Mechanical stretch regulates TRPC expression and calcium entry in human myometrial smooth muscle cells

A.Dalrymple1, K.Mahn2, L.Poston1, E.Songu-Mize3 and R.M.Tribe1,4

1Maternal and Fetal Research Unit, Division of Reproduction and Endocrinology, King’s College London, St Thomas’ Hospital Campus, London, 2Division of Allergy, Asthma and Respiratory Science, King’s College, Guy’s Hospital Campus, London, UK and 3Department of Pharmacology and Experimental Therapeutics, LSU Health Sciences Center, New Orleans, LA, USA
4To whom correspondence should be addressed at: Maternal and Fetal Research Unit, Division of Reproduction and Endocrinology, King’s College London, St Thomas’ Hospital Campus, Lambeth Palace Road, London, SE1 7EH, UK. E-mail: rachel.tribe@kcl.ac.uk

Stretch is known to stimulate myometrial hyperplasia and hypertrophy in early pregnancy and uterine contraction at term. We propose that transduction of the stretch signal involves alteration of intracellular calcium signalling, including changes in transient receptor potential canonical (TRPC) isoform expression. The aim of the present study was to investigate the effect of prolonged mechanical (tonic) stretch in vitro on human myometrial smooth muscle cell calcium signalling and TRPC expression. Cells were cultured from myometrial biopsies, obtained from women undergoing elective Caesarean section at term, grown on FlexiplatesTM and subjected to 25% tonic mechanical stretch for 1, 4 and 14 h. Time-matched control cells were not stretched. Mechanical stretch (14 h) increased basal calcium entry and cyclopiazonic acid (CPA)-induced calcium/Mn2+ entry (P<0.05) in Fura-2 loaded cells. The calcium selectivity of CPA-thapsigargin induced inward currents, measured by patch clamp electrophysiology, was also increased in stretched cells compared with control cells (P<0.05). Real time PCR and Western blot data demonstrated that TRPC3 and TRPC4 mRNA and TRPC3 protein expression were increased by stretch (P<0.05), respectively. These data support the hypothesis that uterine stretch modulates uterine growth and contractility in pregnancy via alterations in calcium signalling.

Key words: cell signalling/ion channels/pregnancy/smooth muscle/uterus

Introduction
To ensure successful reproduction, the uterus has to rapidly adapt in response to the needs of the developing fetus(es). In the early stages of pregnancy, uterine enlargement is mediated by asymmetric uterine smooth muscle cellular hypertrophy and, subsequently, minor hyperplasia (Laguens and Lagrutta, 1964). Conversely, at the end of pregnancy, a period of rapid atrophy must occur for the uterus to return to a non-pregnant state. While a number of these adaptive processes have been examined in the rat, relatively little is known in human pregnancy. In the rat, there appear to be two modulatory influences on uterine growth: hormonal through progesterone (Csapo and Wiest, 1969; Michael and Schofield, 1969, Halme and Woesnner 1975;) and mechanical through uterine stretch (Csapo et al., 1965; Cullen and Harkness, 1968; Alexandrova and Soloff, 1980; Douglas et al., 1988). As pregnancy progresses and as the fetus grows, it is likely that the contribution of prolonged (tonic) mechanical stretch becomes increasingly important (Csapo et al., 1965).

Stretch of myometrial tissue, in vivo and in vitro, stimulates expression of a range of labour-associated proteins, such as COX-2 and prostaglandin as well as oxytocin receptors (Manabe et al., 1992; Ou et al., 1997, 1998; Wu et al., 1999; Korita et al., 2002; Loudon et al., 2004; Sooranna et al., 2004; Terzidou et al., 2005). Myometrial stretch is also associated with activation of AP-1 transcription factors, MAP kinase cascades, ERK1/2 phosphorylation and increased expression of c-fos and cytoskeleton-related genes (Mitchell and Lye, 2002; Oldenhof et al., 2002; Shynlova et al., 2002; Mitchell et al., 2004; Sooranna et al., 2004, 2005).

The transduction mechanisms linking myometrial stretch and downstream events have not been fully explored, but in most cell types mechanotransduction is mediated by integrin signalling and stretch-activated calcium entry (Shaw and Xu, 2003; Iqbal and Zaidi, 2005). A rise in intracellular calcium mediated via stretch-activated channels and release from intracellular calcium stores has been described in cardiac myocytes and vascular smooth muscle cells (McCarron et al., 1997; Zou et al., 2002; Calaghan et al., 2003; Liao et al., 2003). Recent reports suggest that proteins of the TRP superfamily form mechanosensitive calcium channels (Corey et al., 2004; Lin and Corey, 2005; Maroto et al., 2005), which could theoretically play a role, as we and others have shown that transient receptor potential canonical (TRPC) proteins, putative components of store-operated calcium entry, are expressed in human myometrial tissue and cells and regulated during pregnancy (Dalrymple et al., 2002, 2004; Yang et al., 2002; Babich et al., 2004). The aim of this study was to examine the effect of prolonged tonic stretch on TRPC channel gene and protein expression and store-operated calcium entry in human myometrial smooth muscle cells.

© The Author 2007. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved. For permissions, please email: journals.permissions@oxfordjournals.org
Materials and Methods

Subjects

Human myometrial biopsies were obtained at Cesarean section with informed written consent and institutional Ethics Committee approval (Guy’s and St Thomas’ Hospital Trusts, London, UK) in accordance with the principles set out in the Declaration of Helsinki. Biopsies were collected from women without underlying disease at term prior to labour (n = 20, 38–41 weeks’ indications: previous section, breech presentation or maternal request) and used for cell culture.

Cell culture and tonic mechanical stretch protocol

Primary uterine myocytes were dispersed enzymatically from tissue biopsies as described previously from myometrial biopsies from women at term (not in labour) (Tribe et al., 2000; Dalrymple et al., 2002). Cells were incubated (37°C, 95% air, 5% CO2) in 25cm2 culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, Poole, UK) plus 5% fetal calf serum (FCS) (FCS) (Invitrogen, Paisley, UK) and maintained in culture until Passage 1. Cells were then detached using trypsin-EDTA (1×, Sigma) for 5 min at room temperature and cultured (passage 2) in six well-flexible bottom culture plates precoated with collagen type I in 3 ml DMEM plus 5% FCS. The cell solution (100 μl) was placed on glass cover-slips and cells allowed time (1 h) to adhere. Whole cell lysates (taken at t = 14 h) for Western blot analysis were obtained of stretched and control cells (at t = 1, 4 and 14 h) for RNA studies, total RNA (tRNA) was extracted from stretched and control cells (at t = 1, 4 and 14 h) and purified using Trizol (Invitrogen, Paisley, UK, as recommended by the manufacturer).

Digital Ca2+ imaging

Cells exposed to mechanical stretch for 14 h and paired non-stretched time controls were loaded with the fluorescent Ca2+ indicator Fura-2-AM (1 μM, 1 h RT). This time point was chosen to address the functional impact of a period of prolonged tonic stretch (to mimic in vivo events at the end of pregnancy) on intracellular calcium responses. Cells were perfused (15 min) with physiological salt solution (PSS) (140 mM NaCl, 5.9 mM KCl, 1.2 mM NaH2PO4, 5 mM NaHCO3, 1.4 mM MgCl2, 1.8 mM CaCl2, 11.5 mM glucose, and 10 mM Hepes titrated to pH 7.4 with NaOH). Following excitation at 360 and 380 nm, emission was measured at 510 nm. Human myometrial cells were imaged every 4–6 s using a digital camera (Pentaxmark CCD, Princeton Instruments, Trenton, NJ, USA) controlled by Universal Imaging Metamorph computer software (Downington, PA, USA) and the signal normalized for background fluorescence. Images were captured and background corrected 360/380 ratios calculated for all cells within the field of view. Store-operated calcium entry was examined by initiating Ca2+ release from intracellular stores using cyclopiazonic acid (CPA) (5 μM) in the presence or absence of extracellular Ca2+. The peak change in Fura-2 360/380 nm ratio (ΔF360/380/s) from baseline is reported. Store-operated calcium entry was also examined by determining the ratio of Ca2+ re-entry (ΔF360/380/s; assessed using linear regression) on readdition of Ca2+ to Ca2+ free PSS containing CPA. The protocol was then repeated in the presence of the L-type Ca2+ channel inhibitor, diltiazem (20 μM). Experiments were also performed to determine the La2+-sensitivity (50 μM) and Gd3+-sensitivity (10 μM) of basal and store-operated calcium entry in stretch and control cells. Store-operated calcium entry was further investigated by the application of extracellular Mn2+ (200 μM, MnCl2 in nominally Ca2+ free PSS, no EGTA), a surrogate ion for Ca2+ that is not extruded by the plasma membrane calcium ATPase or by Na/Ca exchange. The initial rate of Mn2+ influx [quench of Fura-2 360 nm fluorescence, ΔF360/s] was measured. Modified PSS (sodium phosphate and sodium bicarbonate removed) was used for La3+, Gd3+ and Mn2+ experiments.

Whole-cell current recording

Recordings were made using the tight-seal whole-cell configuration of the patch clamp technique (Hamill et al., 1981). Experiments were performed in single cells, dispersed on a glass coverslip, using the standard patch-clamp technique with Axopatch 200A amplifier and pCLAMP-6 software (Axon Instruments, Union City, CA, USA). The bath solution was either a standard NaCl-PSS (130 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 1.5 mM CaCl2, 10 mM HEPES, 10 mM glucose) or a N-methyl-D-glucamine-PSS (0 Na) and contained TEA and 4-AP, the pipette solution was: 135 mM CsCl, 2.5 mM MgCl2, 10 mM EGTA, 10 mM HEPES, 5 mM NaATP. Neither K+ nor L-type Ca2+ channels were active under these conditions. The membrane potential was clamped at ~70 mV and a combination of CPA (10 μM) and thapsigargin (100 mM) used to activate store-operated currents that were continuously recorded.

Quantitative real-time PCR

After quantification, 1.0 μg of tRNA was reverse transcribed with oligo dT random primers using MULV reverse transcriptase (Promega, Southampton, UK). Validated primer and probe sets for TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7 and 18s rRNA were obtained from Applied Biosysytems Ltd, Warrington, UK. Quantitative PCR was performed in the presence of either VIC (for 18s) or FAM (for TRPCs) (Applied Biosystems Ltd) and amplicon yield monitored during cycling in an ABI Prism™ 7000 Sequence Detection System (Applied Biosystems Ltd), Pre-PCR cycle was 2 min at 50°C and 10 min at 95°C for 1 cycle followed by 40 cycles of 95°C for 15 s and

Figure 1. Calcium entry, following a period of prolonged calcium store depletion, is enhanced in tonically (14 h) stretched human myometrial cells. (A) Representative trace of resting calcium responses and cyclopiazonic (CPA) (10 μM) induced Ca2+ mobilization in control and stretched cells in physiological salt solution (PSS); arrow indicates initiation of Ca2+ re-entry following a period of prolonged store depletion in Ca2+ free PSS containing CPA. (B) Mean ± SEM data demonstrating an increase in the calcium re-entry slope in stretched cells (n = 147) versus control cells (n = 151), ***P = 0.0002.
Data from calcium imaging and patch clamp studies were analysed in obtained from myometrial biopsies originating from at least three subjects). Imaging and patch clamp data when (each originating from a biopsy from a different subject) except for calcium analyses. The cycle threshold (Ct) in each assay was set so that the exponential increase in ampiclon abundance was approximately parallel between all samples. All mRNA abundance data were expressed relative to the amount of the constitutively expressed 18s RNA using the 2−ΔΔCt method of analysis (Livak and Schmittgen, 2001) and, subsequently, normalized to control samples taken at t = 0 h (assigned a value of one).

**Western blot analysis**

Proteins were separated from cell debris by centrifugation (13,000 g, 10 min, 4°C) and the protein concentration determined using BSA as a standard and the DC protein assay kit (Bio-Rad Laboratories Ltd, Herts, UK). Control and stretched HMSM cellular proteins (10 μg) were denatured at 95°C for 10 min in Laemmli sample buffer (Sigma), loaded onto Novex 10% Tris–glycine gels (Invitrogen) and subsequently transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK). The transfer efficiency and equal loading of protein samples was assessed by incubating membranes with Ponceau red solution (Sigma) and then incubated (overnight, 4°C) in blocking buffer (PBS-T, 5% goat serum, Chemicon, Harrow, UK). Membranes were incubated (3 h, room temperature) with TRPC1 (Xu and Beech, 2001; 1:1000), TRPC3, TRPC4 or TRPC6 antibodies (Alomone Labs, Jerusalem, Israel, 1:200) which were diluted in blocking buffer and subsequently incubated (1 h, room temperature) with a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Bio-Rad, diluted 1:2500 in blocking buffer). Protein bands were visualized with ECL solution on hyperfilm (Amersham Pharmacia Biotech Ltd). 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 (1 h, room temperature) with the respective peptides (TRPC3, TRPC4 or TRPC6, Alomone Labs, as recommended by the supplier). TRPC5 and TRPC7 protein expression was not investigated.

**Statistical analysis**

Data are presented as mean ± SEM; n refers to the total number of cell cultures (each originating from a biopsy from a different subject) except for calcium imaging and patch clamp data where n refers to number of cells (cells were obtained from myometrial biopsies originating from at least three subjects). Data from calcium imaging and patch clamp studies were analysed in Microsoft Excel 97 (Bellvue, WA, USA) and two-way ANOVA or Student’s t-test as appropriate. Western blot hyperfilms were analysed using Total Lab software (Nonlinear Dynamics, Newcastle upon Tyne, UK) which allows the calculation of the density of each band and corresponding background values. Final intensity values (arbitrary units, a.u.) were background subtracted. Western blot data were analysed using Student’s t-test. Real time PCR data were analysed using the Box–Cox method, and normal distribution plots were used to confirm that data transformations were not needed. Analysis was by repeated measures ANOVA of treatment by myometrial sample and regression with robust standard errors (Huber, 1967). Planned comparisons gave estimates of the ratios between stretch and control samples for each time point. Smaller P-values in the range 0.05–0.001 are taken as increasing evidence against the null hypothesis, but the totality of evidence is regarded as more important than any single test (Sterne and Davey-Smith, 2001). Marginally significant results (0.05 > P > 0.01) are mentioned, but not discussed in detail. Accordingly, no formal adjustment is made for multiple comparisons.

**Results**

**Basal calcium entry is enhanced in stretched cells**

Stretch did not alter the resting calcium baseline in the presence of calcium (Figure 1A, F360/380, 0.965 ± 0.010, n = 84 cells exposed to 14 h stretch versus F360/380, 0.962 ± 0.015, n = 96 control cells, Figure 3. Basal and CPA-induced Mn2+ influx is enhanced in tonically (14 h) stretched human myometrial cells. Representative plots to show (A) Basal Mn2+ quench of Fura-2 fluorescence at 360 nM (ΔF360) and (B) CPA-induced Mn2+ entry in control and stretched cells. (C) Mean ± SEM data for basal Mn2+ entry in stretched cells (n = 53 cells) versus controls (n = 56 cells; *P < 0.02) and CPA-induced Mn2+ entry in stretched (n = 60 cells) versus controls (n = 76 cells; ***P < 0.0011). CPA (5 μM) induced Mn2+ entry was greater than basal entry in both stretched (#, P < 0.005) and control (†, P < 0.020) groups.

Figure 2. In vitro tonic 14 h stretch does not alter CPA-induced Ca2+ transient peaks in cultured human myometrial smooth muscle cells. Mean ± SEM data show that CPA-induced Ca2+ peaks (change from baseline F360/380) are of similar magnitude in control and stretched cells when measured in PSS (not significant, NS; n = 96–114 cells) and Ca2+-free PSS (NS, n = 84–96 cells).
not significant (NS). However, in the absence of extracellular calcium (Ca\^{2+}-free PSS), the resting baseline in stretched cells was significantly reduced to 0.921 ± 0.010 ($F_{360/380}$, $n = 84, P \leq 0.001$) in stretched cells, but not controls ($F_{360/380}$, 0.936 ± 0.015, $n = 96$ cells, NS).

**CPA-induced calcium responses**

CPA-induced peak calcium responses in PSS were unaltered by 14 h stretch (change from baseline $F_{360/380}$ 0.599 ± 0.055, $n = 95$ cells) versus controls (0.633 ± 0.079, $n = 114$ cells, NS, (Figures 1A and 2). In Ca\^{2+}-free PSS, the mean CPA-evoked Ca\^{2+} peak was of...
similar magnitude in both stretch (change from Ca\(^{2+}\)-free PSS baseline \(F_{560/380}\) = 0.577 ± 0.059, \(n = 84\) cells) versus controls (0.535 ± 0.077, \(n = 96\) cells, NS) (Figure 2). The re-addition of Ca\(^{2+}\) in the presence of CPA elicited a rapid rise in cytoplasmic Ca\(^{2+}\) in control and stretched cells (Figure 1). The initial rate of Ca\(^{2+}\) entry in stretched cells (\(\Delta F_{560/380}\) = 0.0075 ± 0.0006, \(n = 147\) cells) was significantly greater than controls (0.0049 ± 0.0003, \(n = 151\), \(P < 0.0002\)) (Figure 1B).

The CPA-induced Ca\(^{2+}\) peak observed in stretched and control cells was not affected by diltiazem (stretched cells, 0.602 ± 0.066, \(n = 41\) cells; control cells, 0.722 ± 0.099, \(n = 46\) cells, NS). The difference in the initial rate of Ca\(^{2+}\) re-entry between stretched and control cells remained in the presence of diltiazem (\(P < 0.024\)).

**Basal and CPA-induced Mn\(^{2+}\) influx in control and stretched cells**

The basal rate of Mn\(^{2+}\) entry was significantly greater in stretched cells (~0.122 ± 0.005, \(n = 56\) cells, \(P < 0.022\)) (Figure 3A and C). CPA-stimulated Mn\(^{2+}\) entry in Ca\(^{2+}\)-free PSS was also greater in stretched cells (~0.218 ± 0.017, ~60 cells) compared with controls (~0.147 ± 0.008, ~76 cells, \(P < 0.0001\)) (Figure 3B and C). CPA-stimulated Mn\(^{2+}\) entry was also significantly greater than basal entry in stretched (#, \(P < 0.0005\)) and control cells (†, \(P < 0.02\), Figure 3C).

**The effect of Ca\(^{2+}\)-free PSS, La\(^{3+}\), Gd\(^{3+}\) to CPA-induced Ca\(^{2+}\) entry in control and stretched cells**

The effect of calcium removal on the CPA-induced plateau was compared with the addition of La\(^{3+}\) and Gd\(^{3+}\) in stretched and control cells (Figure 4). There was no significant reduction in the CPA-induced plateau in the presence of extracellular calcium (Figure 4A) over 3.5 min in either stretched or control cells (% reduction \(F_{560/380}\) in CPA-PSS plateau, 6.08 ± 0.39, \(n = 117\) stretched cells versus 6.25 ± 0.89, \(n = 102\) control cells, NS) (Figure 4E). However, Ca\(^{2+}\) removal significantly reduced the CPA-induced plateau in stretched cells (percentage reduction \(F_{560/380}\) from CPA plateau, 16.10 ± 0.65, \(n = 71\) cells) versus controls (12.21 ± 0.94, \(n = 72\) cells, \(P < 0.001\)) (Figure 4B and 4E). La\(^{3+}\) addition had a minimal affect on the CPA-induced PSS plateau (Figure 4C), and there was no significant difference between the stretched (2.56 ± 0.66, \(n = 43\) cells) and control cells (1.04 ± 0.47, \(n = 43\) cells, NS) (Figure 4E). Gd\(^{3+}\) also had a minimal effect on the CPA-induced PSS plateau (Figure 4D), but in stretched cells Gd\(^{3+}\) evoked a small but significant reduction in the plateau compared with controls (7.64 ± 0.34, ~83 cells) (5.75 ± 0.36, 96 cells, \(P < 0.0002\)) (Figure 4E).

**Comparison of CPA and thapsigargin induced currents in stretched and control human myometrial cells**

Application of CPA (10 μM) and thapsigargin (100 nM) induced an inward rectifying current in control (\(n = 5\)) and stretched cells (\(n = 4\)) (Figure 5A). The currents were dependent on extracellular Na\(^{+}\) and fractional calcium entry (inward rectifying current in control (\(n = 5\)) and stretched (\(n = 4\)) cells in PSS and in the absence of extracellular Na\(^{+}\) as well as the presence of La\(^{3+}\). *\(P < 0.05\) compared with PSS within experimental group; #\(P < 0.05\) stretched cells versus control cells for current measured in 0 Na\(^{+}\) or presence of La\(^{3+}\).

**TRPC mRNA expression in control and stretched HSMM cells**

TRPC5 or 7 mRNA expression was not detected in either control or stretched HSMM cells (\(n = 8\), data not shown). TRPC1, 3, 4 and 6 mRNA were expressed in both control and stretched HSMM cells (\(n = 8\)) (Figure 6). TRPC1 mRNA expression was transiently increased after 1 h in stretched cells versus the paired time control (\(P = 0.03\), Figure 6A) and TRPC6 mRNA expression was slightly reduced at 14 h (\(P = 0.01\), Figure 6D) compared with the 14 h control. There was a significant up-regulation of TRPC3 mRNA expression in stretched cells 1 h (\(P < 0.0001\)) and 4 h (\(P < 0.0001\)) versus control cells, which returned to basal levels by 14 h (Figure 6B). TRPC4 mRNA expression was enhanced at 1 h (\(P < 0.01\)), 4 h (\(P < 0.001\)) and 14 h (\(P < 0.001\)) in stretched versus controls cells (Figure 6C).

**TRPC protein expression in control and stretched human myometrial cells**

TRPC1 (90 kDa), TRPC3 (90 kDa), TRPC4 (100 kDa) and TRPC6 (100 kDa) proteins were detected by Western blot analysis in control and stretched cells (Figure 7). No bands were observed when the primary antibody was omitted or when primary antibodies
were pre-absorbed with the appropriate peptide (data not shown). TRPC1 (control, 9104 ± 759 a.u. versus stretch 11890 ± 1296 a.u., NS, Figure 7A), TRPC4 (control, 5082 ± 1656 a.u. versus stretch, 7971 ± 1757 a.u., NS, Figure 7C) and TRPC6 (control, 20361 ± 1331 versus stretch, 17631 ± 673, NS, Figure 7D) proteins were expressed at a similar level in control and stretched cells. TRPC3 protein expression was significantly increased following 14 h tonic mechanical strain (control, 4149 ± 1314 versus stretch 11692 ± 2241, *P*/C20 0.05, Figure 7B).

Discussion

Mechanical stretch is hypothesized to be an important regulator of uterine growth and contractility during pregnancy. Several studies report stretch-induced regulation of contraction-associated genes and proteins in human myometrial smooth muscle (Manabe et al., 1992; Ou et al., 1997, 1998; Wu et al., 1999; Korita et al., 2002; Loudon et al., 2004; Soorranna et al., 2004; Terzidou et al., 2005), but functional correlates have not been fully explored. This study has determined the effect of prolonged tonic mechanical strain on intracellular calcium responses and calcium currents in parallel with expression of TRPC isoforms. Tonic mechanical stretch increased basal and store-operated calcium entry in association with an increase in TRPC3 and 4 gene expression. This effect was translated into an increase in TRPC3 protein expression.

Acute stretch of vascular smooth muscle and cardiac muscle in vitro increases intracellular calcium levels in muscle (McCarron et al., 1997; Calaghan et al., 2003; Zou et al., 2002; Liao et al., 2003). Uterine distension, whether in the early or late stages of pregnancy, is more likely to be a tonic stretch, but the influence of prolonged stretch on smooth muscle calcium dynamics is unclear. In our in vitro model, 14 h of tonic stretch induced a rise in basal calcium as evidenced by a drop in resting intracellular calcium on removal of extracellular calcium and stretch-enhanced Mn²⁺ entry. Despite increased basal calcium entry in stretched cells, resting calcium was unaltered in stretched cells compared with controls. This implies that stretch may also facilitate Ca²⁺ sequestration and/or removal. The amplitude of CPA-induced calcium transients (in the presence and absence of extracellular calcium) were similar in control and stretched cells which suggests that the CPA-releasable SR calcium store is unaffected by stretch. However, if calcium removal mechanisms are co-regulated by stretch, then it is possible that a stretch-induced change in the ‘peak CPA’ response is masked.

In contrast, our data clearly support a role for tonic in vitro mechanical stretch in mediating enhanced store-operated calcium entry in cultured human myometrial cells. This to our knowledge has not been reported in other smooth muscle. The plateau phase of the CPA response in both control and stretched cells is dependent on a contribution from store-operated calcium entry. In stretched cells, the reduction in the plateau following calcium removal and the rate...
of CPA-induced Mn$^{2+}$ entry were more rapid and under conditions of prolonged store depletion, the re-addition of Ca$^{2+}$ also elicited a more rapid rise in intracellular calcium in the stretched cells. A contribution of L-type calcium channels to the calcium response is unlikely, as the addition of diltiazem had no effect. Patch clamp data also demonstrated that in vitro mechanical stretch modulated the characteristics of the inwardly rectifying current activated by sarcoplasmic store depletion. In stretched cells, the current was less dependent on extracellular sodium and more dependent on Ca$^{2+}$, compared with controls. Overall, these data indicate that store-operated calcium entry is enhanced by tonic stretch and imply that Ca$^{2+}$ removal/sequestration mechanisms may increase in parallel.

Further characterization of CPA-induced calcium response showed that the plateau phase was insensitive to La$^{3+}$ and only minimally inhibited by Gd$^{3+}$ at concentrations known to inhibit store-operated calcium entry (Shlykov et al., 2003; Bradley et al., 2005). The current activated in stretched cells by store depletion (in the absence of extracellular sodium) was also relatively insensitive to La$^{3+}$, compared with controls. These results differ from our data in primary human myometrial cells (Tribe et al., 2003) in which the CPA-induced calcium response was La$^{3+}$ sensitive. It is possible, that the baseline molecular characteristics of store-operated calcium channels in the cultured cells used in the present study were altered due to different culture conditions (Golovina et al., 2001).

The stretch-induced effects on TRPC isoform protein expression were varied. TRPC1, which is reported to form a mechanosensitive channel that responds to acute stretch (Maroto et al., 2005), was unaffected in the present study by chronic stretch, despite a transient rise in TRPC1 mRNA at 1 h. TRPC6 protein and mRNA expression was unchanged by in vitro mechanical stretch, except for a slight down-regulation in mRNA expression at 4 h. In contrast, our data clearly show a significant increase in TRPC3 and TRPC4 mRNA expression following 1 and 4 h of tonic stretch. This was translated into a significant increase in TRPC3 protein expression at 14 h and a non-significant increase in TRPC4 protein expression. The discrepancy between changes in TRPC4 mRNA compared with protein expression may indicate that TRPC4 expression is regulated post-transcriptionally; indeed we have previously reported a similar lack of correlation between TRPC4 mRNA and protein expression in human myometrial tissue (Dalrymple et al., 2004). Alternatively, the non-significant change in TRPC4 protein expression may reflect the inability of western blot to accurately resolve small changes in protein expression. Interestingly, TRPC4 mRNA expression, unlike TRPC3, remained elevated at the 14 h time point which could

**Figure 7.** In vitro stretch enhances TRPC3 protein expression in cultured human myometrial cells. Representative western blots and graphs depicting mean ± SEM (n=7) of western blot densitometry of (A) TRPC1 (90 M$^{-1}$), (B) TRPC3 (90 M$^{-1}$), (C) TRPC4 (100 M$^{-1}$) and TRPC6 (100 M$^{-1}$) protein expression in control and stretched cells analysis for (A) TRPC1, (B) TRPC3, (C) TRPC4 and (D) TRPC6 protein expression. *P < 0.05; NS, P > 0.05.
suggest that a significant rise in TRPC4 protein might have been detected at a later time point; this observation requires further examination. The impact of TRPC3 and 4 gene silencing on stretch-induced changes in calcium signalling could also provide further insight into the functional role of these TRPC isoforms.

In previous studies, we reported that a similar increase in TRPC3 protein expression was associated with enhanced basal entry and store-operated calcium entry in interleukin (IL)-1β treated HMSC cells (Tribe et al., 2003; Dalrymple et al., 2004). However, there are marked differences in calcium responses activated by the two stimuli. IL-1β treated cells exhibited spontaneous generation of calcium transients and the enhanced store-operated calcium entry was La⁺ sensitive. There was also no indication that IL-1β altered TRPC4 gene expression. This may be explained by (i) differential effects of stretch on TRPC4 isoform expression and (ii) divergence in the activation of other signalling cascades during the period of exposure to stretch or IL-1β (Sooranna et al., 2005). The present study may also reflect differences between primary human myometrial cells and those maintained in longer-term culture. Interestingly, application of stretch or IL-1β results in alterations in TRPC isoforms which are up-regulated in human myometrial tissue taken from women in labour (Dalrymple et al., 2004), providing additional support for a contribution of TRPC3/4 to myometrial function.

Overall, our data suggests that tonic stretch results in co-regulation of calcium entry pathways and TRPC3 and TRPC4 expression in cultured human myometrial cells. Since an up-regulation of calcium entry may lead to increased contractility of smooth muscle, this provides a possible pathway by which uterine function can be altered in response to a growing fetus during pregnancy and at the end of gestation.

Acknowledgements

We thank all the women who kindly participated in this study, Julie Adams and Guy’s and St Thomas’ Hospital Trusts labor ward staff for myometrial sample collection. We are indebted to Jeanette Judah for technical assistance in preparing cells, Professor David Beech (University of Leeds) for kindly supplying the Tie3 antibody, Mr Paul Seed for statistical assistance, and Drs Neil Levinson and Jason Tennant for the real time PCR data. This study was funded by Tommy’s, the baby charity (Reg. Charity No: 1060508).

References


Submitted on August 25, 2006; resubmitted on November 13, 2006; accepted on November 14, 2006.

Mechanical stretch and calcium signals in myometrium