Differential expression of ryanodine receptor RyR2 mRNA in the non-pregnant and pregnant human myometrium

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We describe here the expression of the ryanodine receptor isoforms RyR2 and RyR3 in human non-pregnant and pregnant (non-labouring) myometrium, and in isolated cultured myometrial cells. The mRNA encoding the RyR3 isoform was found in both non-pregnant and pregnant myometrial tissue samples; however, the mRNA for RyR2 was found only in pregnant samples. It can be speculated that the appearance of this additional isoform in the pregnant myometrium may increase the ability of this tissue to contract at term. Control of expression of the RyR2 gene may therefore be another example of an up-regulated signalling system in pregnancy. Although the mRNA for RyR3 was expressed in cultured myometrial cells, the mRNA for RyR2 could not be detected. Thus cultured myometrial cells appear to be similar to the non-pregnant myometrium. The cytokine transforming growth factor β (TGF-β) has been reported to alter RyR mRNA expression in many cell types. After treatment with TGF-β, both RyR2 and RyR3 mRNAs could be detected in cultured myometrial cells. These observations support the idea that the expression of the RyR2 isoform is up-regulated both in pregnancy and in TGF-β-treated cultured myometrial cells. Using measurements of $^{45}Ca^{2+}$ release, we have further demonstrated that cultured human myometrial cells show a significant augmentation of both the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism and ryanodine-induced Ca^{2+} release after treatment with TGF-β. Additionally, caffeine was able to induce Ca^{2+} release and sensitize the CICR mechanism to ryanodine. Thus we suggest that the appearance of RyR2 mRNA leads to the expression of this receptor/channel protein with identifiable pharmacological characteristics. These results are discussed in the context of the potential role of gene activation in the process of maturation of the human myometrium during pregnancy.

INTRODUCTION

There is now a considerable body of evidence suggesting that, as term approaches and before normal labour can begin, several key structural and functional changes take place within the myometrium. For example, there is a down-regulation of the adenylate cyclase membrane signalling system, in conjunction with the up-regulation of oxytocin receptor numbers, cyclo-oxygenase enzymes, gap junctions, ion channels and G-protein-linked membrane signalling systems [1–4]. In this way the myometrium is transferred from a state of quiescence to one primed for activation. Recently we have reported preliminary observations suggesting that elements of the intracellular Ca^{2+}-mobilizing system in the human myometrium may also be specifically regulated during pregnancy [5,6]. The factors which trigger these changes are not well understood, but the major elements of these changes are likely to involve the control of gene expression. From this point of view, parturition can only be initiated effectively when all of these changes are in place. Failure to activate any of these systems at the correct time in pregnancy, either early or late, could lead to complications, e.g. premature labour or poorly co-ordinated contractions.

A key element in smooth muscle activation is cytoplasmic Ca^{2+}. A rise in the intracellular Ca^{2+} concentration is essential for the initiation of contraction. Hormones, such as the prostaglandins and oxytocin, have been implicated in the activation of uterine contractility via a mechanism involving inositol 1,4,5-trisphosphate ($Ins P_3$) [7,8]. The binding of $Ins P_3$ to specific receptors on the sarcoplasmic reticulum leads to the release of Ca^{2+} from intracellular stores and a subsequent rise in the intracellular Ca^{2+} concentration [7–9].

A second family of intracellular Ca^{2+} release channels, known as the ryanodine-sensitive receptor channel complex (RyR), has also been found on the internal membranes of a number of cell types, including smooth muscle [9–11]. These Ca^{2+}-release channels are activated by Ca^{2+} entering the cell as a result of electrical activity at the surface membrane. In smooth muscle, this Ca^{2+}-induced Ca^{2+} release (CICR) mechanism is thought to play a key role in the amplification of the intracellular Ca^{2+} transient needed to initiate contraction.

Three genes encoding RyRs have been detected in mammalian tissue, giving rise to isoforms RyR1, RyR2 and RyR3 [10,12,13]. The RyR1 isoform was initially identified in skeletal muscle [14], RyR2 is primarily associated with cardiac muscle [15] but is also present in some smooth muscles, and RyR3, originally identified in mink lung [16] and human Hep 3B cells [17], is also found extensively in neural tissue. Although each isoform is responsible for activating Ca^{2+} release from internal membranes, the mechanisms triggering release appear to be different for each isoform. RyR1 is activated by depolarization of the surface membrane, while RyR2 and RyR3 are activated by a rise in the cytoplasmic Ca^{2+} concentration [18]. Furthermore, it is emerging that the different isoforms have different pharmacological sensitivities. RyR1 and RyR2 are activated by caffeine [13], whereas RyR3 is reported to be caffeine-insensitive [16,19].

We have reported preliminary data showing the expression of RyR3 in the human myometrium and in cultured myometrial cells [11]. Until recently the human RyR2 gene sequence was

Abbreviations used: CICR, calcium-induced calcium release; RyR, ryanodine-sensitive receptor/channel complex; TGF-β, transforming growth factor β; RT-PCR, reverse transcriptase-PCR.

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unavailable, and consequently the complete analysis of RyR isoform expression by reverse transcriptase-PCR (RT-PCR) could not be carried out. In the present study we have used specific oligonucleotide probes designed from the porcine RyR2 sequence [20] to identify a partial DNA sequence of the RyR2 isoform isolated from human cardiac tissue [6] (EMBL accession no. X91869), which was subsequently used to design specific oligonucleotide probes to determine the expression of the RyR2 isoform in human myometrial tissue and cultured cells. Analysis of the sequences obtained from human myometrium reveals 100 % identity with the published cardiac RyR2 sequence in this region [21] (EMBL accession no. X98330).

Our data show that the RyR2 isoform could not be detected in the non-pregnant myometrium, but could be detected in samples of pregnant myometrium. Thus the RyR2 system appears to be a further example of a key cell signalling mechanism that is up-regulated in pregnancy. As yet, the mechanisms that trigger RyR2 gene expression *in vivo* are not known.

RyR2 was not detected in cultured myometrial cells. Cytokines, including transforming growth factor β (TGF-β), have been reported to alter RyR mRNA expression in many cell types [16,22–25]. In this paper we also present data to show that TGF-β can induce the expression of RyR2 in cultured myometrial cells. Thus activation by cytokines appears to mimic the changes in RyR2 seen in the myometrium *in vivo*. The importance of these observations for the maturation of the uterus, in preparation for parturition, is discussed.

MATERIALS AND METHODS

Tissue preparation and culture techniques

Myometrial tissue was taken from the lower uterine segment, with informed consent, from patients undergoing either hysterectomy (women under 40 years of age) for non-malignant disorders or elective caesarean section (non-labouring). Ethical approval was obtained from Newcastle Area Health Authority. Myocytes were prepared by dispase/collagenase digestion [26] and maintained in M199 (Gibco) supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) glutamine and 2 % (v/v) penicillin/streptomycin. Cells were grown to confluence in 75 cm^2 culture flasks before being passaged on to plastic 12-well multiwell plates for $^{45}Ca^{2+}$ efflux studies. Cells were not used beyond passage number 6. Direct immunofluorescent staining for α-actin was carried out routinely on cultured cells to check the purity of preparations, as previously described [26]. Data were analysed using Lysys II (Becton Dickinson). Typically, preparations demonstrated 80–90 % positive staining for α-actin.

Synthetic oligonucleotides

All oligonucleotides were obtained from the Molecular Biology Service Unit, University of Newcastle upon Tyne.

Set A

A set of RyR2 specific primers designed by Ledbetter et al. [20] based on the published rabbit cardiac RyR2 sequence was used to amplify a product of 490 bp. Sense, 5′ GGAAGTCCCATTCTGAACTCTC 3′; antisense, 5′ GCTGTCCACAAACGGCTCTGTG 3′.

Set B

A second RyR2 antisense primer was designed from the human RyR2 sequence reported in the present paper and incorporating an *Eco*RI site: 5′ GGTCCACCATGAATTCATAGTA 3′. This antisense primer, used in conjunction with the sense primer of RyR2 set A, amplified a 466 bp fragment.

Set C

These oligonucleotides were used to generate a 292 bp fragment from the small ribonuclear protein S14: sense, 5′ ATGGCACCTCGAAAGGGGA 3′ (nt 1940–2090); antisense, 5′ GGAGTTTGATGTGTAGGGC 3′ (nt 2703–2845). The oligonucleotides span an intron and the co-ordinates given in parentheses correspond to the numbering in the published sequence [27].

Specific oligonucleotides for the RyR1 and RyR3 isoforms are as previously reported [11].

cDNA synthesis and PCR

Total RNA was extracted from both cultured myometrial cells and myometrial whole tissue by the guanidine isothiocyanate/lithium chloride method, with 0.1 % lithium dodecyl sulphate added to the initial extraction buffer for cultured cells [28]. Samples of 1 μg of total RNA were treated with DNase I Amplification Grade (Gibco-BRL) at room temperature for 15 min in 10 × DNase I reaction buffer (Gibco-BRL), followed by heat inactivation of the DNase I at 65 °C for 10 min. A portion of 1 μg of DNase I-treated RNA was annealed with 0.5 μg of random primers (Gibco-BRL) at 65 °C for 5 min and allowed to cool to room temperature for 30 min. First-strand cDNA synthesis was carried out using Superscript reverse transcriptase (Gibco-BRL) at 200 units per μg of RNA at 42 °C for 50 min in a reaction volume of 20 μl; negative controls were carried out in the absence of reverse transcriptase. One-tenth of the reverse transcriptase reaction product was amplified, using standard PCR methods, in a 100 μl reaction volume using *Taq* polymerase (Promega). Amplification conditions were as follows: denaturation at 94 °C for 3 min, primer annealing at 45–55 °C for 2 min and extension at 72 °C for 2 min. This was followed by 34 cycles of denaturation at 94 °C for 1 min, primer annealing at 45–55 °C for 2 min and extension at 72 °C for 2 min. The annealing temperature was determined for the following specific primer sets: the RyR2 primer sets A and B were annealed at 50 °C, the S14 primer set C was annealed at 55 °C, the RyR1 primers were annealed at 45 °C and the RyR3 primers were annealed at 55 °C, as previously described [11].

Nucleotide sequencing of PCR-derived RyR2 clones

The RT-PCR products derived from cardiac RNA, using RyR primer set A, were cloned into the pGEM-T vector (Promega) following the manufacturer's recommendations, and the plasmids were purified by the modified method of Birnboim and Doly [29]. The clones were sequenced using both forward and reverse universal primers. Internal sequencing primers were used to produce a complete double-stranded sequence for this fragment. The internal primers were: 5′ TGTCAGCAGCATGAGAC 3′ and 5′ GTCTCATGCTGCTGACA 3′.

The RT-PCR products from pregnant myometrial samples generated using RyR2 oligonucleotide set B were cloned into M13mp18 and M13mp19 vectors on PCR products digested with *Hin*dIII and *Eco*RI. Internal sequencing primers as described above were used to produce double-stranded sequences for these fragments. An RT-PCR-derived product of 292 bp, using oligonucleotide S14 primer set C, was cloned into the pGEM-T

vector (Promega) and plasmid-purified as above. The clones were sequenced using both forward and reverse universal primers. Automated DNA sequencing of the PCR-derived clones was carried out by the Molecular Biology Service Unit, University of Newcastle upon Tyne.

Total protein assay

Cells grown in 6-well multiwell plates were treated with TGF-β (1 ng/ml) for 24 h; three untreated wells in each plate served as internal controls. A Bio-Rad protein assay was used [30] with a slight modification incorporating Coomassie Brilliant Blue as a dye. BSA was used as a standard solution at concentrations ranging from 1 to 25 μg/ml. Cells were solubilized by adding 0.5 ml of 5 % (v/v) trichloroacetic acid/well. Aliquots of 20 μl of the dye reagent concentrate were added to 80 μl of either standard or sample in prelabelled wells of a 96-well multiwell plate (Becton Dickinson). After a period of 10 min the A_{595} was measured using a microplate reader (Dynatech Laboratories). Concentrations were plotted against A_{595}, and the unknown concentrations were read from the standard curve.

$^{45}Ca^{2+}$ efflux from saponin-permeabilized cultured cells

Confluent cells, grown in 12-well multiwell plates, were washed with a balanced salt solution containing (mM): 140 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4, 5 D-glucose and 10 Hepes buffered to pH 7.4, to remove the culture medium, before being fixed to a mechanical shaker. Cells were permeabilized with 15 μg/ml saponin for 15 min in the following skinning solution (mM): 20 KCl, 10 Hepes, 2 $MgCl_2$, 1 ATP and 1 EGTA, pH 7.0. The efficiency of skinning was routinely checked using Trypan Bue staining. Loading of non-mitochondrial intracellular stores with $^{45}Ca^{2+}$ and subsequent efflux experiments were carried out as previously described [31]. Cultured cells on multiwell plates were treated with 1 ng/ml TGF-β1 (Sigma) for 24 h prior to $^{45}Ca^{2+}$ efflux studies or total RNA isolation.

Statistics

Data are expressed as means $\pm$ S.E.M. Where appropriate, data were analysed using paired t tests, and a P value of < 0.05 was considered significant.

RESULTS

mRNA expression of the RyR1 and RyR3 isoforms in human myometrium

Preliminary data have suggested that the RyR1 isoform can be detected in myometrial samples by RT-PCR in which a specific RyR1 oligonucleotide primer is used in the reverse transcriptase reaction to produce first-strand cDNA [11]. This approach may have led to the detection of low basal levels of transcripts. When these experiments were repeated using random primed cDNA, RyR1 expression could not be detected in any of the samples (Figure 1a). The present data therefore suggest that RyR1 expression in the human myometrium is likely to occur at very low basal levels or to be restricted to a small subpopulation of cells.

Specific primers for the RyR3 isoform [11] were used to amplify RT-PCR products of the predicted size (Figure 1b). The identity of these products was verified by restriction enzyme analysis (results not shown). mRNA analysis was carried out on a total of three samples from pregnant myometrium, three samples from non-pregnant myometrium and two samples from cultured cells. RyR3 was detected in all samples studied. Thus we

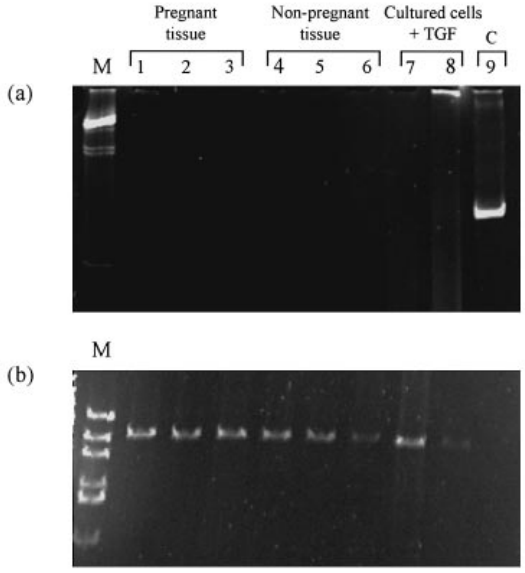

Figure 1 PAGE (6% gel) of RT-PCR products from pregnant tissue (lanes 1–3), non-pregnant tissue (lanes 4–6) and cultured myometrial cells treated with TGF-β (lanes 7 and 8)

Total RNA was reverse-transcribed using random hexamer primers, and the cDNA was subjected to amplification by PCR using specific primers. (**a**) RT-PCR products obtained using RyR1 primers [11]. The sample labelled C indicates a control sample of this segment of RyR1 DNA previously cloned into M13mp18 and subjected to DNA sequence analysis [11]. Lane M contains molecular size markers obtained by digestion of λ with the restriction enzyme *Hin*dIII. The molecular sizes from the top are 23 kb, 9.3 kb and 6.4 kb running as a triplet, 4.3 kb, 2.3 kb and 2 kb. The expected molecular size of the RT-PCR product is 999 bp. (**b**) RT-PCR products obtained using RyR3 specific primers [11].

suggest that this RyR isoform is constitutively expressed in the human myometrium.

mRNA expression of the RyR2 isoform

Human DNA sequence data, to allow the design of specific human RyR2 oligonucleotide primers, were unavailable at the time of this study. In the absence of such information, we have used specific RyR2 oligonucleotide primers taken from the published porcine RyR2 sequence [20] in RT-PCR reactions with mRNA from human cardiac tissue. The PCR product was subcloned into the pGEM-T vector and subjected to DNA sequence analysis (EMBL accession no. X91869). The human cardiac RyR2 cDNA sequence was found to show 93 % identity to the rabbit RyR2 cDNA sequence [15]. Comparison of the deduced amino acid sequences of human and rabbit RyR2 indicate 100 % identity between the two species.

The human sequence was then used to design oligonucleotide primer set B in order to determine the expression of the RyR2 isoform in non-pregnant (hysterectomy samples) and pregnant non-labouring (elective caesarian section samples) human myometrial tissue. The results of the RT-PCR analysis for expression of the RyR2 isoform in human myometrium are presented in Figure 2. Figure 2(a) shows that expression of the RyR2 isoform could not be detected in any of the five non-pregnant myometrial whole-tissue samples studied. However, expression of mRNA encoding the small ribonuclear protein S14 was detected in these samples by RT-PCR (Figure 2c), indicating that the RNA was not degraded and the resulting cDNA was synthesized correctly. In contrast, expression of the RyR2 isoform was detected in all

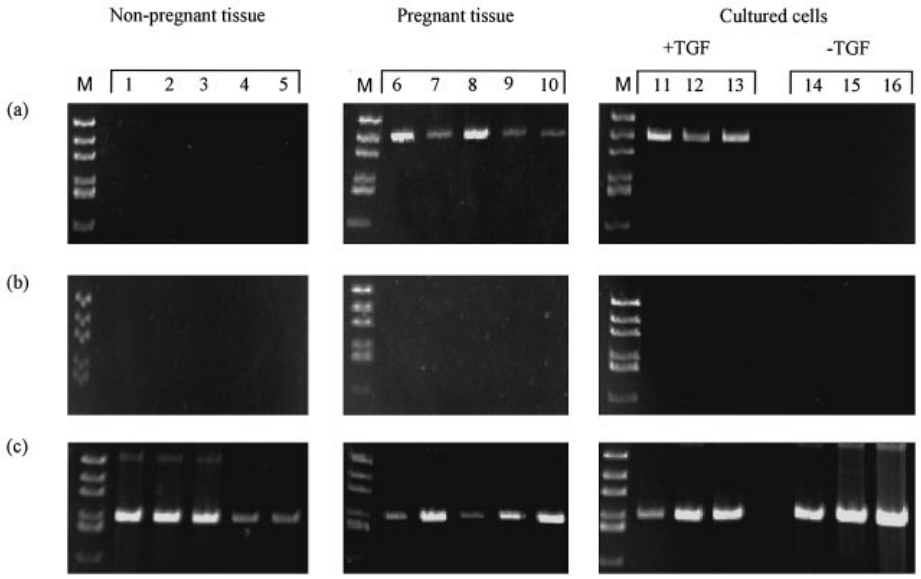

Figure 2 PAGE (6% gel) of RT-PCR products from non-pregnant tissue (lanes 1–5), pregnant tissue (lanes 6–10) and cultured cells treated (lanes 11–13) or not (lanes 14–16) with TGF-β

Total RNA was reverse-transcribed using random hexamer primers and the cDNA was subjected to amplification by PCR using specific primers. (**a**) RT-PCR products obtained using RyR2 primers (set A; see the Materials and methods section) giving the expected fragment size of 466 bp. (**b**) Control samples in which RNA was not subjected to reverse transcription. PCR amplification of these samples did not produce detectable levels of products using the same RyR2 primers. (**c**) RT-PCR products obtained using S14 primers (set D). Lane M contains molecular size markers which were produced by digestion of pUC18 with the restriction enzyme *Hae*III following the manufacturer's recommendations (Gibco-BRL). Molecular size markers from the top of the gel are: 587 bp, 458 bp, 438 bp, 298 bp, 277/267 bp running as a doublet and 178 bp.

five pregnant myometrial whole-tissue samples studied. The results suggest that RyR2 expression appears to be 'switched on' in the myometrium at some point during pregnancy.

RyR2 expression in cultured myometrial cells

mRNA for the RyR2 isoform could not be detected in cultured human myometrial cells (Figure 2a, lanes 14–16). These results suggest that cultured cells may be similar to the non-pregnant tissue. In many cell systems, stimulation with growth factors such as TGF-β has been shown to alter the expression of RyR isoforms [16,22–25]. Consequently we treated cells with TGF-β for 24 h to determine whether a similar system operates in myometrial smooth muscle. Figure 2(a) (lanes 12 and 13) shows that expression of RyR2 mRNA was detected in cultured myometrial cells after TGF-β treatment. Thus it would appear that we may be able to mirror the changes taking place between non-pregnant and pregnant myometrium with respect to the expression of RyR2.

The RyR2 fragment obtained from pregnant tissue and TGF-β-treated cultured cells was of the predicted size (466 bp). The identity of the PCR product was verified for three of the pregnant tissue samples by DNA sequence analysis. Using a predicted internal *Hin*dIII site present within the PCR-generated fragments and the *Eco*RI site in the antisense primer (set B), the fragments were cloned into M13mp18/19. The sequence was identical to the published human RyR2 cardiac sequence (EMBL accession no. X91869). The identity of the PCR-generated fragments from the TGF-β treated cells was also verified by restriction enzyme digestion using internal recognition sites for the restriction enzymes *Hin*dIII and *Ssp*I. Restriction enzyme patterns were identical to the PCR product generated from the human cardiac RyR2 fragment cloned into pGEM-T [6]. Control samples not treated with reverse transcriptase did not produce a PCR product (Figure 2b), indicating that the RT-PCR product was not obtained from contaminating genomic DNA. The oligonucleotides used to detect expression of the S14 protein (set C) were designed to span an intron [27], so that any samples contaminated with genomic DNA could be readily identified by the presence of a 905 bp product. A product of this size was not detected in any of the myometrial tissues or cultured cell samples used in this study. The predicted 292 bp product of the S14 RT-PCR was detected in all samples studied, and one sample was cloned into pGEM-T. DNA sequencing verified the product of the S14 gene. Some of the non-pregnant myometrial samples and cultured myometrial cells produced an additional PCR product of 720 bp, which may represent a splice site variant or may be related to S14, which is a member of a large multigene family encoding ribosomal proteins [27].

The alterations in RyR2 mRNA levels, assessed from RT-PCR analysis, do not give any insight into levels of protein expression or the functional consequences of RyR2 expression. However, the ability to induce the expression of RyR2 *in vitro* presents a unique opportunity to determine the physiological consequences of the co-expression of two isoforms of RyR. Therefore we have examined the effects of TGF-β treatment on the physiological and pharmacological responses of cultured human myometrial cells to Ca^{2+}, ryanodine and caffeine.

Effects of TGF-β on CICR and the response to ryanodine in permeabilized human myometrial smooth muscle cells

We have previously shown that increasing concentrations of Ca^{2+}, in the range 100 nM–10 μM, induce the release of $^{45}Ca^{2+}$

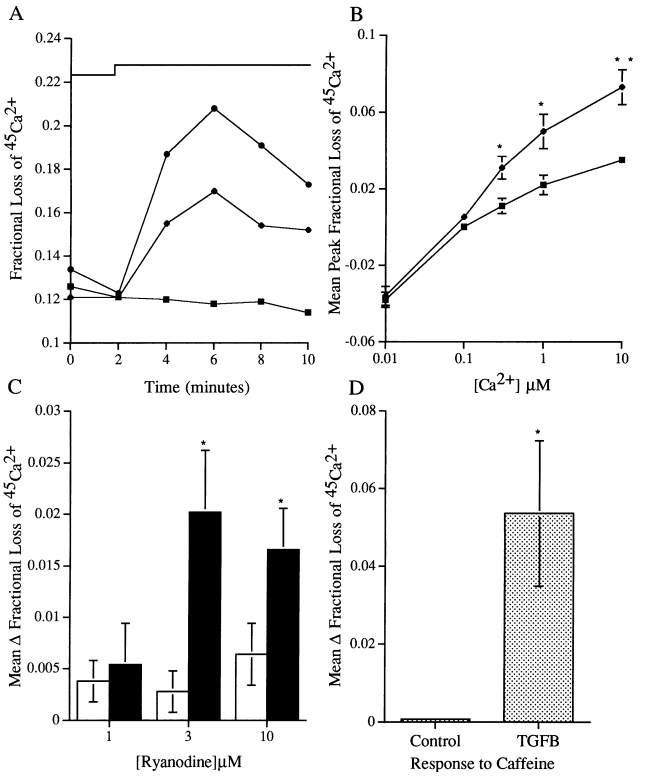

Figure 3 Effect of TGF-β on the ryanodine-sensitive Ca^{2+} release system

(**A**) Representative data from a single experiment illustrating an augmentation of Ca^{2+}-induced $^{45}Ca^{2+}$ release at 10 μM free Ca^{2+} in cells pretreated with TGF-β (◆) compared with untreated control cells (●). The bottom trace (■) shows basal efflux at 100 nM free Ca^{2+}, and the step is indicated by the bar. (**B**) Mean data from four paired experiments (cells from four patients) in which the free Ca^{2+} concentration was changed in the range 10 nM–10 μM. TGF-β-treated cells (◆) showed a significant increase in Ca^{2+}-induced $^{45}Ca^{2+}$ release above the control level (■) in the range 300 nM–10 μM free Ca^{2+} (* denotes statistical significance where $P < 0.05$; bars represent S.E.M.). (**C**) Bar graph of mean responses from five paired experiments (cells from five patients) to illustrate the augmentation of ryanodine-induced $^{45}Ca^{2+}$ release in TGF-β-treated cells (solid bars) as compared with untreated control cells (open bars). Data are means ± S.E.M. (**D**) Significant increase in $^{45}Ca^{2+}$ release in response to 10 mM caffeine in TGF-β-treated cells compared with controls. Data represent means ± S.E.M. of six paired experiments (three batches of cells from three patients).

from the internal membranes of permeabilized human myometrial smooth muscle cells [31]. These responses are not due to $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange caused by stimulation of the Ca^{2+} pump, since the CICR response is observed in the presence of the Ca^{2+} pump inhibitor thapsigargin [32]. In order to determine the effect of TGF-β on the CICR response, one-half of a 12-well multiwell plate was incubated for 24 h with 1 ng/ml TGF-β prior to experimentation. Figure 3(A) shows raw data from a single paired experiment illustrating the effect of TGF-β on the response to stepped changes in Ca^{2+} concentration from 100 nM to 10 μM Ca^{2+} in permeabilized cells loaded with $^{45}Ca^{2+}$. Control efflux from untreated cells was carried out in 100 nM Ca^{2+}. A step change in Ca^{2+} concentration from 100 nM to 10 μM (indicated by the bar) was applied to both control and treated cells. TGF-β augmented the release of $^{45}Ca^{2+}$ during the Ca^{2+} step. Figure 3(B) shows mean data from four experiments (four batches of cells from four patients) showing the effect of TGF-β in the presence of stepped changes in Ca^{2+}, from a basal level of 100 nM through the range 10 nM–10 μM. Interestingly, the response to a stepped change in Ca^{2+} from 100 nM to 10 nM did not seem to be affected by TGF-β, whereas the response was significantly increased over the control level between 300 nM and 10 μM Ca^{2+}. Additionally, it can be seen that TGF-β treatment did not affect basal Ca^{2+} release measured at 100 nM Ca^{2+}.

Table 1 Effects of TGF-β on cell proliferation

Measurement of total protein, total $^{45}Ca^{2+}$ uptake and $InsP_3$-induced $^{45}Ca^{2+}$ release was carried out in cultured cells pretreated with TGF-β and in untreated control samples. Values represent means ± S.E.M.; no significant differences were found between the two groups.

	Control	+ TGF-β
Total protein (μg/ml)	5.55 ± 0.94	7.67 ± 1.13
Total $^{45}Ca^{2+}$ uptake (absolute c.p.m.)	379129 ± 21292	398746 ± 13398
$InsP_3$-induced responses (fractional loss)	0.329 ± 0.011	0.373 ± 0.041

Ryanodine can activate the release of $^{45}Ca^{2+}$ from the sarcoplasmic reticulum of human myometrial cells [31]. We therefore examined the effect of TGF-β on Ca^{2+} release using various concentrations of ryanodine (Figure 3C). Mean data from five paired experiments (five batches of cells from five patients), over the range 1–10 μM ryanodine, are shown. In all cells pretreated with TGF-β an augmentation of the response to ryanodine could be demonstrated, which was significant at both 3 and 10 μM ryanodine ($P < 0.05$).

The sensitivity of permeabilized cultured cells to the methylxanthine caffeine was also examined in control and TGF-β-treated cells; 10 mM caffeine was applied for 2 min during the efflux period. Figure 3(D) shows mean data from six experiments (three batches of cells) illustrating the augmentation of caffeine-induced $^{45}Ca^{2+}$ release in TGF-treated cells.

Since TGF-β is known to induce cell growth and differentiation in many cell types, a series of control experiments was carried out to determine whether the effects of TGF-β on the physiological responses to ryanodine and Ca^{2+} were simply the result of an increase in cell number within each well. Paired experiments were carried out in which the total $^{45}Ca^{2+}$ uptake ($n = 24$; four batches of cells from four patients), the response to 300 nM $InsP_3$ ($n = 6$; three batches of cells from three patients) and total protein content per well ($n = 9$, three batches of cells from three patients) were determined for both control and TGF-β-treated cells (Table 1). Statistical analysis indicated that there was no significant increase in cell number after 24 h of treatment with TGF-β.

DISCUSSION

In this paper we report the cloning and partial sequence analysis of the RyR2 isoform from human cardiac (accession no. X91869) and pregnant myometrial tissue. The sequences were found to be identical to the recently published human cardiac RyR2 sequence, and the fragment corresponds to base pairs 1718–2164 [21]. We have shown that the RyR2 isoform, which may be involved in intracellular Ca^{2+} signalling in human myometrial tissue, is up-regulated in non-labouring pregnant human myometrial tissue. Conversely, the mRNA for RyR2 could not be detected in non-pregnant whole tissue. The significance of this gene activation is not known; however, one strong possibility is that, in late pregnancy, the expression of an additional RyR isoform may serve to augment intracellular Ca^{2+} transients involved in contractile activation by the process of CICR. In this way, the amplitude and time course of each myometrial contraction might be increased.

It would therefore appear that the RyR Ca^{2+}-release system is another example of a cell signalling mechanism whose expression may be activated or regulated in the myometrium in preparation for parturition. Various systems, including gap junctions [3,33], oxytocin receptors [1,34], specific G-proteins [2,35], K^+ channels [36,37] and L-type voltage-dependent Ca^{2+} channels [38], have all been implicated in the priming of the uterus for the onset of labour. It is probable that such cell signalling mechanisms have to be in place in the myometrium at term in order that the myometrial smooth muscle cells can produce the appropriate co-ordinated contractions needed to expel the fetus [39].

There is little information available on the mechanisms that initiate any of these changes *in vivo*, or the precise point at which they occur during pregnancy. Further analysis of these changes is complicated by the difficulty in obtaining isolated human myometrial tissue during the various stages of pregnancy. It is also obviously inappropriate, and a potential danger to both mother and foetus, to attempt any pharmacological manipulation of the myometrium *in vivo*. Thus one alternative is to use isolated myometrial cells maintained in tissue culture in order to identify the hormonal influences which may be involved in the regulation of gene expression. We have used this model system to investigate the RyR isoforms at both the molecular and the functional level. Myometrial cells in culture express mRNA encoding the RyR3 isoform, and this receptor/channel complex has been shown to function as a CICR mechanism [11,31]. This isoform was also shown to be sensitive to Ca^{2+}, ryanodine and Ruthenium Red, but was not directly activated by caffeine [11,31].

It is well documented that the expression of the RyR system in many cell types can be modified by growth factors and cytokines. For example, fibroblast growth factor regulates the expression of the mRNA encoding the RyR in BC3H1 cells [22]. Similarly, the induction of B4 mRNA, encoding the RyR3 isoform, has been reported in mink lung epithelial cells after treatment with TGF-β [16]. For these reasons we examined the effects of TGF-β treatment on the expression of the RyR system in cultured human myometrial cells. Cultured cells treated with TGF-β expressed the RyR2 isoform in addition to RyR3, demonstrating the activation of RyR2 gene expression by TGF-β.

The link between these observations with cultured cells and the altered expression of the RyR2 system in pregnant tissue is, at present, tenuous. However, stimulation of cultured cells with TGF-β may prove to be a useful model with which to follow the time course and functional consequences of the up-regulation of the RyR system and to probe the mechanisms linking surface membrane stimulation and activation of the genome to express a specific Ca^{2+} signalling system.

The expression of TGF-β and TGF-β receptors has been reported to be influenced by hormones [40,41]. For example, corticotropin ('ACTH') up-regulates TGF-β receptors in bovine adrenocortical cells [40], and parathyroid hormones have been reported to alter the affinity of the TGF-β receptor for TGF-β in osteoblast-enriched cell cultures from rat parietal bone [41]. There is direct evidence for the involvement of steroid hormones in the regulation of expression of TGF-β [42] and the TGF-β receptor [43]. There is also compelling evidence for the expression of TGF-β isoforms throughout the female reproductive tract, including the ovaries, fallopian tubes and endometrium [44–46]. Additionally, expression of three isoforms of TGF-β (TGF-β1, 2 and 3), as well as of the type II TGF-β receptor, has been reported in the non-pregnant human myometrium and decidua [47]. The degree of expression appears to change during the different stages of the menstrual cycle, supporting the suggestion that ovarian steroids may be involved in the regulation of these events [47].

It can be speculated that TGF-β and TGF-β receptors may be regulated by other factors in the human pregnant myometrium. If this were to be the case, then the preparation of the uterus would involve a cascade in which the hormones of pregnancy may influence the TGF-β system. As described above, this could facilitate the activation of a number of specific genes involved in priming the uterus for parturition and making it receptive to external excitatory stimulation. However, it is unlikely that TGF-β is the sole activator of the priming process in the uterus, and other cytokines and hormones are almost certainly involved. The nature of these factors and the interactions between these various signals have yet to be characterized.

The data presented here suggest that TGF-β does not influence cell proliferation within the time course of our experiments. It is also unlikely that the $InsP_3$-dependent Ca^{2+}-release system or the sarcoplasmic Ca^{2+}-ATPase are affected by TGF-β treatment. Thus TGF-β stimulation does not appear to reflect a generalized activation of gene expression relating to Ca^{2+} signalling in these cultured myometrial cells. We have also shown that the expression of the additional RyR2 isoform has clear effects on the ability of the cells to mobilize intracellular Ca^{2+}. The magnitude of the CICR response is increased significantly, together with an increased responsiveness of the cells to ryanodine. These physiological changes support the conclusion that the increased expression of RyR2 mRNA is also reflected at the functional level. It is interesting to note that TGF-β-treated cells demonstrate Ca^{2+} release in direct response to caffeine, which is not seen in untreated cells. It has been reported that the RyR3 isoform is not directly activated by caffeine, and this was confirmed by our data on control untreated cells, which express only the RyR3 isoform [11,31]. The cardiac RyR2 isoform is reported to be caffeine-sensitive [10,13]. The caffeine-sensitivity observed in TGF-β-treated cells must therefore reflect a caffeine-sensitivity of the RyR2 isoform. Interestingly, although other workers have reported that caffeine is unable to induce contracture in pregnant rat myometrial tissue as a result of its ability to inhibit phosphodiesterase activity and increase cAMP levels [8,48], investigations on pregnant human myometrial strips show that caffeine is able to release Ca^{2+} from intracellular stores in skinned preparations [49]. It has been suggested that the responsiveness of human pregnant tissue to caffeine may be due to the suppression of cAMP activity by some other means, leading to activation of the contractile apparatus [49]. Alternatively, we suggest that caffeine-sensitivity may be conferred on human pregnant tissue by the presence of the RyR2 isoform reported in this study.

We are grateful to The Physiological Society for financial support. We also thank the British Heart Foundation, who supported that element of the work associated with the isolation and sequencing of RyR2. We are indebted to Professor A. Hawkins (Department of Biochemistry and Genetics, University of Newcastle) for the use of molecular biology facilities, Dr. L. Mertens and Dr. H. De Smedt (University of Leuven, Belgium) who provided a sample of human cardiac mRNA, and Dr. H. A. Otun for his help in the preparation of figures.

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Received 2 July 1996/8 November 1996; accepted 15 November 1996

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