
PA
MA
expression (KCNQx/GAPDH x 10³)
12
10
8
6
4
2
0
*
*
*
KCNQ1
KCNQ4
KCNQ5
relative expression
25
15
5
0.8
0.4
0
KCNQ1 vs heart
KCNQ4 vs brain
KCNQ5 vs brain
G14
S18
N10
control

Figure 6