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Expression of mRNA transcripts for ATP-sensitive potassium channels in human myometrium

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The molecular mechanisms regulating human uterine quiescence and parturition are poorly understood. Potassium channels are central to regulation of cell membrane potential and contractility of smooth muscle. The aim of this study was to examine the expression of ATP-sensitive potassium channel (K_{ATP} channel) subunits in human myometrium, and to investigate for possible differential expression of these subunits in myometrium obtained from three different functional states: (i) non-pregnant (NP); (ii) late pregnant not in labour (PNL); and (iii) late pregnant in labour (PL). RT–PCR detected the presence of mRNA for four subunits of K_{ATP} channels (Kir6.1, Kir6.2, SUR1 and SUR2B) in the three tissue types. Quantitative analysis of these subunits was achieved with real-time RT–PCR using Lightcycler™ technology. This analysis showed that there were significantly higher levels of Kir6.1 and SUR2B transcripts in NP myometrium compared with those measured in myometrium obtained during pregnancy ($P < 0.001$). Lower levels of Kir6.2 and SUR1 mRNA expression were found, although higher transcript levels in NP myometrium ($P < 0.05$) were still observed. Our results indicate that the major K_{ATP} channel expressed in human myometrium is composed of Kir6.1 and SUR2B, and that down-regulation of this channel may facilitate myometrial function during late pregnancy.

Key words: ATP-sensitive potassium channels/human myometrium/real-time RT–PCR/sulphonylurea receptor

Introduction

The factors regulating myometrial function during human pregnancy and labour are poorly understood. An understanding of these processes, at the molecular and cellular level, is essential to developing novel therapeutic strategies for management of associated clinical problems such as preterm labour, which is the largest cause of perinatal mortality and morbidity in the developed world (Byrne and Morrison, 2001; Challis *et al*., 2001). During most of pregnancy, the myometrium remains in a relatively quiescent state with an increase in contractility occurring with labour onset to facilitate birth. Ion channels play an important role in the regulation of cell membrane potential, which is central to myometrial contractility (Wray, 1993; Kawarabayashi, 1994). Potassium (K^+) channels constitute a superfamily of integral membrane proteins that comprise the largest category of ion channels in the cell (Rudy, 1988; Bolton and Beech, 1992). The opening of these channels results in an outward flow of K^+ ions, drawing the cell membrane potential closer to the K^+ equilibrium potential, and thereby reducing cellular excitability and contractility (Khan *et al*., 2001). Similarly, K^+ channel opening compounds are known potent smooth muscle relaxants (Hamilton and Weston, 1989), and have been reported as potent inhibitors of human myometrial contractility (Morrison *et al*., 1993).

ATP-sensitive potassium channels (K_{ATP} channels) are present in many tissue types and are known to regulate a variety of cellular functions by coupling cell metabolism with membrane potential (Ashcroft and Ashcroft, 1990; Inagaki *et al*., 1995; Shyng and Nichols, 1998). The channels comprise heteromultimers of an inwardly rectifying K^+ channel (Kir) subunit and a regulatory sulphonylurea receptor (SUR) subunit (Aguilar-Bryan *et al*., 1998). Two Kir genes (Kir6.1 and Kir6.2) and two SUR genes [SUR1 and SUR2A/SUR2B (different isoforms of SUR2)] have been identified, and combinations of these subunits give rise to the classic K_{ATP} channel subtypes found in various tissue types (Aguilar-Bryan *et al*., 1998). Previous transfection studies on mammalian cell lines have suggested that combinations of Kir6.2/SUR2B or Kir6.1/SUR2B form functional K_{ATP} channels characteristic of smooth muscle type (Isomoto *et al*., 1996; Yamada *et al*., 1997). Little is known about the presence or role of K_{ATP} channels in human myometrium. While mRNA transcripts for Kir6.1 and SUR2B have recently been identified in rat myometrium (Chien *et al*., 1999), there are no reports of expression in human uterus. However, the relaxant effect of K^+ channel openers on human myometrium is antagonized by the sulphonylureas glibenclamide and tolbutamide, indicating involvement of K_{ATP} channel activity (Morrison *et al*., 1993; Khan *et al*., 1998a,b), yet there is minimal information in relation to the electrophysiological properties of K_{ATP} channels in human myometrium (Khan *et al*., 2001), questioning their presence and functional significance. The objectives of this study were to investigate for expression of K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1 and SUR2B) in human myometrium obtained in the non-pregnant state, and during pregnancy, prior to and after the onset

Table I. Specific primer pairs

Primer name	Primer sequence	Product size (bp)	GenBank Acc. No.
Kir6.1	F: CATCTTTACCATGTCCTTCC R: GTGAGCCTGAGCTGTTTTCA	336	NM_004982
Kir6.2 3′	F: ACTCCAAGTTTGGCAACACC R: CTGCTGAGGCCAGAAATAGC	353	D50582
Kir6.2 5′	F: GCTTTGTGTCCAAGAAAGG R: CCAAAGCCAATAGTCACTTG	301	D50582
SUR1	F: ATGAGGAAGAGGAGGAAGAG R: TCGATGGTGTTACAGTCAGA	492	L78207
SUR2B	F: TGTGATGAAGCGAGGAAATA R: TGACACTTCCATTCCTGAGAGA	434	AF061324
β-Actin	F: CAACTCCATCATGAAGTGTGAC R: GCCATGCCAATCTCATCTTG	377	M10277

of labour, and to examine for possible differential expression of these subunits between the tissue types studied.

Materials and methods

Patient recruitment and tissue collection

Patient recruitment took place in the Department of Obstetrics and Gynaecology, University College Hospital Galway (UCHG) between October 1999 and April 2001. The study was approved by the Research Ethics Committee, UCHG, and recruitment was carried out by provision of information sheets and by obtaining written informed consent. Biopsies of myometrium were excised from the midline of the upper lip of the uterine incision made at Caesarean section, at elective and emergency (i.e. intrapartum) procedures. Women who had received prostaglandins or oxytocin were excluded from the study. Samples of non-pregnant myometrium were excised from the body of the uterus of hysterectomy specimens from pre-menopausal women. Women with malignant conditions and those receiving exogenous hormone therapy (e.g. progestagens) were excluded from the study. Immediately upon removal, tissue samples were rinsed in sterile saline, snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction.

RNA preparation and purification

RNA was isolated from frozen tissue by homogenization in TRIzol Reagent (Life Technologies, Paisley, UK) (Chomczynski, 1993). RNA concentration was determined by absorbance at A_{260}, and samples were stored at –80°C. To eliminate any residual contaminating genomic DNA, all RNA samples were treated with the DNA-free™ DNA removal kit (Ambion, Huntingdon, Cambridgeshire, UK). Sample aliquots containing 25 µg RNA were incubated for 30 min at 37°C with 2 IU DNase I in DNase reaction buffer [10 mmol/l Tris–Cl (pH 7.5), 2.5 mmol/l $MgCl_2$, 0.1 mmol/l $CaCl_2$] in a total volume of 50 µl. The reaction was stopped by addition of 5 µl resuspended DNase Inactivation Reagent and incubation at room temperature for 2 min. This reagent, as well as the DNase I and divalent cations, was removed by centrifugation at 10 000 *g* for 1 min. The supernatant, containing DNA-free RNA, was transferred to fresh tubes. RNA concentration was measured again by absorbance at A_{260}, after removal of DNA, and adjusted to a final concentration of 500 ng/µl.

RT–PCR

RT–PCR was used to examine the expression of messenger RNA for the ATP-sensitive potassium channel subunits, Kir6.1, Kir6.2, SUR1 and SUR2B, in the three tissue types. DNase I-treated RNA samples were reverse-transcribed in a 20 µl reaction volume containing 50 mmol/l Tris–HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 500 ng oligo$(dT)_{15}$ primer and 200 IU M-MLV reverse transcriptase (Promega, Madison, WI, USA) for 1 h at 42°C. Control samples, where no reverse transcriptase was added, were included in all experiments to show that all products were RNA-derived and not the result of genomic DNA contamination.

PCR amplification was carried out with 5 µl cDNA product in a 50 µl reaction volume containing 20 pmol of each specific oligonucleotide primer (Table I), 50 nmol/l dNTP, 1.25 IU Taq DNA Polymerase (5 IU/µl) (Roche Diagnostics, Mannheim, Germany) in 10 mmol/l Tris–HCl, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl (pH 8.3). After an initial pre-heat at 95°C, PCR amplification was carried out for 22 to 45 cycles of denaturation at 95°C (20 s), annealing at 52°C (45 s), and extension at 72°C (1 min), followed by a final extension at 72°C for 10 min. The number of cycles used was limited to ensure product amplification remained in the log-linear range. β-actin was adopted as an internal control as it had been shown by Northern blot analysis to be constant in the three tissue sets being assayed (data not shown). In general, primer pairs were not designed across introns because appropriate genomic information on the human genes was not available. However, β-actin primers were designed across introns. PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized after ethidium bromide staining over UV light.

Quantitative analysis of expression using real-time RT–PCR

Real-time RT–PCR amplification was performed using the Lightcycler™ RNA Amplification kit SYBR Green I (Roche Diagnostics). This kit is specially adapted to perform one-step RT–PCR using the Lightcycler™ instrument. Prior to quantitative analysis, several titration experiments, for $MgCl_2$, primer concentration and RNA concentration were performed to determine optimum amplification conditions. Standard curves containing a specific number of cDNA copies were generated for each of the gene transcripts analysed (1×10^8 cDNA copies, 1×10^6 cDNA copies, and 1×10^4 cDNA copies). The following master mix of the components of the Lightcycler™ RNA amplification kit was prepared to the indicated end-concentration: 9 µl water, 4 µl Lightcycler™ RT–PCR reaction mix, 2 µl resolution solution, 2.4 µl $MgCl_2$ (6 mmol/l), 0.6 µl sense primer (0.3 µmol/l), 0.6 µl antisense primer (0.3 µmol/l) and 0.4 µl Lightcycler™ RT–PCR Enzyme mix. The master mix (19 µl) was aliquoted into Lightcycler™ glass capillaries (Roche Diagnostics) and 1 µl RNA (500 ng/µl) was added to the respective capillaries. The experimental protocol used for one-step RT–PCR consisted of four stages: reverse transcription (55°C for 10 min), an initial denaturation step (95°C for 45 s), followed by 45 cycles of denaturation (94°C for 5 s), annealing (55°C for 20 s), and extension (72°C for 20 s). Fluorescence data was acquired at the end of each extension cycle. A melting curve was carried out as follows: 95°C for 0 s, 65°C for 15 s, followed by a temperature increase of 0.1°C/s to 95°C for 0 s. Fluorescence was measured continually during this melting curve cycle. The temperature transition rate was 20°C/s in all cases, except for extension (2°C/s).

Analysis of real-time RT–PCR data was carried out using Lightcycler™ 'Fit Point Method' software, which utilizes a three-step mRNA quantification method for measurement of mRNA copy number. PCR products were isolated from the capillaries after 45 cycles of amplification on the Lightcycler™ and visualized after electrophoresis on 1.5% agarose gels.

Statistical analysis

All statistical tests were performed using the SPSS computer software package (Statistical Package for the Social Sciences, v.10, SPSS Inc., Chicago, IL, USA). Multiple group comparisons were analysed by analysis of variance, followed by individual group comparison using Tukey's HSD test, where appropriate. Comparisons between related genes (i.e. Kir6.1 versus Kir6.2, and SUR1 versus SUR2B) were analysed using a paired *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

Results

Tissue samples

Biopsies of myometrium during pregnancy were obtained at elective ($n = 7$) and intrapartum ($n = 4$) Caesarean sections. The mean age of the women was 32.9 years (range 26–37) of whom five were primagravida and six were multigravida. All women delivered at between 38 and 41 weeks gestation. There was no significant difference between those undergoing Caesarean section electively or intrapartum in terms of age, gestation or parity. Samples of non-pregnant myometrium ($n = 6$) were obtained at the time of hysterectomy. The mean age of women undergoing hysterectomy was 42 years (range 34–46).

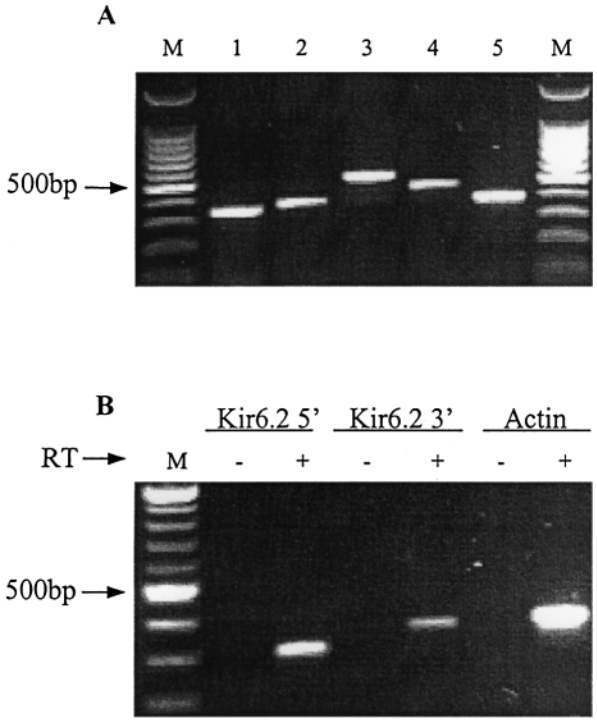

Figure 1. Agarose gel electrophoresis of RT–PCR products. (**A**) (1) Kir6.1, (2) Kir6.2 3', (3) SUR1, (4) SUR2B, and (5) β-actin RT–PCR products after 40 cycles amplification, using RNA extracted from non-pregnant human myometrium. PCR performed on RNA samples not reverse-transcribed gave no bands (data not shown). M = 100 bp marker. (**B**) RT negative (–) and positive (+) PCR products of two Kir6.2 primer sets, Kir6.2 5' F/R and Kir6.2 3' F/R, and β-actin after 40 cycles amplification in non-pregnant human myometrium. The absence of bands in RT– lanes confirms that contaminating DNA is absent in the samples, and that the amplification present is from mRNA. Similar results were obtained for late pregnant not in labour (PNL) and late pregnant in labour (PL) myometrial samples (results not shown). M = 2-log DNA ladder (0.1–10 kb).

mRNA expression analysis by RT–PCR

RT–PCR analysis of DNase I-treated RNA samples showed expression of all four K_{ATP} channel subunits assayed, namely Kir6.1, Kir6.2, SUR1 and SUR2B (Figure 1A). Two different primer sets for Kir6.2, designed to either end of the predicted mRNA transcript, were used to confirm expression of this subunit, as it had not been previously identified in human myometrium. Figure 1B shows expression of the mRNA transcript for Kir6.2 in non-pregnant human myometrium. The absence of these transcripts in reactions performed without reverse transcriptase confirmed that the signals were the result of RNA expression and not of DNA contamination.

Quantitative analysis of K_{ATP} channel subunit mRNA using real-time fluorescence RT–PCR

After demonstration that mRNA for all K_{ATP} channel subunits was present, quantitative analysis was performed to assess the expression levels of these transcripts, as outlined. In order to correct for random errors from sources such as pipetting inaccuracies, three separate real-time RT–PCR amplifications were carried out for each of the five genes. The specificity of RT–PCR products was confirmed by melting curve analysis, which showed single product-specific melting temperature peaks. Furthermore, agarose gel electrophoresis of the RT–PCR products yielded single product bands of the expected size (data not shown). Negligible primer-dimer bands were produced during the 45-cycle amplification. For each experiment, a baseline was set just above fluorescence background. Quantitative results were obtained by determination of crossing point (CP) values, which mark the cycle number at which sample fluorescence crosses a predetermined value. This value was nominally set at fluorescence level 10 for all five genes analysed, as it calculated results within the initial phase of exponential amplification. An example of this, for SUR1, is demonstrated in Figure 2. The inter-assay coefficient of variation for all genes was <6.4%.

Mean cDNA copy number and CP values, for each gene, in each myometrial sample (0.5 μg total starting RNA), were measured and compared, in order to identify any significant differential gene expression. Graphic representations of the findings, including the results for β-actin expression levels, are shown in Figure 3, while the copy number values for each gene in each condition are provided in Table II. β-actin expression showed no significant difference between the three tissue types ($P > 0.05$). This confirmed our previous Northern blot analysis (data not shown), and justifies the use of β-actin as a housekeeping gene in these tissues. Multiple comparisons (i.e. of cDNA copy numbers for each transcript in each of the three different myometrial tissues) revealed significant differences in the expression of Kir6.1, Kir6.2, SUR1 and SUR2B. Post-hoc analysis identified significantly higher expression of Kir6.1 ($P < 0.001$), SUR1 ($P < 0.05$) and SUR2B ($P < 0.001$) transcripts in NP samples compared with both PNL and PL samples, while Kir6.2 was expressed at significantly higher levels in NP samples compared to PNL samples only ($P < 0.05$). Statistical analysis using a paired *t*-test showed that Kir6.1 and SUR2B were expressed at significantly higher levels than were Kir6.2 and SUR1, respectively, in all three tissue sets ($P < 0.01$ in all cases).

Discussion

We have identified expression of the four main subunits for the K_{ATP} channel of smooth muscle type (Kir6.1, Kir6.2, SUR1 and SUR2B) in human myometrial tissue, in its non-pregnant state, and during pregnancy at term, prior to and after the onset of labour. This is the first report of such expression in human myometrium, and, for Kir6.2, the first illustration of its expression in any myometrial tissue type. In addition, our findings demonstrate that there is a 2–3-fold down-regulation in mRNA levels for all K_{ATP} subunits in human myometrium in late pregnancy [as measured prior to labour or after labour onset (except for Kir6.2)], compared with non-pregnant myometrium. We are unable to delineate the exact time point at which the down-regulation occurs, since, due to obvious ethical constraints, it is not possible to do serial sampling. While the average age of the women in the non-pregnant cohort was greater than that of the pregnant cohorts, there was significant overlap in age between all three groups, and no trend beween age and mRNA expression levels was detected. We are also unaware of any data indicating an association between ageing and ion channel expression pattern. Potassium channels are closely involved in reducing cellular excitability and contractility, because in the open state they draw the cell membrane potential closer to the K^+ equilibrium potential (Khan *et al*., 2001). While there is little known about the exact physiological role of the K_{ATP} channel in myometrium (Khan *et al*., 2001), our findings indicate that a decrease in the level of K_{ATP} channel expression in late pregnancy may facilitate enhanced excitability of the myometrium, hence paving the way for synchronised contractions throughout the uterine smooth muscle mass in preparation for, and at the time of, labour. We did not observe any alteration in channel expression in association with labour itself, suggesting that such changes occurred prior to labour onset.

A previous study (Chien *et al*., 1999) investigated K_{ATP} channel subunit expression in rat myometrium. They identified expression of Kir6.1, SUR1 and SUR2B and suggested that the complex of Kir6.1

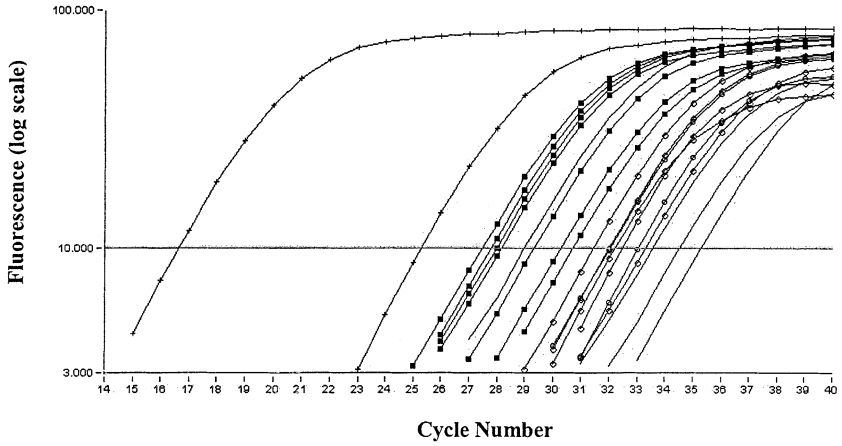

Figure 2. Representative real-time RT–PCR plot of logarithmic fluorescence versus cycle number for SUR1. The horizontal line corresponds to the crossing point (CP) determination line, set at fluorescence level 10, from which copy number and CP values were determined. Only standard values representing 10^6 and 10^4 gene-specific copy numbers are shown. Lines shown represent non-pregnant samples (closed squares), late pregnant not in labour samples (open circles), late pregnant in labour samples (continuous lines), and gene-specific standards (lines with crosses).

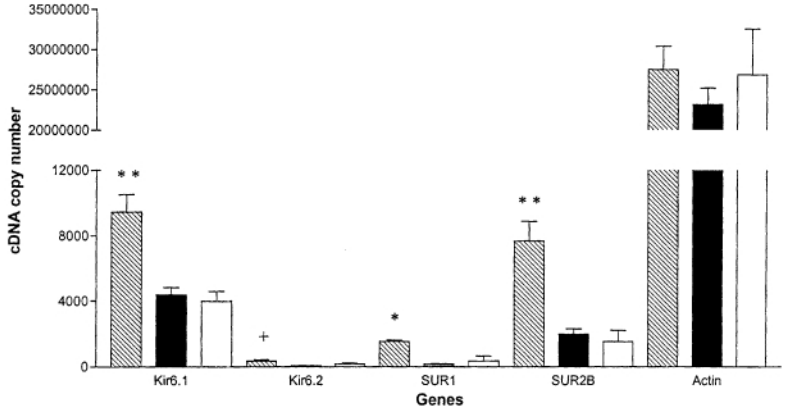

Figure 3. Tissue-averaged K_{ATP} channel subunit mRNA expression analysed by quantitative real-time RT–PCR. The overall mean (± SEM) copy number values, per 0.5 μg total RNA, were calculated for each K_{ATP} channel subunit, and β-actin. Copy number values are based on internal standard dilutions of product-specific cDNA transcripts. Tissue sets are represented by striped columns (non-pregnant, NP), grey columns (late pregnant not in labour, PNL), and open columns (late pregnant in labour, PL). $^*P < 0.05$ versus PNL, PL; $^{**}P < 0.001$ versus PNL, PL; $^+P < 0.05$ versus PNL.

Table II. cDNA copy numbers (± SEM) per 0.5 μg total RNA for each gene transcript in non-pregnant (NP), late pregnant not in labour (PNL), and late pregnant in labour (PL) samples

	NP ($n = 6$)	PNL ($n = 7$)	PL ($n = 4$)
Kir6.1	9442 ± 1083	4380 ± 444	3976 ± 622
Kir6.2	337 ± 95	77 ± 16	169 ± 64
SUR1	1528 ± 83	161 ± 27	346 ± 301
SUR2B	7687 ± 1183	1978 ± 324	1526 ± 680
β-actin	27 466 667 ± 2 873 870	23 134 048 ± 2 036 319	26 826 000 ± 5 676 105

and SUR2B results in the predominant K_{ATP} channel subtype. Our findings also suggest that the complex of Kir6.1 and SUR2B constitutes the predominant K_{ATP} channel subtype in human myometrium, as we have demonstrated significantly higher levels of Kir6.1 and SUR2B mRNA than those measured for Kir6.2 and SUR1 in all myometrial tissues studied. This subunit combination has been described previously as being an actively expressed subtype in vascular smooth muscle cells (Yamada *et al.*, 1997). However, the electrophysiological significance of this subtype in myometrial tissue is not clear. It is possible that there is a small but significant amount of the Kir6.1/SUR1 complex in the tissue sets assayed, since SUR2B levels were consistently lower than Kir6.1 mRNA levels and may not account for all the Kir6.1 subunits present. Unlike Chien *et al.* (1999), we identified expression of Kir6.2 mRNA, albeit at a much lower level of expression. The significance of this is not clear but it may be that Kir6.2 combines with either one of the two SUR subunits to form functional channels.

While the expression of K_{ATP} channel subunits in human myometrium has been demonstrated in this study, there remains a lack of understanding of the electrophysiological properties of these channels and their potential role in regulating myometrial contractility during pregnancy and labour (Khan *et al.*, 2001). In contrast, for the large conductance calcium-activated K^+ channel (BK_{Ca}), its pharmacological properties (in terms of sensitivity to Ca^{2+} and voltage) are altered by the onset of human labour (Khan *et al.*, 1993). In rat myometrium, down-regulation of mRNA and protein for the

α-subunit of the BK_{Ca} channel has been similarly suggested as a possible mechanism underlying enhanced myometrial excitability at term (Song *et al*., 1999). Likewise, for voltage-gated K^+ channels, their current characteristics have been suggested as having a possible function in the control of myometrial membrane potentials (Knock *et al*., 1999), with potential for modulation by estradiol and progesterone (Knock *et al*., 2001). Our study highlights the need for further research investigating the electrophysiological function of the K_{ATP} channel in human myometrium.

The use of real-time RT–PCR enabled accurate quantification of these subunit transcripts in the human myometrium. A major advantage of this method is that it allows rapid analysis of absolute template amounts, and accuracy of quantification is assured by analysis of amplification in the log-linear phase. Analysis was carried out for both copy number and CP values obtained from the experiments, and between-sample variation was minimized by performing the experiments in triplicate for each gene assayed. The intercalating dye, SYBR Green I, which was used to detect double-stranded DNA, is a more specific dye than ethidium bromide, and is widely used in real-time RT–PCR.

In conclusion, the identification of transcripts for Kir6.1, Kir6.2, SUR1 and SUR2B potassium channel subunits, as well as their decreased expression in late pregnancy, in the human myometrium, provides novel information outlining their potential role in myometrial modulation. Further studies are required to fully assess and understand the level of contribution of these channels to the process of parturition. Such studies are important in understanding myometrial physiology and in the development of novel and better therapeutic interventions for preterm labour management.

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