Molecular identification and localization of \textit{Trp} homologues, putative calcium channels, in pregnant human uterus

A.Dalrymple\textsuperscript{1}, D.M.Slater\textsuperscript{2}, D.Beech\textsuperscript{3}, L.Poston\textsuperscript{1} and R.M.Tribe\textsuperscript{1,4}

\textsuperscript{1}Parturition Research Group, Maternal and Fetal Research Unit, Department of Women’s Health, 10th Floor North Wing, Guy’s, King’s and St Thomas’ School of Medicine, St Thomas’ Hospital Campus, Lambeth Palace Road, London SE1 7EH, \textsuperscript{2}Department of Biological Sciences, The University of Warwick, Coventry CV4 7AL and \textsuperscript{3}School of Biomedical Sciences, Worsley Building, Level 9, University of Leeds, Leeds LS2 9JT, UK

\textsuperscript{4}To whom correspondence should be addressed. E-mail: rachel.tribe@kcl.ac.uk

The mechanisms underlying the switch from uterine quiescence to contractile activity in labour are not clearly understood. Increasing evidence suggests that pathways of myometrial calcium homeostasis, including store-operated calcium entry (SOCE), may play an important role. The molecular basis of the membrane-associated calcium channels contributing to SOCE in pregnant human myometrium is not known, but they are likely to be hetero- or homo-oligomeric assemblies of transient receptor potential channels (TrpC), encoded by the mammalian homologues of \textit{Drosophila} Trp genes. This study has therefore determined \textit{Trp} gene expression and also TrpC protein expression and localization in term pregnant human myometrial tissue and primary cultured human myometrial smooth muscle (HMSM) cells. RT-PCR amplified fragments of \textit{Trp1}, \textit{Trp3}, \textit{Trp4}, \textit{Trp6} and \textit{Trp7}. PCR products were 100\% homologous to published human sequences. Western blot analysis detected \textit{TrpC1}, \textit{TrpC3}, \textit{TrpC4} and \textit{TrpC6} proteins, which were of expected size. Immunolocalization revealed \textit{TrpC1}, \textit{TrpC3}, \textit{TrpC4} and \textit{TrpC6} protein expression in myometrial tissue and HMSM cells. \textit{TrpC} protein immunostaining in HMSM cells was distributed in a distinct reticular fashion. \textit{TrpC} proteins may be candidate proteins forming SOCE channels in term pregnant human myometrium.

\textbf{Key words:} calcium/myometrium/smooth muscle/SOCE/\textit{TrpC}

\section*{Introduction}

Extracellular calcium is essential for the generation of spontaneous and agonist-induced contractile activity in human myometrium at term (Szal et al., 1994; McKillen et al., 1999; Tribe, 2001) and during labour (Parkington et al., 1999). It has been generally assumed that voltage-gated calcium channels are the predominant calcium entry pathway. However, there is increasing functional evidence for a contribution of store operated calcium entry (SOCE) to calcium signalling in the human myometrium (Monga et al., 1999; Tribe et al., 2000; Tribe, 2001). Indeed, SOCE across the plasma membrane appears to be augmented in response to the depletion of intracellular sarcoplasmic reticulum calcium stores in contracting human myometrium during labour (Tribe et al., 2000; Tribe, 2001).

SOCE and non (n)SOCE have been reported in various smooth muscle types (Freichel et al., 1999; Nilius and Droogmans, 2001; Tribe, 2001). SOCE is triggered upon calcium release from inositol triphosphate (IP\textsubscript{3})-sensitive sarcoplasmic reticulum stores, whereas nSOCE is activated by agonists and intracellular messengers including arachidonic acid, diacylglycerol and phospholipase C. SOCE can also be activated pharmacologically by cyclopiazonic acid and thapsigargin (Parekh and Penner, 1997; Broad et al., 1999). SOCE and nSOCE play a role in calcium store replenishment, calcium oscillations and calcium-sensitive gene expression, and contribute to the maintenance/amplification of agonist-induced responses (Parekh and Penner, 1997).

In many cell types, SOCE and nSOCE are now considered to be mediated in part by plasma membrane protein channels, and it is proposed that assemblies of transient receptor potential channel (TrpC) proteins may form these channels (Freichel et al., 2001; Inoue et al., 2001; Xu and Beech, 2001). TrpC proteins were first described in \textit{Drosophila}, where they form light-sensitive ion channels in photoreceptor cells (Montell and Rubin, 1989). Since then, several mammalian homologues, \textit{Trp1}–7 (Harteneck et al., 2000; review), \textit{Trp}-p8 (Tsavaler et al., 2001) and \textit{Trp}12 (Wissenbach et al., 2000), have been cloned. At the molecular level, TrpC homologues share a common structure, being between 700 and 1000 aa long and composed of six transmembrane helices with a pore-forming loop between the fifth and sixth helix (Nilius and Droogmans, 2001). Four TrpC proteins form a functional channel and it is proposed that different assemblies of hetero- or homo-oligomeric protein channels can confer tissue/cell specificity and individual regulation (Nilius and Droogmans, 2001).

Recent studies have suggested that endogenous TrpC1, TrpC3 and TrpC4 are associated with SOCE, whereas TrpC6 is associated with nSOCE in vascular smooth muscle and endothelial cells from a variety of different sources (Groschner et al., 1998; Brough et al., 2001; Freichel et al., 2001; Inoue et al., 2001; Xu and Beech, 2001). However, the membrane channels that facilitate SOCE and nSOCE in pregnant human myometrium are unknown. This study aimed to determine the array of \textit{Trp} genes expressed in myometrium obtained from pregnant women at term and to examine TrpC protein expression...
and localization. In addition, Trp gene and protein expression was investigated in primary cultured human myometrial smooth muscle cells to assess the suitability of cultured cells as an in-vitro model for the study of TrpC protein function.

Materials and methods

Subjects
Human myometrial biopsies (n = 35) were obtained at elective Caesarean section prior to labour (38–40 weeks) from women with uncomplicated pregnancies (breech presentation or maternal request), with informed written consent and institutional Ethics Committee approval (Guy’s and St. Thomas’ Hospitals, London, UK, EC3/94/037). All biopsies were taken from the midline of the upper edge of a lower uterine segment incision. Following collection, samples were snap frozen for RNA or protein isolation or were used immediately for cell culture. For immunohistochemistry, biopsies were fixed for 4 h in 4% paraformaldehyde, incubated for 24 h in 3% sucrose and then 70% ethanol until processing and paraffin embedding.

Cell isolation and primary culture of human myometrial smooth muscle (HMSM) cells
Human myometrial smooth muscle (HMSM) cells were isolated as described previously (Tribe et al., 2000). Briefly, small sections of myometrium (n = 17) were incubated (37°C, 30 cyc/min) with 1 mg/ml collagenease 1A and 1 mg/ml collagenase XII (Sigma, Poole, UK). Isolated myometrial smooth muscle cells were resuspended in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 5% fetal calf serum (Invitrogen Life Technologies, Paisley, UK), 40 IU/ml penicillin and 40 µg/ml streptomycin (Sigma). Myocytes were incubated (37°C, 95% air, 5% CO2) and maintained as a primary culture until confluent (on average 7 days). The culture medium was changed every 2–3 days. To assess the purity of myocyte cultures, immunocytochemistry was routinely performed using α-actin and calponin monoclonal antibodies as previously described (Tribe et al., 2000).

RT–PCR
RT–PCR was performed to determine Trp gene expression in human myometrial tissue and primary cultured HMSM cells. Total RNA was isolated from tissue (n = 6) and primary cultured HMSM cells (n = 6) using a guanidine isothiocyanate and phenol–chloroform method (Chirgwin et al., 1979). Brain total RNA (Invitrogen) was also purchased. cDNA was synthesized by incubating total RNA (100 ng) with 0.2 µg random hexanucleotide primers (Promega, Southampton, UK) and 200 IU Superscript II (RT conditions as recommended by Invitrogen). To determine whether RNA contained contaminating genomic DNA, RT negative controls were prepared as above without the addition of Superscript II. For PCR, synthesized cDNA was incubated with 1.25 IU AmpliTaq (conditions as recommended by Applied Bioscience, Cheshire, UK) and 125 ng of 5′ and 3′ primers (Sigma Genosys, Cambis, UK). PCR primers were designed to amplify fragments of Trp1 (sense 5′-ACAGCAGAAGGGGGAAGCTTTTGCAAA-3′, antisense 5′-AAGTCCCAAAGGGC- AAGTAAA-3′), Trp3 (sense 5′-GTGTGGGGAATGTGTTGACT-3′, antisense 5′-GAGTTGATAGGCTTTGAC-3′), Trp4 (sense 5′-CCCCATGAGTCTTCTCA-3′, antisense 5′-GAACTTCCCTGCAA-3′), Trp5 (sense 5′-CATCTCCCTCTGCAA-3′, antisense 5′-TTTGGGCTCAGTCT-3′), Trp6 (sense 5′-AGGATGAGGCTGATGTTGAG-3′, antisense 5′-TCCCTCGAGCTCTCCCTTTTGT-3′) and Trp7 (sense 5′-ATGGCTTGGCCTCTGCTC-3′, antisense 5′-TTGTCTCCATTTGATGTAC-3′). Cycling parameters used were: 36 cycles of denaturing at 94°C for 1 min; annealing at 44°C (Trp5), 48°C (Trp7), 51°C (Trp4), 54°C (Trp1) or 57°C (Trp3 and Trp6) for 1 min; extension at 72°C for 2 min, followed by 72°C for 5 min. Following amplification, PCR products were analysed by agarose gel electrophoresis, submerged into the pGEM-T Easy vector (Promega) and the sequence was verified.

Microsomal membrane protein isolation from human myometrial tissue
Human myometrial biopsies (n = 6, 250 mg) were homogenized using an Ultra Turrax T50 homogenizer in 5 ml of homogenization buffer [10 mmol/l HEPES–KOH, pH 7, 1 mmol/l DTT and protease inhibitor cocktail (COMPLETE™ tablets; Boehringer Mannheim Biochemical Lewes, UK)]. Following homogenization, microsomal membrane proteins were isolated as previously described (Tribe et al., 2000). Protein concentrations in all samples were determined using bovine serum albumin (BSA) as a standard and the DC protein assay kit (BioRad Laboratories Ltd, Herts, UK).

Total protein isolation from primary cultured HMSM cells
Confluent primary cultured HMSM cells (n = 6) were detached from culture dishes using 500 µl of homogenization buffer (as above) containing 1% (v/v) nonidet-P40 (British Drug House, BDH, Poole, UK). Cell lysates were homogenized (as above) and proteins were separated from cell debris by centrifugation (13 000 g, 10 min, 4°C). Protein concentration was determined in all samples as described above.

Western blotting
Myometrial tissue microsomal membrane proteins (10 µg) and primary cultured HMSM cells total proteins (10 µg) were denatured at 95°C for 10 min in 2× laemmli sample buffer (Sigma) and then size separated using a 10% sodium dodecyl sulphate–polyacrylamide gel. Thereafter, proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech Ltd, Little Chalfont, UK) as previously described (Towbin et al., 1979). Following transfer, equal loading of protein samples and transfer efficiency was assessed by incubating membranes with Ponceau red solution (as directed by manufacturer, Sigma). Membranes were then washed with PBS–T (PBS, 0.1% Tween-20; Sigma) and incubated (1 h, room temperature) in blocking buffer (PBS–T, 5% goat serum; Chemicon, Harrow, UK) and subsequently incubated (2 h, room temperature) with the desired rabbit polyclonal primary antibody. Primary antibodies used were TrpC1 (Xu and Beech, 2001; 1:1000) TrpC3, TrpC4 or TrpC6 (1:1400, 1:400 and 1:2000, respectively; Alomone Labs, Jerusalem, Israel) which were diluted in antibody buffer (PBS–T, 1% BSA, 0.1% sodium azide; Sigma). For the negative controls, membranes were incubated with a 1:1000 dilution of rabbit pre-immune serum (TrpC1) or incubated with primary antibodies that were pre-absorbed with the respective peptides (TrpC3, TrpC4 or TrpC6; Alomone Labs, as recommended by the supplier). Briefly, the required concentration of antibody and respective peptide were diluted in antibody buffer and incubated (1 h, room temperature). Following incubation with the primary antibody or negative control, membranes were incubated (1 h, room temperature) with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:2500 in blocking buffer; BioRad). Thereafter, membranes were washed in PBS–T (4×15 min) and protein bands were visualized with enhanced chemiluminescence (Amersham). Western blotting was not performed for TrpC5 or TrpC7 as antibodies were not commercially available.

Immunolocalization
Wax embedded myometrial tissue sections (5 µm, n = 6) were dewaxed in Histoclear (National Diagnostics, Hull, UK), rehydrated with decreasing concentrations of ethanol (100, 96 and 70%) and then washed (2×5 min in Tris-buffered saline (TBS; 50 mmol/l Tris–HCl, pH 7.4, 0.85% NaCl). Primary cultured HMSM cells (n = 5) were also used for immunolocalization studies and fixed for 20 min in 4% paraformaldehyde (Sigma). To localize TrpC proteins, the Envision™ detection kit (Dako, High Wycombe, UK) was used as recommended by the manufacturer. Briefly, endogenous hydrogen peroxide was eliminated by incubating (5 min) tissue section and HMSM cells with the supplied peroxidase block. Tissue sections and HMSM cells were then washed (2×5 min) with TBS and incubated (30 min) with the required primary antibody or negative control, an identical dilution of pre-immune serum (TrpC1) (Xu and Beech, 2001) or pre-absorbed primary antibody (TrpC3, TrpC4 and TrpC6; Alomone Labs, prepared as described for Western blotting). Primary antibodies, TrpC1 (1:500), TrpC3 (1:200), TrpC4 (1:50) and TrpC6 (1:400), were diluted in TBS containing 5% goat serum (Chemicon). Thereafter, tissue sections and HMSM cells were washed (2×5 min) with TBS, incubated (30 min) with the supplied peroxidase-labelled polymer (Dako), washed (2×5 min) with TBS and colour reaction was developed by incubating with the supplied 3,3′-diaminobenzidine chromogen solution (Dako). Samples were counterstained using Mayer’s haematoxylin (BDH), dehydrated with increasing concentrations of ethanol (70, 96 and 100%).
Discussion

This study provides direct evidence that *Trp* mRNA and *Trp* proteins are expressed in term pregnant human myometrium and primary cultured HSMC cells. Of the *Trp* family of genes, *Trp1*, *Trp3*, *Trp4*, *Trp6* and *Trp7* mRNA were expressed in term pregnant human myometrium. PCR failed to amplify *Trp5* in the human myometrium; however, the *Trp5* primers designed for these studies amplified *Trp5* in the human brain, which suggests that *Trp5* is not expressed in the human myometrium. PCR products obtained for each *Trp* gene were 100% homologous to published sequences. The data obtained is comparable with other investigations which have confirmed that *Trp1*, *Trp3*, *Trp4*, *Trp6* and *Trp7* are expressed by a number of tissues, whereas *Trp5* is predominantly expressed by the brain (Clapham, 1995; Okada et al., 1998; Freichel et al., 1999; Nilius and Droogmans, 2001; Riccio et al., 2002). In addition to *Trp1* and *Trp3–7*, *Trp2* and *Trp*-p8 genes have also been cloned. *Trp2* is expressed by rodent and bovine tissues and is regarded to be a pseudogene in humans (Liman et al., 1999); *Trp*-p8 is exclusively expressed by tumour tissue (Tsavaler et al., 2001), and therefore the expression of these genes was not investigated in human myometrium.

*Trp7* was the only isoform expressed in human myometrial tissue but not in primary cultured HSMC cells. It is possible that the *Trp7* mRNA detected in myometrial tissue may be of vascular origin and not from myometrial smooth cells, or alternatively, a factor in the culture medium may have induced a switch in *Trp* gene expression. Culture-induced changes in gene expression have previously been documented for other calcium regulatory proteins; in passaged term human myometrial tissue, *SERCA3* and IP 

\[ 3 \] 

receptor type 1 gene expression is reduced (Mountian et al., 1995). Moreover, others have shown up-regulation of *Trp1* gene expression in growth-arrested human pulmonary arterial smooth muscle cells following incubation with serum and growth factors (Golovina et al., 2001) and down-regulation of *Trp4* in endothelial cells by \( \beta \)-estradiol (Chang et al., 1997).

Western blotting data confirmed the translation of a number of *Trp* mRNA to proteins. *Trp1* and *Trp3* proteins were ~90 kDa, which is similar to *Trp1* in human aorta (Xu and Beech, 2001) and *Trp1* (Li et al., 1999) and *Trp3* (Wang et al., 1999) proteins expressed in rat brain. Human myometrial *Trp4* and *Trp6* proteins were ~100 kDa, which is comparable with bovine adrenal...
Trp expression and localization in human myometrium

Figure 3. Representative immunolocalization of TrpC protein isoforms in term pregnant myometrial tissue and HMSM cells. Intense immunostaining of TrpC1, TrpC3, TrpC4 and TrpC6 was observed in human myometrial tissue sections (A, TrpC1; F, TrpC3; K, TrpC4; P, TrpC6) and primary cultured HMSM cells (C and D, TrpC1; H and I, TrpC3; M and N, TrpC4; R and S, TrpC6). No immunostaining was observed when human myometrial tissue sections and HMSM cells were incubated with pre-immune serum (B and E, TrpC1) or pre-absorbed primary antibodies (G and J, TrpC3; L and O, TrpC4; Q and T, TrpC6). Arrow 1 = TrpC1 immunostaining in vascular tissue. Arrow 2 = limited immunostaining when nucleus is visible. Arrow 3 = intense immunostaining when the nucleus was not in the plane of focus. Arrow 4 = reticular staining in HMSM cells. Scale bars = 50 µm.

gland (Philipp et al., 2000), mouse vascular endothelial cells TrpC4 (Freichel et al., 2001) and rat brain TrpC6 (Tesfai et al., 2001). Recently, two TrpC4 isoforms have been described, α and β; TrpC4-β occurs due to alternative exon splicing, is 84 aa shorter and lacks amino acids 785–868 (Mery et al., 2001). Indeed, Western blotting analysis using the anti-TrpC4 antibody appeared in some cases to detect two TrpC4 protein bands in HMSM primary cultured cells, which may correspond to TrpC4-α and -β isoforms. In myometrial tissue, only one band was visible; however, the abundant expression of TrpC4 in myometrial tissue possibly masks discrimination of the doublet. TrpC7 and TrpC5 protein expression in human myometrium was not investigated, as suitable antibodies were not commercially available.

Immunolocalization confirmed the expression of TrpC1, 3, 4 and
6 proteins in term pregnant human myometrial tissue and primary cultured HMSM cells. Moreover, these data clarified that expression was predominately in myometrial smooth muscle cells, although some immunostaining was observed in vascular smooth muscle cells. Immunostaining was less apparent in myometrial tissue sections when the nucleus was in the plane of focus, which could suggest that TrpC proteins are localized within the plasma membrane. Furthermore, the Western blotting data confirmed that human myometrial TrpC isoforms were present in the microsomal membrane protein fraction, strongly supporting plasma membrane localization. The TrpC1 antibody used in the present study has been used to localize TrpC1 to the plasma membrane of human, mouse and rabbit vascular smooth muscle cells (Xu and Beech, 2001). In addition, the TrpC3, TrpC4 and TrpC6 antibodies have localized these proteins to the plasma membrane of mouse sperm (Trevino et al., 2001) and rat pulmonary artery smooth muscle cells (Ng and Gurney, 2001). Fluorescent and confocal microscopic studies would verify TrpC protein localization to the plasma membrane of myometrial cells.

TrpC1, 3, 4 and 6 immunostaining in HMSM cells appears to be distributed in a reticular fashion and is comparable with IP3 receptor immunostaining, a sarcoplasmic reticulum-associated protein, in human myometrial cells (Young and Mathur, 1999). In addition, the pattern of TrpC protein immunostaining is reminiscent of the staining observed when HMSM cells are incubated with DiCO6, a membrane potential dye that stains the sarcoplasmic reticulum in living cells (data not shown). TrpC protein reticular distribution in HMSM cells strengthens the suggestion that plasma membrane-bound TrpC proteins may associate with the underlying sarcoplasmic reticulum. Indeed, recent immunoprecipitation studies have demonstrated that endogenous TrpC1 couples with endogenous sarcoplasmic reticulum-associated IP3 receptor type 2 in human platelets (Riccio et al., 2001).

The physiological functions of TrpC proteins and association with SOCE or nSOCE have been studied in other tissues. Functional overexpression studies have produced conflicting data, whereas a greater consensus has been achieved by the transfection of cells with N-terminal or antisense Trp cDNA fragments, and also after incubation with antibodies specific for TrpC protein extracellular domains. These approaches have clarified the association of endogenous TrpC1 (Brough et al., 2001; Xu and Beech, 2001) and TrpC3 (Groschner et al., 1998) proteins with SOCE and TrpC6 (Inoue et al., 2001) with nSOCE in vascular smooth muscle and endothelial cells. In addition, in-vivo studies, using a Trp4 knockout mouse model, have clearly confirmed TrpC4 association with SOCE in the mouse aorta (Freichel et al., 2001). The association of endogenous TrpC7 with SOCE or nSOCE has yet to be confirmed. Recent studies have suggested that transfected TrpC7 is associated with SOCE, as the transfection of antisense human Trp7 into cells stably expressing transfected human TrpC7 inhibits SOCE (Riccio et al., 2002).

The data from the present study have demonstrated Trp gene and protein expression by term pregnant human myometrial tissue and primary cultured HMSM cells. Confirmation of expression strengthens the hypothesis that TrpC1, 3, 4 or 6 individually, as homomeric or heteromeric channels, may facilitate SOCE and nSOCE in the human myometrium. Moreover, TrpC protein expression in primary cultured HMSM cells, with the exception of Trp7, reflects that of human myometrial tissue and hence provides a good in-vitro model to study TrpC protein function. Future studies aim to clarify the potential physiological and pathophysiological roles of myometrial TrpC proteins.

Acknowledgements
We thank Ruth Brucker, Julie Adams, the Maternal and Fetal Research Unit Myometrial collection team, Guy’s and St Thomas’ Hospitals labour ward staff and all the women who kindly participated in this study. We also thank Vasia Dakou for technical assistance in preparing HMSM cells. This study was funded by the Wellcome Trust (Grant No.: 061138) and Tommys’ the baby charity (Reg. Charity No.: 1060508).

References


