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Investigation of the role of TASK-2 channels in rat pulmonary arteries; pharmacological and functional studies following RNA interference procedures

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1 In the present study, we investigated the ability of RNA interference technology to suppress TASK-2 potassium channel expression in human embryonic kidney (HEK293) cells stably transfected with TASK-2 cDNA and in rat isolated intact pulmonary arteries.

2 Lipofectamine-induced transfection of a specific siRNA sequence targeted against TASK-2 resulted in a dose- and time-dependent decrease in TASK-2 channel protein expression. In siRNA-transfected cells the TASK-2 peak currents were significantly smaller than in control cells at every investigated pH, while the pH sensitivity was not altered. Using scrambled siRNA as a negative control, there were no significant changes in TASK-2 protein expression or current compared to mock-transfected cells.

3 In TASK-2 siRNA-transfected small pulmonary arteries, but not in scrambled siRNA-treated vessels, myocyte resting membrane potential at pH 7.4 was significantly less negative and the hyperpolarisations in response to increasing pH from 6.4 to 8.4 were significantly smaller compared with control.

4 The application of levcromakalim (10 μM), NS1619 (33 μM) and a potassium channel inhibitor cocktail (5 mM 4-aminopyridine, 10 mM tetraethylammonium chloride, 30 μM Ba^{2+} and 10 μM glibenclamide) had similar effects in control and in siRNA-transfected vessels. The TASK-1 (anandamide-sensitive) contribution to resting membrane potential was comparable in each group. Clofilium (100 μM) generated significantly smaller responses in transfected artery segments.

5 These results suggest that RNA interference techniques are effective at inhibiting TASK-2 channel expression in cultured cells and in intact vessels and that TASK-2 channels have a functional role in setting the membrane potential of pulmonary artery myocytes.
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Abbreviations: 4-AP, 4-aminopyridine; BK_{Ca}, calcium-activated potassium channel; DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's Modified Eagle Medium; E_m, membrane potential; ΔE_m, membrane potential changes; EGTA, ethylene-bis(oxyethylenenitrilo)tetraacetic acid; FBS, foetal bovine serum; GFP, green fluorescent protein; HEK293, human embryonic kidney cells; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; K_{ATP}, adenosine triphosphate-activated potassium channel; K_{IR}, inward-rectifying potassium channel; K_{2P}, two-pore domain potassium channel; K_V, voltage-sensitive potassium channel; LK, levcromakalim; MEM, minimal essential medium; PASMC, pulmonary artery smooth muscle cell; PKC, protein kinase C; RNAi, RNA-mediated interference; siRNA, small interfering RNA; TASK, TWIK-related acid-sensitive K^+ channel; TEA, tetraethylammonium chloride; TREK, TWIK-related K^+ channel

Introduction

Two-pore domain potassium (K_{2P}) channels are dimers of α-subunits, each of which contains two pore-forming regions (Coetzee *et al*., 1999; Lesage & Lazdunski, 2000; Goldstein *et al*., 2001). One such channel subfamily designated by the acronym TASK (TWIK-related acid-sensitive K^+ channel) produces K^+ currents that possess many of the characteristics of background or baseline conductances of various cells (Duprat *et al*., 1997; Leonoudakis *et al*., 1998; Kim *et al*., 1999). Current flow through TASK channels is strongly dependent on external pH in the physiological range (Duprat *et al*., 1997; Reyes *et al*., 1998; Leonoudakis *et al*., 1998; Kim *et al*., 1999; Kim *et al*., 2000). There is evidence that the TASK family member known as TASK-1 has an important role in setting the resting membrane potential (E_m) in vascular myocytes (Gurney *et al*., 2003; Gardener *et al*., 2004) and in neuronal (Millar *et al*., 2000; Talley *et al*., 2000), cardiac (Barbuti *et al*., 2002) and adrenal glomerulosa cells (Czirják *et al*., 2000). However, the possible role of TASK-2 channels in modulating cell E_m has only been demonstrated in rat cerebellar granule neurones (Cotten *et al*., 2004).

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Owing to their similar kinetics and pharmacology, unambiguous identification of individual K_{2P} channels within a subfamily is problematic. However, the analysis of gene function in animals and plants was revolutionised in 1998 by the discovery of the mechanisms underlying RNA-mediated interference (RNAi) in *Caenorhabditis elegans* (Fire *et al*., 1998; Tuschl, 2003). During RNAi, long transcripts of double-stranded RNA (dsRNA) are rapidly processed into small interfering RNAs (siRNAs) consisting of RNA duplexes of specific length and structure that induce sequence-specific mRNA degradation (Elbashir *et al*., 2001). The transfection of siRNAs into animal cells results in potent, long-lasting, post-transcriptional silencing of specific genes (Caplen *et al*., 2001; Harborth *et al*., 2001), allowing analysis of gene function over extended periods of time.

In the present study, the siRNA technique has been used to inhibit TASK-2 channel expression, and the effects of gene silencing have been investigated using a combination of molecular biology and electrophysiological studies. We provide evidence that TASK-2 protein expression and current are substantially downregulated in a TASK-2 stably transfected human embryonic kidney (HEK293) cell line following siRNA treatment. In addition our data suggest that TASK-2 channels are involved in both setting resting E_m and modulating electrical responses to local changes in pH in small pulmonary artery myocytes. Certain aspects of these results have been presented at the XXXIII Congress of the Spanish Society of Physiological Sciences (Gönczi *et al*., 2005).

Methods

Generation of a stably transfected HEK293 cell line

HEK293 cells were continuously cultured in a Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% foetal bovine serum (FBS), 1% minimal essential medium (MEM) nonessential amino-acids, 50 units ml^{-1} penicillin, 50 units ml^{-1} streptomycin and were incubated at 37°C in a humidified incubator with 5% CO_2 /95% O_2.

PCR products (from rat kidney) corresponding to the full-length coding sequences of TASK-2 or TASK-1 channels were ligated into the pcDNA3.1/V5-His TOPO expression vector. Following transformation of competent *Escherichia coli*, colonies were selected, cultured and sufficient quantities of plasmid DNA were produced with a Plasmid Maxi Kit. Transfection was performed using a mix of linearised plasmid DNA with calcium–phosphate for 30 h to allow expression of the transferred gene and the medium was then changed to growth medium containing the selecting agent geneticin (800 $\mu g\,ml^{-1}$).

RNA interference technique

The siRNA cassette is a PCR product, which consists of a promoter (e.g. U6 or H1 promoter) and terminator sequence flanking a DNA insert encoding a hairpin siRNA. After transfection, the target-specific DNA insert is expressed and induces gene-specific silencing. Four different siRNA cassette sequences against the TASK-2 gene (KCNK5) were designed by Genscript using their target-finding strategy. BLAST filtering ensured that these sequences had homology only with TASK-2 and not with any other known gene. Lipofectamine 2000 transfection of stably expressing TASK-2 HEK293 cells (TASK-2 cA1) was carried out according to the manufacturer's instructions. The efficacy of these sequences (the concentration- and time-dependent effect on TASK-2 protein levels) was investigated over the 4 days following transfection. The most effective siRNA cassette sequence pair (sense: 5′ TTGGA TATGG CAATG TGGCTC and antisense: 5′ GAGCC ACATT GCCAT ATCCAA) was inserted into a pRNAT-U6.1/Hygro expression vector (siRNA-U6/Hygro). At 24 h after transfection, 100 $\mu g\,ml^{-1}$ hygromycin B was used to select competent transfectants.

Silencer 5′-carboxyfluorescein (FAM)-labelled negative control siRNA was used to demonstrate that the target-specific siRNA did not induce a nonspecific effect on gene expression. The product consisted of a 19 bp scrambled sequence with 3′ dT overhangs and had no significant homology to any known gene sequence from mouse, rat or human.

Plasmid siRNA transfection was repeated in HEK293 cells expressing TASK-1 (TASK-1 cC).

Molecular biology

The effectiveness of the RNAi technique was investigated both at protein (Western blot analysis and immunostaining) and RNA levels (RT-PCR) using methods as previously described (Papp *et al*., 2003; Gardener *et al*., 2004). Specific primary antibodies against TASK-2 and TASK-1, protein kinase C-α (PKC-α) and protein kinase C-δ (PKC-δ) and gene-specific primers (TASK-2 IntFL-5p: 5′ CAAGT TCTTC GGGGG ACGTGC and 3p: 5′ GCCTG TGCCC CAGCC CTCCTCA – 64°C annealing temperature) were used for these experiments. The immunoblot results were analysed with a ChemiGeniusQ chemiluminescence analysis system (Syngene, Cambridge, U.K.) measuring the band volume on light-sensitive films, which could then be used as a quantitative estimate of sample protein levels.

Voltage clamp measurements

Currents were recorded using the whole-cell patch clamp technique with an Axopatch 2A amplifier (Axon Instruments, CA, U.S.A.). The pipettes had resistances of 3–4 MΩ when filled with an artificial internal solution, containing (in mM) 140 KCl, 1 $MgCl_2$, 10 EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid), 10 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 1 Na_2ATP and 0.1 GTP (pH was set to 7.3 using KOH). Cells were maintained in physiological salt solution (in mM: 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 30 sucrose; pH was set to 7.4 with NaOH; Niemeyer *et al*., 2001). Solutions were made more acidic (pH 6.4) or alkaline (pH 8.4) by the addition of 3 M HCl or 3 M NaOH, respectively. Electrical signals were digitised at 1 kHz (Digidata 1200, Axon Instruments, Union City, U.S.A.) and data were analysed using the pClamp 8.0 (Axon Instruments) software.

Functional experiments in isolated pulmonary arteries

Pulmonary arteries (approximately 250–350 μm internal diameter) were dissected on ice from male Sprague–Dawley rats (225–250 g) previously killed by increasing concentrations of

CO_2 followed by exsanguination. Prepared vessels were transfected – with 0.8 μg TASK-2 plasmid siRNA and 2 μl Lipofectamine 2000 transfection reagent in 250 μl Opti-MEM medium or with 15 pmol scrambled siRNA and 2 μl Lipofectamine 2000 in 250 μl Opti-MEM medium – for 4 h at 37°C in a humidified incubator with 5% CO_2/95% O_2. Pulmonary artery segments were then placed and carefully fixed in a sterilised chamber and continuously superfused with oxygenated DMEM medium supplemented with FBS and antibiotics for 72 h.

Microelectrode studies were performed in Tyrode solution (in mM): NaCl 118, KCl 3.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, gassed with O_2. 4-aminopyridine (4-AP, 5 mM), tetraethylammonium chloride (TEA, 10 mM), barium chloride (Ba^{2+}, 30 μM) and glibenclamide (10 μM) were added to Tyrode solution. The pH of the different solutions was set to 6.4, 7.4 or 8.4. Levcromakalim (LK, 10 μM), NS1619 (33 μM), anandamide (10 μM) and clofilium (100 μM) were each dissolved in Tyrode solution and experiments were carried out as previously described (Gardener *et al*., 2004).

Reagents and drugs

DMEM, MEM, FBS, geneticin, penicillin, streptomycin, pcDNA3.1/V5-His TOPO expression vector, TOPO-1 transformation Kit, hygromycin, Lipofectamine 2000 from Invitrogen (Paisley, U.K.); Calcium phosphate transfection unit from Promega (Southampton, U.K.); Plasmid Maxi Kit from Qiagen (West Sussex, U.K.); siRNA cassette sequences, pRNAT-U6.1/Hygro (siRNA-U6/Hygro) expression vector from Genscript (Piscataway, U.S.A.); Silencer 5′-carboxy-fluorescein (FAM)-labelled negative control siRNA from Ambion (Huntington, U.K.); ExTaq PCR-kit from TaKaRa Biomedicals (U.K.); primary antibodies against TASK-2 and TASK-1 (Alomone, Jerusalem, Israel); all other compounds were from Sigma (Dorset, U.K.).

Statistical evaluation

Data are displayed as mean ± s.e.m. To assess the significance of differences (each test group was individually compared with control in all cases), Student's *t*-test, for paired or unpaired data as appropriate, was used. A difference was regarded as significant when $P<0.05$ (marked with *) or $P<0.005$ (marked with **).

Results

The assessment of four different siRNA cassette sequences on TASK-2 channel protein expression in stably-transfected TASK-2 HEK293 cells was compared to mock-transfected TASK-2 cA1 cells using chemiluminescence analysis. As the changes in TASK-2 protein expression were transient using siRNA cassettes, the most effective sequence (shown in Methods) was inserted into a pRNAT-U6.1/Hygro expression vector in an attempt to increase the stability of protein knockdown and this was used in 0.8 $\mu g \cdot well^{-1}$ concentration for further studies.

Plasmid siRNA transfection caused a significant decrease in TASK-2 protein levels

Western blot analysis was used to study the ability of siRNA/Hygro vectors to reduce TASK-2 protein expression in comparison to scrambled and mock-transfected cells. A 56 kDa band corresponding to that predicted for TASK-2 channel protein was evident in all preparations (TASK-2cA1, the plasmid and the scrambled siRNA-transfected cells) (Figure 1a). In contrast no such bands were visible in native HEK293 cells indicating a lack of endogenous channel expression in the cell line itself. Densitometry (Figure 1b) showed that siRNA plasmid-transfections decreased TASK-2 protein expression by 64.2 ± 5.9% ($n=8$) compared with the mock-transfected cell line (100%, $n=8$). The protein level of scrambled-siRNA transfected cells was 98.7 ± 5.3% ($n=8$) of control, showing that only our siRNA sequence was capable to knockdown effectively the TASK-2 channel expression.

Plasmid siRNA transfection was also carried out in TASK-1 cC cells at the same concentration as used above. No significant protein expression changes (band at ≈50 kDa) were observed over the 6 days following siRNA transfection using Western blot analysis (Figure 1c). In addition, in TASK-2 cA1 cells, a primary antibody against PKC-α and PKC-δ demonstrated that nontargeted gene expression was unaffected following the siRNA transfection (data not shown).

The effect of the RNA-silencing methodology was also investigated using fluorescent immunocytochemistry (Figure 2). The intensity of the TASK-2 fluorescent signal was reduced after the plasmid transfections, whereas there was no obvious difference between control and scrambled siRNA-transfected cells.

RNA was extracted from HEK293, mock-transfected, plasmid- and scrambled-siRNA transfected cells and reverse-transcribed prior to PCR (Figure 3a). The RNA concentration of the HEK293 cell line increased following the transfection of

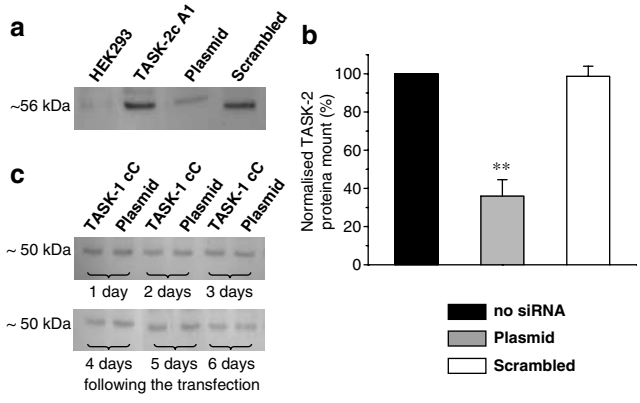

Figure 1 Effect of RNAi on TASK-2 and TASK-1 protein expression. Each sample, containing the same amount of protein (20–30 $\mu g\ lane^{-1}$) was probed using specific polyclonal rabbit TASK-2 (1 : 1000), TASK-1 (1 : 1000) primary and goat anti-rabbit IgG (1 : 20,000) secondary antibodies. The bands at 56 and 50 kDa represent TASK-2 (a) and TASK-1 (c), respectively. Note that TASK-1 protein was unaffected by the anti-TASK-2 RNAi. Chemiluminescence analysis after three different transfections (b) showed that TASK-2 protein expression was significantly decreased ($P<0.005$) following the plasmid transfections ($n=8$), whereas the scrambled siRNA sequence ($n=8$) had no effect compared with controls (defined as 100%, $n=8$).

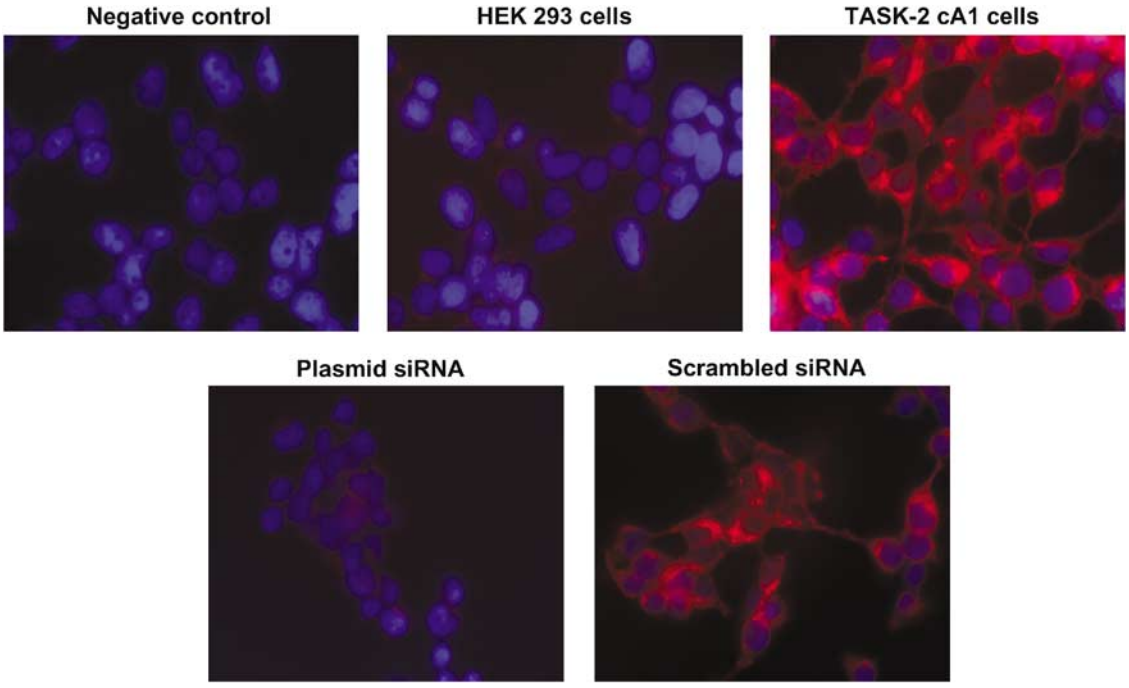

Figure 2 Immunocytochemical analysis of siRNA treatment. Control and siRNA-transfected cells were incubated with primary TASK-2 antibody (1 : 250 in blocking solution) and Texas-red conjugated goat anti-rabbit secondary antibody (1 : 200 in PBS). For negative controls, control peptide was added to the primer reaction. Vectashield mounting medium with DAPI was used to stain the nuclei. Fluorescent microscope images (63 × oil immersion) clearly show that TASK-2 protein expression in the host HEK cells increased after the cDNA transfection. Using plasmid-siRNA transfection in mock-transfected cells, TASK-2 channel expression decreased, whereas the scrambled sequence had no effect.

TASK-2 cDNA (TASK-2cA1) and then significantly decreased after the plasmid siRNA transfection. RNA levels of control and scrambled siRNA-transfected cells showed no differences and corroborated the results obtained for Western blots above. cDNA integrity was assessed using h-GAPDH primers (5′ AAGGT CGGAG TCAAC GGATT TGG and 3′ AATGA GCCCC AGCCT TCTCC AT) at an annealing temperature of 56°C (Figure 3b).

pH-sensitive potassium currents in HEK293 cells were altered by transfection with the specific siRNA sequence

The presence of TASK-2 channels in HEK293 cells was responsible for an outward-rectifying potassium current. To describe the functional effect of siRNA transfection on channel expression, membrane currents were measured using the whole-cell configuration of the patch clamp technique. Only those cells that showed a green fluorescence owing to the presence of the green fluorescent protein (GFP) gene in the plasmid were clamped to −60 mV with the external solution at pH 7.4. During the exposure to acidic (pH 6.4) or alkaline (pH 8.4) solutions, current changes were followed with a 400 ms long depolarising pulse from −60 to +20 mV applied every second. Figure 4a shows representative potassium currents measured in control, scrambled siRNA- and plasmid-transfected cells at different pH levels. RNAi-induced changes are clearly noticeable at pH 7.4 and pH 8.4, whereas smaller differences were detected at pH 6.4. The time-course of current changes in response to acidic and alkaline conditions was rapid (within 10–15 s) and reversible. Currents were stable throughout the experiments and this was the criterion used to determine the *I–V* relationship under differing pH conditions. To illustrate that RNAi technology was effective, we normalised the potassium currents measured in siRNA-transfected cells to the peak current determined in control cells (Figure 4b). In plasmid-transfected cells, currents were significantly smaller at every pH and every voltage step above −40 mV than those measured in control cells. In test cells, at pH 7.4, currents decreased by 43.2% at +50 mV. At pH 6.4, siRNA transfection caused 26.1% current decrease, while at pH 8.4 it generated a 43.3% decrease. We could not observe any difference between the mock-transfected and scrambled siRNA-treated cells, (4% increase at pH 7.4, and 6.1 and 3.9% decrease at pH 6.4 and pH 8.4, respectively) (Figure 4c).

The *I–V* characteristics and reversal potential (approximately −70 mV) of TASK-2 currents were not altered by application of either acidic or alkaline external solutions or by using the siRNA sequence to knockdown channel protein expression (Figure 5a and b). In mock-transfected cells (Figure 5c), the potassium current measured at pH 6.4 and +50 mV decreased by 63.3±3.2% ($n=13$), while at pH 8.4 it increased by 73.6±8.6% of that measured at pH 7.4. In siRNA/Hygro-treated cells peak current at +50 mV was reduced by 50.3±2.5% ($n=18$), and raised by 70.9±6.5% following exposure to pH 6.4 and 8.4, respectively. Similarly, in scrambled siRNA-transfected cells, currents decreased by 66.2±3.8% ($n=10$) in acidic conditions, whereas at pH 8.4 they increased by 74.6±12.1% of the original current. These changes were significant ($P<0.005$) at every voltage step greater than −40 mV.

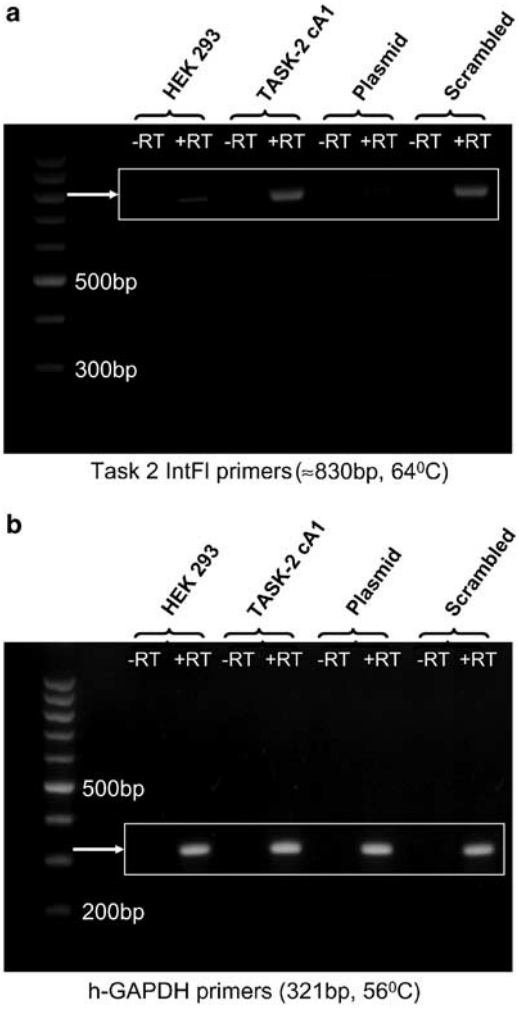

Figure 3 Effect of RNAi on mRNA levels. Extracted and reverse-transcribed RNA samples (−RT: without reverse transcriptase, +RT: with reverse transcriptase) from HEK293 cells, mock-transfected, plasmid- and scrambled siRNA-transfected cells were used for the PCR reactions. The PCR products, amplified with TASK-2 specific primers, were electrophoresed on a 1.5%wv^{-1} agarose gel containing ethidium bromide and visualised under UV light. The TASK-2 RNA level of plasmid siRNA-transfected cells, (band at approximately 830 bp in the +RT samples), was significantly weaker than in the control cells, but not in the negative control siRNA-treated (scrambled) cells (a). The integrity of cDNAs was investigated using h-GAPDH primers. (b) All the +RT-samples had a unique and identical band at 321 bp.

Membrane potential changes in pulmonary artery myocytes were altered following siRNA transfection

The resting E_m of pulmonary artery smooth muscle cells (PASMC) and the membrane potential changes (ΔE_m) caused by different drugs and pH alterations were continuously recorded using sharp microelectrodes in nontransfected control and in TASK-2 siRNA-transfected vessels (Figure 6a). The adenosine triphosphate-activated potassium channel (K_{ATP}) opener LK (10 μM) was added directly to the bath to hyperpolarise the vessels towards the potassium equilibrium potential to assess myocyte viability. The calcium-activated potassium channel (BK_{Ca}) opener NS1619 and a potassium channel inhibitor cocktail (5 mM 4-AP + 10 mM TEA + 30 μM Ba^{2+} + 10 μM glibenclamide) were used to investigate the TASK channel-specific effect of plasmid siRNA in vessels. The hyperpolarisation caused by the application of NS1619 was not significantly different in control and plasmid siRNA-treated vessels (14.2 ± 0.9 mV; $n=4$ and 13.9 ± 0.4 mV; $n=4$, respectively) (Figure 6b). ΔE_m values generated by the inhibitor cocktail were also similar (12.5 ± 0.8 mV in control and 10.3 ± 0.5 mV in the test vessels, $n=4$ in both cases). Although the effects of LK and NS1619 were totally abolished by the cocktail, the pH sensitivity of the myocytes was unchanged under these conditions. The depolarisation induced by anandamide (a putative inhibitor of TASK-1 channels; Maingret *et al*., 2001) in control and siRNA-treated PAMSCs in the presence of the cocktail was similar (9.3 ± 0.6 and 9.6 ± 0.9 mV, respectively). However, a significantly smaller ΔE_m was observed in siRNA-treated cells (1.6 ± 0.3 mV, $n=3$) than in control vessels (6.4 ± 0.5 mV, $n=4$) following exposure to 100 μM clofilium (a putative inhibitor of TASK-2 channels; Niemeyer *et al*., 2001) (Figure 6b). By using the data from these experiments and from the measurements shown in Figure 7, we observed that the resting E_m of control vessels (-54.7 ± 0.3 mV, $n=12$) was not different from that seen after scrambled-siRNA treatment (-55.7 ± 0.2 mV, $n=4$). However, in TASK-2 siRNA-transfected vessels, the basal E_m was significantly depolarised (-51.5 ± 0.5 mV, $P<0.005$, $n=12$) (Figure 6c). This result was the first indication that TASK-2 channels are likely to have a functional role in setting basal E_m.

A protocol involving a series of changes in pH of the solutions was applied to investigate further the presence of functional TASK channels (Figure 7a and b). Depolarisation and hyperpolarisation of the pulmonary artery myocytes mediated by changing to acidic (pH 6.4) and alkaline (pH 8.4) solutions revealed no differences in response between control and scrambled siRNA-transfected vessels (Figure 7b). However, a significantly smaller ΔE_m ($P<0.05$) was seen upon changing from acidic to alkaline solutions in TASK-2 siRNA-transfected vessels (-13.8 ± 0.8 mV, $n=8$) compared to control (-18.3 ± 0.7 mV, $n=8$) or scrambled-siRNA treated cells (-16.8 ± 1.3 mV, $n=4$). Similarly, upon changing back to an acidic solution, the induced depolarisation was significantly smaller ($P<0.005$) in TASK-2 siRNA-transfected vessels (12.9 ± 0.7 mV) than that measured in the other two groups (17.6 ± 0.8 mV in control and 17.2 ± 1.1 mV in scrambled siRNA-transfected cells).

In the presence of 10 μM anandamide, the ΔE_m values measured in the plasmid-treated myocytes were also significantly different at both pH 6.4 and pH 8.4 (15.9 ± 0.9 and -6.3 ± 0.9 mV, respectively) compared with the control (21.9 ± 0.6 and -13.4 ± 0.9 mV) or scrambled siRNA-transfected cells (21 ± 1.3 and -11.6 ± 1.4 mV). Finally, the ΔE_m generated by addition of 100 μM clofilium at pH 8.4 in the plasmid-treated vessels (5.4 ± 0.7 mV) was significantly smaller than in the other two groups (8.9 ± 0.9 in control and 8.1 ± 0.6 mV in scrambled siRNA-transfected cells).

Discussion

Background to the investigation

In earlier studies, we obtained evidence of a functional role for TASK-1 channels in rat pulmonary arteries (Gardener *et al*.,

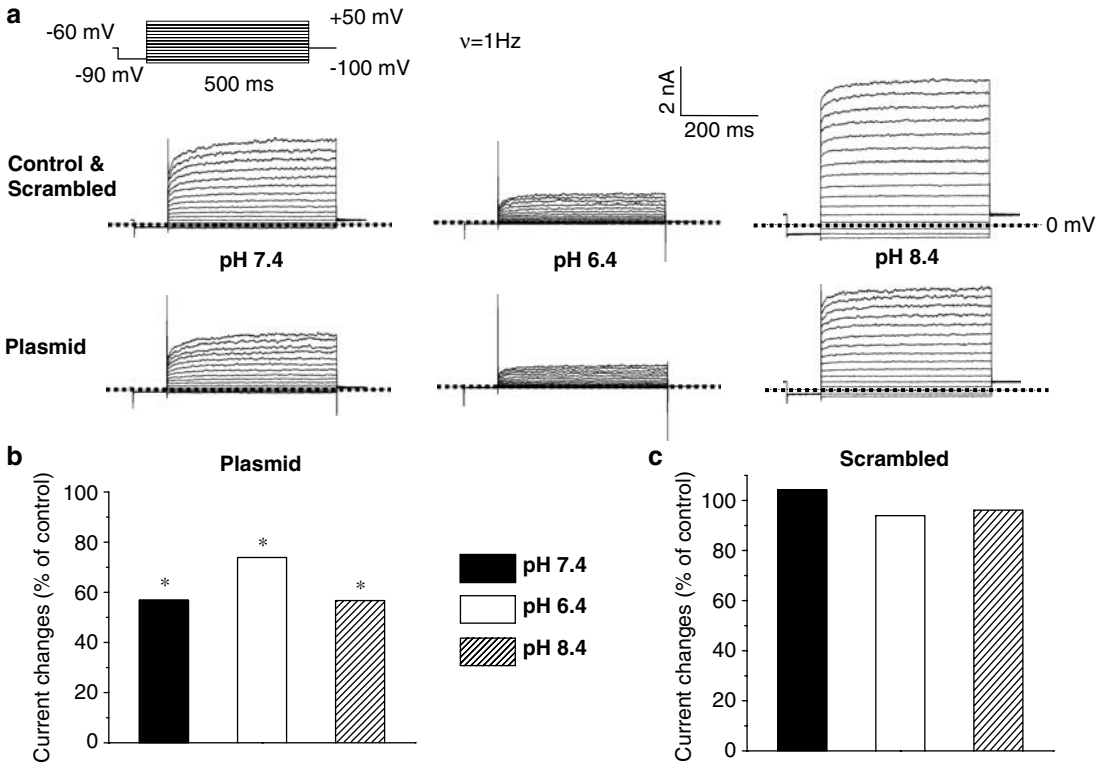

Figure 4 Whole-cell patch clamp analysis of RNAi methodologies. Wavelengths of 395 nm excitation and 500 nm emission were used to detect the siRNA expression vector (containing a fluorescence GFP marker) in the plasmid-transfected cells. The TASK-2 potassium currents were measured with the pulse protocol shown and the data clearly show the effect of pH changes and RNA interference (a). The currents in siRNA-(plasmid or scrambled) transfected cells were normalised to those measured in control cells in every pH condition. The specific siRNA sequence (siRNA-U6/Hygro, $n=18$) significantly decreased ($P<0.05$) the measured currents compared with mock-transfected cells ($n=13$) (b), while the TASK-2 currents were not significantly different in control cells and the cells transfected with the scrambled sequence ($n=10$) (c).

2004). Since the presence of TASK-2 channels was also detected in this study, the objective of the present investigation was to determine whether TASK-2 channels also had a function similar to that associated with TASK-1. As the pharmacology of the TASK family is at an early stage of development (only putative activators are available), the initial strategy adopted was to 'knock out' TASK-2 channels using RNA interference techniques (Gönczi *et al*., 2005). Although there are numerous reports of the effectiveness of siRNA transfection into mammalian cell lines, there are very few published functional studies using this technology (Hasuwa *et al*., 2002; Kunath *et al*., 2003; Giladi *et al*., 2003; Gratsch *et al*., 2003; Sorensen *et al*., 2003). siRNA techniques have been previously applied to whole organs (Gurney & Hunter, 2004), but we believe that the present study is the first to examine the functional consequences of ion channel deletion in a whole isolated blood vessel.

Efficacy and selectivity of the TASK-2 siRNA/Hygro vector in HEK cells

Although the RNAi technique efficiently decreases gene post-transcription, many factors affect the success of gene silencing. The most important consideration is the choice of siRNA sequence and the method of its incorporation into cells. We thus designed and evaluated a number of sequences before proceeding with the most effective one. The time-dependence of the effectiveness of gene silencing is also dependent on the biophysical properties of the protein encoded by the target gene. Thus, with short half-life proteins (48 h or less), siRNA will be more effective than against those with longer half-lives. In our stably transfected TASK-2 cell-line, the knockdown effects of our cassette sequences were reversible. Therefore, in order to generate a more stable inhibition of TASK-2 protein expression and to select transfected cells, the most effective cassette sequence was built into a pRNAT-U6.1/Hygro expression vector.

Transfection using the selected TASK-2-specific siRNA/Hygro vector resulted in a remarkable decrease in TASK-2 protein expression in our stably-transfected TASK-2 cells compared with the mock-transfected cell line. Both immunocytochemistry and RT-PCR confirmed the specificity of our chosen siRNA sequence whereas the nonspecific, scrambled siRNA sequence failed to inhibit TASK-2 channel expression. To prove the selectivity of the chosen siRNA sequence, possible expression changes in general proteins are often also investigated (Harborth *et al*., 2001; Hasuwa *et al*., 2002; Kunath *et al*., 2003). Thus, the TASK-2 siRNA/Hygro vector had no effect on either PKC-α or PKC-δ protein expression (data not shown). Furthermore, HEK293 cells stably expressing TASK-1 channel proteins (closely related to TASK-2), were also transfected with the TASK-2 siRNA/Hygro vector, but no significant changes in TASK-1 protein levels were observed. These 'controls' clearly indicate that the chosen

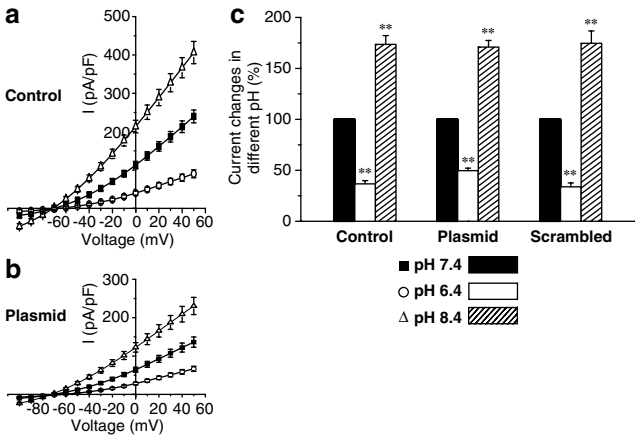

Figure 5 Effect of RNAi on pH-sensitive currents. The peak currents were determined at the end of each of the 500 ms steps shown in Figure 4, normalised to cell capacitance and plotted as a current–voltage relationship (representative curves from control (a) and plasmid-transfected cells (b)). The mean currents measured following exposure to acidic and alkaline solution were significantly different ($P<0.005$) from those at pH 7.4 at every voltage step above -40 mV and therefore only the current changes at $+50$ mV are presented (c). The pH sensitivity of the potassium currents was not significantly different in any of the tested cell types.

siRNA sequence was selectively silencing TASK-2 expression without affecting the expression of closely-related TASK-1 channels or other proteins.

The presence of TASK-2 channels in a HEK293 cell line generated an outwardly rectifying potassium current. In both mock-transfected and siRNA-transfected cells, potassium currents at pH 7.4 decreased on exposure to acidic (pH 6.4) solution, while peak currents increased at pH 8.4. RNAi induced a clear reduction in potassium current at pH 7.4 and 8.4, conditions under which TASK-2 channels are more likely to be open (Duprat *et al*., 1997; Reyes *et al*., 1998; Lesage & Lazdunski, 2000). Although the slopes of the TASK-2 *I–V* relationships were altered at these pH levels, no change in reversal potential was observed. At pH 6.4, RNAi treatment also significantly reduced TASK-2 currents. However, the reduction was smaller than that at pH 7.4 and 8.4, perhaps because the open probability of TASK-2 is lower at this pH. The specificity of our RNAi sequences was also demonstrated at a functional level, using transfection with scrambled siRNA. After this procedure, potassium currents were not significantly changed either in their magnitude or pH-sensitivity compared with mock-treated controls.

The current reductions caused by RNAi (approximately 40%) were relatively smaller than anticipated from measure-

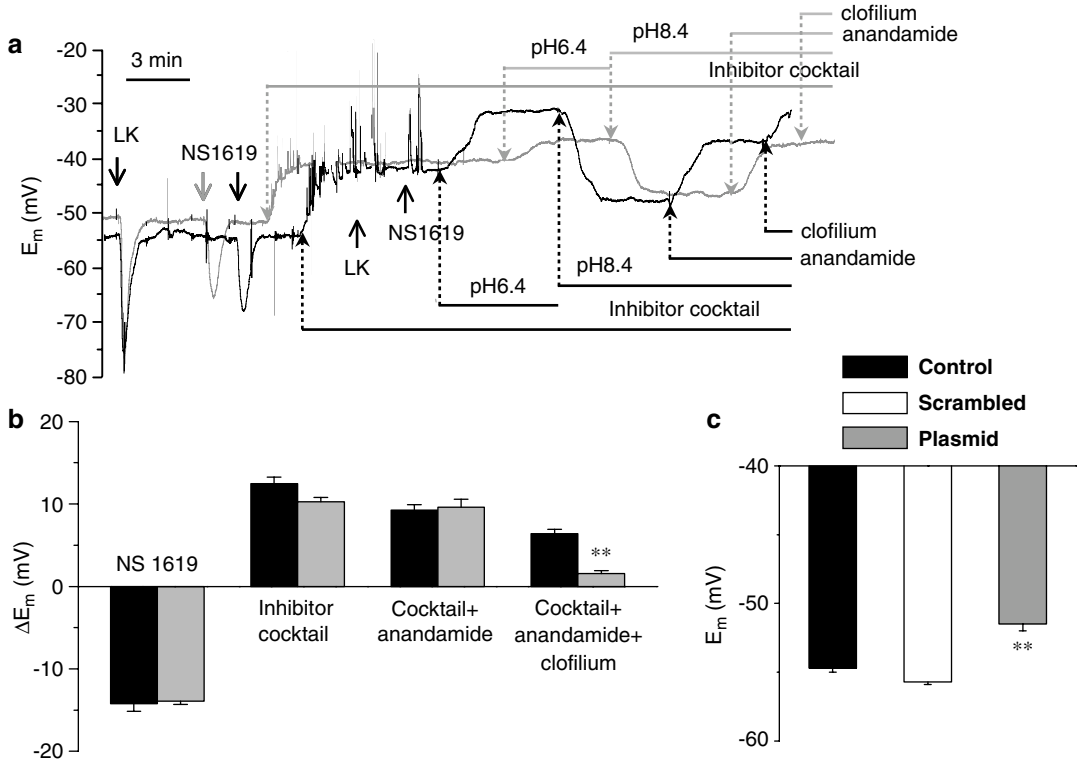

Figure 6 Microelectrode experiments in pulmonary artery segments. The membrane potential of pulmonary artery myocytes was monitored using microelectrodes filled with 3 M KCl (tip resistance 50–80 MΩ). Representative recordings (a) from control and plasmid siRNA-transfected vessels show membrane potential changes following transient exposure to 10 μM LK, 33 μM NS1619 in the absence and presence of the potassium channel inhibitor cocktail (containing 5 mM 4-AP, 10 mM TEA, 30 μM Ba^{2+} and 10 μM glibenclamide) in pH 7.4 PSS. Then different pH solutions, 10 μM anandamide and 100 μM clofilium were used in the presence of the cocktail. Horizontal lines indicate the duration of different solution applications, whereas vertical, dotted lines mark the starting points of the solution changes. The mean membrane potential changes ($n=4$ in every case) caused by NS1619, the inhibitor cocktail, by anandamide and clofilium (applied together with the cocktail in pH 8.4 solution) are represented in panel b. The mean basal membrane potential of control ($n=12$), plasmid siRNA-transfected ($n=12$) and scrambled siRNA-treated ($n=4$) PASMCs following five separate transfections is represented in panel c.

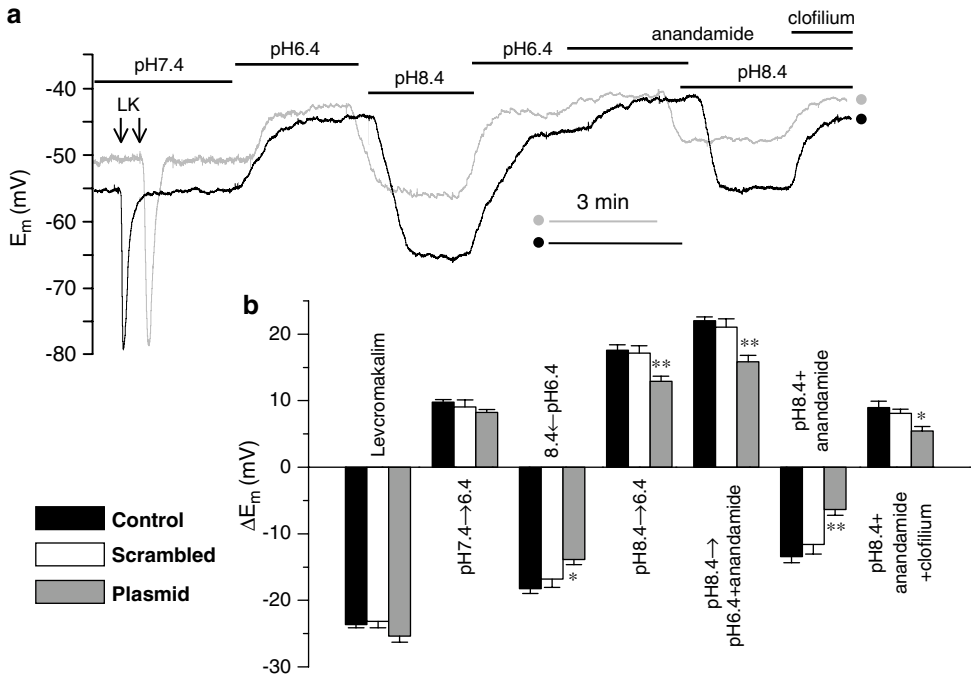

Figure 7 Altered pH responses in plasmid siRNA-transfected arteries. Representative recordings from control and plasmid siRNA-transfected vessels clearly show that the myocyte resting membrane potentials were different, whereas LK hyperpolarised cells in both groups to similar levels. (a). The scrambled siRNA sequence had no effect (not shown). Membrane potential changes caused by acidic and alkaline solutions were significantly smaller in plasmid-transfected arteries than in control or scrambled vessels in the presence or absence of 10 μM anandamide and 100 μM clofilium (b).

ments of TASK-2 protein (reduced by approximately 65%), but several factors could account for this. Firstly, K_{2P} channels form functional dimers (Lesage & Lazdunski, 2000), so proteins detected by immunolabelling techniques are not necessarily just the functionally active ones. Conversely, RNAi technology functions at an mRNA level, but has no effect on proteins already translated and membrane-bound. Also, there could be monomer channel proteins in the cytoplasm ready to form functional channels. Presumably cells react to the decreased protein transcription and raise the rate of self-dimerisation of TASK-2 units. Collectively, such possibilities could account for the relatively small discrepancy between the observed reduction in channel protein and in TASK-2 currents.

Functional effects of the TASK-2 siRNA/Hygro vector in isolated pulmonary arteries

The resting E_m of myocytes from vessels exposed to scrambled siRNA-transfection and superfused over a 72 h period were not different from our previously published values, indicating that the experimental procedures themselves did not produce any functional effect on resting E_m. In siRNA-transfected vessels, myocyte resting E_m was significantly more positive (mean 4 mV) than in scrambled siRNA-treated and control vessels. Furthermore, compared to the effects in control vessels, there were significantly smaller de- and hyperpolarisations in plasmid-transfected vessels following exposure to acidic and alkaline solutions, respectively. In contrast, control hyperpolarisations using NS1619 and LK showed that TASK-2 specific siRNA transfection did not decrease or modulate the function of BK_{Ca} and K_{ATP}. The contribution of other K^+ channels such as voltage-sensitive potassium channels (K_v) and inward-rectifying potassium channels (K_{IR}) (also K_{ATP} and BK_{Ca}, as judged by the change in E_m in the presence of the inhibitor cocktail), were not different in either control or siRNA-treated vessels. Collectively, therefore, these results strongly suggest that the removed TASK-2 channels contributed approximately 4 mV to basal E_m levels. Assuming that the TASK-2 channel expression was not totally abolished by plasmid siRNA transfection (based on the observations that the TASK-2 protein expression and current were partially reduced in HEK293 cells), the contribution of TASK-2 channels to basal E_m could indeed be greater than 4 mV. However, since other types of K_{2P} channels (KCNA and KCNQ) also play an important role in the regulation of resting E_m in the vasculature, it is very difficult to be precise about the total contribution of TASK-2 channels in this complex system.

Selectivity of TASK-2 siRNA interference techniques in whole vessels

We have previously shown that TASK-1 channels contribute to setting resting E_m in both pulmonary and mesenteric artery smooth muscle cells (Gardener *et al*., 2004). It was thus important to show that TASK-2 plasmid siRNA transfection did not alter the function of the closely related TASK-1 channel in pulmonary artery myocytes. Although the endocannabinoid, anandamide has a complicated pharmacological profile involving TASK-1 channel inhibition, actions at cannabinoid receptors and possibly other targets (Maingret *et al*., 2001; Randall, 2005), the effect of pH changes in the presence of anandamide was significantly smaller in the

TASK-2 siRNA-treated group than in scrambled siRNA or control vessels in which TASK-2 channels were fully present. Moreover, the magnitude of the anandamide-sensitive component, that is, the putative TASK-1 contribution, was the same in scrambled- and siRNA-treated arteries. These observations collectively suggest that the tiny pH-induced ΔE_m seen in TASK-2 siRNA-transfected vessels (in the presence of anandamide to block TASK-1 channels) were indeed due to the specific reduction of TASK-2 channel expression.

The quaternary ammonium derivative, clofilium, inhibits a variety of K^+ channels including TASK-2 (Niemeyer *et al*., 2001; Steidl & Yool, 2001; Perry *et al*., 2004). Thus, our finding that the clofilium-induced depolarisation was also significantly smaller in siRNA plasmid-treated vessels than in the other two groups (even in the presence of the K^+ channel inhibitor cocktail) supports the conclusion that our siRNA procedures had indeed selectively reduced the number of functional TASK-2 channels.

This pharmacological evidence, coupled with the lack of effect of TASK-2 siRNA on TASK-1 (in the stably-transfected TASK-1 cells), PKC-α and PKC-δ expression provides additional evidence that TASK-2 channel expression was markedly and selectively inhibited following gene-specific siRNA transfection. The RNAi protocols adopted in the present study demonstrate that the technology to inhibit TASK-2 channel expression in a heterologous system can be transferred to a whole isolated vessel to estimate the contribution and function of the targeted ion channel. This is the first time, to our knowledge, that the functional consequences of ion channel deletion using this technique have been reported and was achieved without affecting the sensitivity or characteristics of the response to pH or the function of other K^+ channels such as BK_{Ca} or K_{ATP}.

K_{2P} channels and their role in cellular excitability

Several subclasses of K^+ channel have been postulated to control resting E_m in pulmonary arteries. The classical view is that voltage-sensitive channels, with contributions of Ca^{2+}-sensitive and ATP-dependent K^+ channels, play major roles in controlling resting E_m. However, this view has been questioned since the voltage-, ionic- and metabolic dependencies of these channels do not make them ideal candidates for setting E_m levels over the physiological range (Gurney *et al*., 2002). In contrast, the properties of the K_{2P} (KCNK) family make them highly likely candidates for involvement as 'leak' or background conductance pathways (Lesage & Lazdunski, 2000). Indeed, strong evidence of a role for TASK-1 channels in setting resting E_m in both rabbit and rat pulmonary myocytes has recently emerged (Gurney *et al*., 2003; Gardener *et al*., 2004).

Limited data are available regarding a physiological role for TASK-2. Speculatively, such channels may be involved in regulating cell volume in mouse Erlich cells (Niemeyer *et al*., 2001), primary cultured mouse proximal tubules (Barriere *et al*., 2003) and also murine spermatozoa (Barfield *et al*., 2005). TASK-2 may also contribute to bicarbonate transport in the renal proximal tubule (Warth *et al*., 2004), may act as a background current in taste receptor cells (Lin *et al*., 2004) and in small and large intestinal smooth muscle (Cho *et al*., 2005). The data from the present study on TASK-2 channels additionally attribute a physiological role for TASK-2 in vascular myocytes. Together with our previous observations on TASK-1 (Gardener *et al*., 2004), the results suggest that these closely-related channels could each contribute several millivolts to basal E_m values in PASMC. Thus, the opening or closing of either channel could significantly affect the excitability of the pulmonary vasculature and the recent development of a putative opener (BL-1249; [(5,6,7,8-tetrahydro-naphtolen-1-yl)-[2-(1H-tetrazol-5-yl)-phenyl]-amine]) of the K_{2P} channel, TWIK-related K^+ channel (TREK-1), illustrates the therapeutic potential of K_{2P} modulation in irritable bladder syndrome (Tertyshnikova *et al*., 2005). The development of a similar TASK-2 modulator could potentially represent an exciting development for the treatment of pulmonary vascular disease.

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