Expression of TASK and TREK, two-pore domain K⁺ channels, in human myometrium

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Abstract

Two-pore domain K⁺ channels are an emerging family of K⁺ channels that may contribute to setting membrane potential in both electrically excitable and non-excitable cells and, as such, influence cellular function. The human uteroplacental unit contains both excitable (e.g. myometrial) and non-excitable cells, whose function depends upon the activity of K⁺ channels. We have therefore investigated the expression of two members of this family, TWIK (two-pore domain weak inward rectifying K⁺ channel)-related acid-sensitive K⁺ channel (TASK) and TWIK-related K⁺ channel (TREK) in human myometrium. Using RT-PCR the mRNA expression of TASK and TREK isoforms was examined in myometrial tissue from pregnant women. mRNAs encoding TASK1, 4 and 5 and TREK1 were detected whereas weak or no signals were observed for TASK2, TASK3 and TREK2. Western blotting for TASK1 gave two bands of approximately 44 and 65 kDa, whereas TREK1 gave bands of approximately 59 and 90 kDa in myometrium from pregnant women. TASK1 and TREK1 immunofluorescence was prominent in intracellular and plasmalemmal locations within myometrial cells. Therefore, we conclude that the human myometrium is a site of expression for the two-pore domain K⁺ channel proteins TASK1 and TREK1.

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Introduction

Two-pore domain K⁺ (K_{2P}) channels are an emerging family of K⁺ channels. They are different from other classic K⁺ channels in that they possess two pore-forming domains and exhibit different pharmacological properties (Patel & Honoré 2001). A key biophysical feature of these channels is their relative voltage-insensitivity and, as such, they are thought to contribute to resting membrane potential and have been termed leak or background channels. However, it is becoming more evident that these channels have diverse mechanisms of modulation. Factors influencing these channels include extracellular pH (Duprat *et al.* 1997), membrane stretch, arachidonic acid (Bang *et al.* 2000, Miller *et al.* 2003) and oxygen tension (hypoxia; Lewis *et al.* 2001, Miller *et al.* 2003).

Since the cloning of the first member of the K_{2P} channel family, TWIK1 (two-pore domain weak inward rectifying K⁺ channel) (Lesage *et al.* 1996), numerous other members have been described including TASK (TWIK-related acid-sensitive K⁺ channels)1–5 and TREK (TWIK-related K⁺ channels)1 and 2. TASK channels have been localised to both electrically excitable and non-excitable tissues (e.g. brain, pancreas, lung and kidney; reviewed in

O'Connell *et al.* 2002). TREK channels have a predominantly neural location but their mRNA has also been found in small intestine, kidney and pancreas (Medhurst *et al.* 2001). Functionally, TASK channels are exquisitely sensitive to perturbations in extracellular pH: acidosis inhibits TASK currents whereas alkalosis increases the current (Duprat *et al.* 1997). TREK channels, on the other hand, are modulated by membrane stretch and arachidonic acid (Bang *et al.* 2000, Miller *et al.* 2003). One common regulatory mechanism, described for TASK1, TASK3 and TREK1 is their inhibition by hypoxia (Hartness *et al.* 2001, Lewis *et al.* 2001, Miller *et al.* 2003).

The K⁺ channel superfamily contains over 100 different subunits/proteins (Coetzee *et al.* 1999) yet current understanding of uterine K⁺ channel expression is very limited. Large-conductance Ca²⁺-activated K⁺ channel mRNA, protein and function has been documented (Khan *et al.* 2001). Voltage-gated K⁺ channel currents have been studied, although there is scant evidence regarding molecular identities (Knock *et al.* 1999). Alternatively, K_{ATP} mRNA expression has been studied during gestation (Curley *et al.* 2002) but little functional evidence documented. Clearly,

there is a need for a more complete analysis of K⁺ channel expression in uterine tissue.

To this end, we decided as an initial step to study the expression of K_{2P} channels in pregnant human myometrium. This would give important information on the expression of these channels and, if present, offer insights into their possible roles in determining uterine excitability. Although there are likely to be many K⁺ channel subtypes responsive to pH, the K_{2P} channels, as mentioned above, do have a uniqueness as a K⁺ channel sub-family in their reported sensitivities to extracellular pH, experimental hypoxia and stretch – all stimuli that influence animal and human myometrial contractility (Taggart *et al.* 1997, Bugg *et al.* 2002a, Monir-Bishty *et al.* 2003). For both these reasons, we focused our attention on examining the expression and localisation of TASK and TREK in human myometrium.

Materials and Methods

Myometrium was taken from women undergoing elective Caesarean section at term after informed written consent and following local ethical committee approval. Expression studies were performed on myometrial biopsies taken from the mid-upper anterior lip. Samples were snap-frozen in liquid nitrogen and stored at –80 °C until required.

RT-PCR

Total RNA was isolated from homogenised myometrium using Trizol reagent (Invitrogen). RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (RT) according to the manufacturer's instructions (Invitrogen) in a Perkin-Elmer Cetus thermal cycler (Perkin-Elmer, Beaconsfield, South Bucks, UK). An initial RT-PCR, using β -actin, in which the RT was omitted was performed to ensure that all RNA samples were not contaminated with genomic DNA. Subsequent RT-PCR, using standard techniques, with a hot start was employed using primer pairs already utilised in other studies or designed by us: TASK1, annealing temperature (Ta) 59 °C (Hamilton *et al.* 2000); TASK2, Ta 62 °C (Reyes *et al.* 1998); TASK3 5'-cggatcaaggggaagtacaa-3' (forward) with 5'-tgccataacctatgggtggt-3' (reverse), Ta 60 °C; TASK4 5'-gcattctggaaccttctgctc-3' (forward) with 5'-atagctgcacccagcctcta-3' (reverse), Ta 62 °C; TASK5, Ta 62 °C (Kim & Gnatenco 2001); TREK1 and 2, Ta 55 and 56 °C respectively (Lesage *et al.* 2000). BLAST searches were performed to ensure primers had no homology with any other known gene products. The number of cycles was 35 for each primer pair. An appropriate positive (human kidney and brain; Becton Dickinson Bioscience, Oxford, UK) and negative control (water replacing the template) were used. β -Actin was routinely amplified from all samples, confirming sample integrity and amplification capacity. PCR products were purified and subsequently sequenced using in-house facilities to confirm identity. The sequenced products had 98% (TASK1, 2, 3), 99% (TASK5) and 100% (TASK4 and

TREK1) homology with the known published sequences when compared using BLAST (not shown).

Western blotting

Myometrial tissues, different from those used for RNA extraction, were homogenised on ice in homogenisation buffer (in mol/l): 0.01 Hepes, 0.001 EDTA, 0.25 sucrose (pH 7.4) with an anti-protease inhibitor cocktail (4-(2-aminoethyl)benzenesulphonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin; Sigma-Aldrich). Rat brain was used as a positive control for TASK and TREK expression. Barrier bred male Wistar rats were housed at BSU, University of Manchester and Schedule I killed (Stunning followed by cervical dislocation) according to UK Home Office guidelines. We routinely used the post-nuclear supernatant, obtained after centrifugation at 4000 *g* for 10 min, for our blotting experiments.

In a subset of experiments, we examined the sub-cellular distribution of TREK1 protein. After homogenisation of myometrial tissue, an initial centrifugation at 1000 *g* for 10 min was performed to yield a pellet predominately containing nuclear protein. The resulting supernatant was further centrifuged at 100 000 *g* for 1 h, after which a crude membrane pellet and supernatant with cytosolic protein were obtained. Both the nuclear and membrane pellets were resuspended in homogenisation buffer. All sample protein concentrations were determined using a commercial protein assay kit (Bio-Rad). Samples were stored at –80 °C.

Protein from myometrium (elective Caesarean section, 50 or 100 μ g as indicated) and rat brain (50 μ g) was mixed with a reducing, non-boiling loading buffer containing 8 mol/l urea, 5% SDS (w/v), 0.04% bromophenol blue (v/v) and 0.455 mol/l dithiothreitol, in 0.05 mol/l Tris-HCl (pH 6.9). Proteins were electrophoretically separated in 10% polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked for 1 h using blocking buffer (3% dried milk powder (w/v) in 0.05% Tween 20 (v/v), Tris-buffered saline (TBS (in mol/l): 0.015 Tris, 0.150 NaCl; pH 8.0)). The membrane was probed for 2 h at room temperature with anti-TASK1 or anti-TREK1 antibody (Alomone Labs, Jerusalem, Israel) at a 1:100 or 1:200 dilutions respectively in blocking buffer. TASK1 rabbit polyclonal antibody was raised against the peptide (C-terminus) corresponding to residues 252–269 of the human TASK1 channel. TREK1 rabbit polyclonal antibody was raised against residues 8–28 of human TREK1 channel. Three washes in TBS/0.05% Tween 20 were followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a 1:2000 dilution (DAKO, Ely, Cambs, UK) for 1 h. After washing in TBS, membranes were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). Appropriate negative and pre-absorption controls were also performed. These controls consisted of omission of the primary antibody and pre-absorption of the primary

antibody for 1 h at ambient room temperature with a 3-fold excess of the epitope to which it was raised.

Immunocytochemistry

Frozen myometrial tissue sections (4 µm thick, sampled randomly from three separate biopsies) were air-dried and fixed with 2% paraformaldehyde (v/v), 4% sucrose (w/v) in PBS (in mol/l: 0.137 NaCl, 0.0027 KCl, 0.0081 Na₂HPO₄, 0.0019 NaH₂PO₄; pH 7.4) for 10 min, then permeabilised with 0.3% Triton X-100 (v/v; Sigma) in PBS for 10 min. Non-specific binding sites were blocked with 4% fetal calf serum (v/v; FCS), 0.1% Tween 20 (v/v) in PBS for 30 min. Anti-TASK1 or anti-TREK1 was applied at a 1:100 dilution for 1 h at room temperature, then washed in 4% FCS in PBS and incubated further for 30 min at room temperature with a 1:50 dilution of the secondary antibody (DAKO), which was FITC-labelled anti-rabbit IgG. The sections were washed with PBS and mounted with Vectashield (Vector Labs, Peterborough, Cambs, UK) containing propidium iodide. Sections were then viewed using confocal laser-scanning microscopy (Bio-Rad Radiance 2000). The localisation of TASK1 and TREK1 within myometrial tissue was determined using an ×40 oil immersion objective. In negative control and pre-absorption experiments, an equivalent volume of PBS replaced the primary antibody or the primary antibody was pre-absorbed for 1 h at ambient room temperature with a 3-fold excess of the epitope to which it was raised.

Results

Figure 1A demonstrates that the amplification products from control tissues (human kidney or human brain) using gene-specific primers for TASK1-5 or TREK1-2 appear as single amplicons at the predicted molecular size, validating the primers used in this study. These primers were then used to screen myometrial biopsies taken from four individual women (Fig. 1B). Detectable signals, which co-migrated with those in the positive controls, were observed for TASK1, TASK4, TASK5 and TREK1, weaker and more variable signals were observed for TASK2 and TASK3. TREK2 mRNA was undetected.

The presence of mRNA, however, does not necessarily mean that translation into protein occurs. To examine this further, the expression of TASK1 and TREK1 at the protein level was studied, selected on the basis that TASK1 is the main K_{2P} channel reported to be present by both Western blotting and immunolocalisation in other tissues (see Discussion) and that the message for TREK1 was qualitatively similar to TASK1. Using anti-TASK1, a single band of approximately 55 kDa was observed in rat brain (Fig. 2A). However, in myometrium from individual pregnant women we observed two bands of ~44 kDa and 65 kDa (Fig. 2A). Using anti-TREK1, two bands of ~60 and 90 kDa were observed (Fig. 2D) in myometrium and a single band of ~60 kDa in rat brain. In negative control and competition experiments, when

Figure 1 mRNA expression of TASK and TREK in human myometrium. (A) RT-PCR of positive control tissues for TASK and TREK. Amplicons of the predicted size were observed for TASK1-5 and TREK1 and 2. For TASK1-5 predicted sizes were (in bp) 521, 566, 155, 169 and 419 respectively and for TREK1 and 2, 356 and 482 bp. The molecular ladders (L) are shown on both the left and right hand sides. The lanes correspond to the following cDNA samples: KI, kidney; HB, human brain. (B) Screening of myometrial strips from four independent Caesarean sections (lanes 1–4). –VE, cDNA template replaced with water; +VE, positive control cDNA template, which was either human brain (HB) or kidney (KI) as indicated. In (B) every sample exhibited amplification for β-actin, confirming sample cDNA integrity and amplification capacity.

either the primary antibody was omitted or pre-absorbed with the epitope against which it was raised, no signals were observed (Fig. 2B and E respectively). Comparable signals were obtained for β-actin in all samples confirming equal protein loading between samples (Fig. 2C and F). In a separate series of experiments, we examined the sub-cellular distribution of TREK1 protein (Fig. 2G). As can be seen, TREK1 was localised to all three cellular fractions, with a more predominate signal observed in the cytosol.

We decided to examine sites of possible expression using immunostaining, the results of which are shown in Fig. 3. Figure 3A shows the pattern of TASK1 staining observed in pregnant myometrium. TASK1 was observed throughout plasmalemmal and intracellular locations. TREK1 also exhibited staining consistent with an intracellular location, as evidenced by a near-nuclear distribution (Fig. 3E). In similar negative control and

Figure 2 Detection of TASK1 and TREK1 proteins in human myometrium. Western blotting analyses of TASK1 and TREK1 in three myometrial samples from pregnant women. (A) Images were derived from identically treated gels loaded with 100 µg protein and exposed to a 1:100 dilution of anti-TASK1. (D) A separate membrane treated as (A) but loaded with 50 µg protein and exposed to a 1:200 dilution of anti-TREK1. The same samples exhibited no bands when the primary antibody was omitted or when the primary antibody was pre-absorbed with a 3-fold excess of the epitope against which it was raised (B, E). Rat brain (RB, 50 µg) was used as positive control samples for TASK1 and TREK1. β-Actin bands (43 kDa) support the loading of comparable amounts of protein between each myometrial sample (C, F). (G) Characterisation of the sub-cellular distribution of TREK1 protein. Cellular fractions were prepared as described and 50 µg protein loaded per lane. The membrane was exposed to a 1:200 dilution of anti-TREK1. MEM-membrane fraction. CTT-Cytosolic fraction NUC nuclear fraction.

competition experiments to those described for Western blotting, when the primary antibody was omitted (Fig. 3D, TASK1 shown) or pre-absorbed with a 3-fold excess of the epitope (Fig. 3F, TREK1 shown), no staining was observed.

Discussion

We have investigated the expression of TASK1-5 and TREK1 and TREK2 within human myometrium. mRNA for TASK1, 4 and 5 and TREK1 were observed. At the

Figure 3 TASK1 and TREK1 immunofluorescence within myometrial tissue sections. (A) Representative example of immunofluorescence staining for TASK1. (B) Nuclear staining in the cells shown in panel (A). (C) A merged image of the signals from (A) and (B). (D) Negative control in which the TASK1 primary antibody was omitted and nuclei were subsequently stained. (E) Representative example of immunofluorescence staining for TREK1 (green) and nuclei (red). (F) Competition control where the primary antibody to TREK1 was pre-absorbed with a 3-fold excess of the epitope against which it was raised and nuclei were subsequently stained. For all experimental manoeuvres, *n* > 3 tissue samples. Anti-TASK1 and anti-TREK1 were used at a dilution of 1:100. Secondary FITC-labelled anti-rabbit IgG was used at 1:50. Propidium iodide was used to stain nuclei. The scale bar in all panels is 10 µm.

level of protein, TASK1 was detected in myometrium, as was TREK1. Immunocytochemistry revealed a relatively uniform plasmalemmal and intracellular distribution of TASK1 and TREK1 in myometrial cells.

Using RT-PCR and Northern blotting, both TASK1 and TASK2 have been shown to be relatively weakly expressed in whole mouse and human uterus (Reyes *et al.* 1998, Medhurst *et al.* 2001), as has TASK3 (Medhurst *et al.* 2001). The screening of myometrial strips (Fig. 1B) gave us consistently detectable signals for TASK1, 4 and 5, with mRNA for the latter two channels being identified in the human myometrium for the first time. TREK1 mRNA has been shown to be expressed in human uterus (Reyes *et al.* 1998, Meadows *et al.* 2000) and our data suggest TREK1 expression is at the level of the myometrial cells and we document, for the first time, the lack of a detected signal for TREK2 in human myometrium, suggesting that myometrium is unlikely to be a significant site of expression of TREK2.

Western blotting for TASK1 revealed the presence of two bands, one of 65 kDa and another of 44 kDa in myometrium (Fig. 2A). Other studies in various tissues using this commercial antibody, whose epitope is conserved between human, rat and mouse, have documented bands of differing sizes. Lopes *et al.* (2000) found a single band of approximately 55–60 kDa in mouse heart. In rat heart, bands of 65 and 130 kDa have been observed (Jones *et al.* 2002), as has a band of 45 kDa (Rajan *et al.* 2002). In rat brain, TASK1 has been reported to be 50 kDa (Rajan *et al.* 2002). Clearly variability exists in the reported molecular size of TASK1. A core molecular mass for rat TASK1 has been suggested to be 45 kDa (Leonoudakis *et al.* 1998).

There are also limited Western blotting and immunostaining data for TREK1. Using an antibody corresponding to residues 5–21 of human TREK1, Hervieu *et al.* (2001) demonstrated the presence of bands that were 56 kDa and, more predominately, 112 kDa in size. In cells overexpressing TREK1, Maingret *et al.* (2000) used a polyclonal antibody directed against residues 1–44 and 71–114 to visualise a band of 84 kDa under non-reducing conditions that subsequently migrated to an apparent molecular mass of 45 kDa under reducing conditions (β -mercaptoethanol). However, Western blotting of murine and rat brain revealed a single band of $\sim$ 92 kDa. This antibody recognised only a single band of $\sim$ 45 kDa when subsequently used to blot rat heart microsomes (Terrenoire *et al.* 2001). A core size for TREK1 has been estimated to be 44 kDa (Hervieu *et al.* 2001). The bands we document here are $\sim$ 60 and 90 kDa in pregnant human myometrium and $\sim$ 60 kDa in rat brain. These bands are comparable to those already reported in other tissues. Although we detected messages for TASK4 and 5 in human myometrium, there are as yet no antibodies available to TASK4 or TASK5, therefore we could not test for protein expression.

The origins of different sized molecular mass bands for TASK1 and TREK1 are unknown, but may reflect different states of post-translational modification, e.g. there are putative glycosylation sites on TASK1 (Duprat *et al.* 1997,

Leonoudakis *et al.* 1998) and TREK1 (Fink *et al.* 1996, Meadows *et al.* 2000). The 44 kDa band in myometrium could represent an unprocessed form of core TASK1 protein with the 65 kDa band reflecting a fully post-translationally modified protein (Fig. 2A). A similar situation may exist for TREK1. However, the higher molecular mass band might represent some form of dimerisation of TASK or TREK channels. Collectively, these observations imply differential TASK1 and TREK1 processing in different tissues, both within the same species and between different species.

To date there are no reports on immunostaining of TASK1 in the human myometrium. Our novel results (Fig. 3A) show TASK1 in myometrial cells with both a plasmalemmal and predominantly intracellular non-nuclear location. Staining for TREK1 (Fig. 3E) also exhibited plasmalemmal and intracellular staining, the latter evidenced as a prominent near-nuclear location. Taken together, the immunofluorescence and Western blotting data on the sub-cellular localisation of TREK1 (Fig. 2G) question a role for TREK in the determination of membrane potential at late term in myometrium.

The data we describe within the current study, therefore, provide a platform on which to base future functional studies that will address the role of TASK and TREK in the control of uterine excitability and contractility. This presents a considerable challenge in freshly isolated, fully differentiated human myometrial cells due to the absence of specific pharmacological inhibitors of these channel subtypes. The putative TASK1 channel blocker anandamide has been reported to inhibit K_{2P} -like currents in other smooth muscle cells by $\sim$ 25% (Gurney *et al.* 2003).

As TASK1, at least in other tissues, is a functional K^+ channel, a plasma membrane localisation in myometrial cells may have an impact upon cellular function. Hypoxia or metabolic inhibition has been shown to reduce human myometrial contractility (Bugg *et al.* 2002b, Monir-Bishty *et al.* 2003). As myometrial contractility is in part dependent upon electrical excitability and membrane potential, the presence of ion channels that are sensitive to hypoxia and pH may influence contraction. As TASK1 (Lewis *et al.* 2001) and TASK3 (Hartness *et al.* 2001) are sensitive to hypoxia and pH, this makes them attractive candidates for roles in the control of myometrial function. A role for TREK1 in myometrial contractility is, at this moment, harder to define. Our data suggest a predominantly non-plasmalemmal location for TREK1 protein in term tissue, thus making it difficult to predict an impact upon myometrial contractility. However, the intracellular localisation might represent a functional down-regulation. As the myometrium becomes distended during pregnancy, the presence of a stretch-modulated K^+ channel such as TREK1 (Bang *et al.* 2000, Miller *et al.* 2003) may contribute to the mechanisms by which the cell membrane remains hyperpolarised, dampening tissue excitability. Functional down-regulation of stretch-sensitive K^+ channels by intracellular redistribution may result in the setting of a membrane potential that favours contractile activity.

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