Relaxin signalling in primary cultures of human myometrial cells

Kee Heng1, Richard Ivell1, Prabhath Wagaarachchi2 and Ravinder Anand-Ivell1,3

1School of Molecular and Biomedical Science, University of Adelaide, Adelaide SA5005, Australia; 2Department of Perinatal Medicine, Women’s and Children’s Hospital, North Adelaide SA 5006, Australia
3Correspondence address. E-mail: ravinder.anand-ivell@adelaide.edu.au

In myometrium of pigs and rats, though not humans, relaxin appears to mediate an inhibition of spontaneous and oxytocin-induced contractility, presumably acting through a G-protein coupled receptor (RXFP1) to generate cAMP. In humans, circulating relaxin is highest in the first trimester, including the time of implantation, when transitory uterine quiescence could help a blastocyst to implant. We investigated whether relaxin can activate adenylate cyclase in primary human myometrial cells from non-pregnant tissue, and we show that relaxin is able to stimulate the generation of cAMP in a manner, which is dependent upon a tyrosine phosphorylation activity, as in the endometrium. We identified transcripts for the relaxin receptor RXFP1 as full-length variants, though a minor splice variant missing exon 2 was also present in low amounts. These cells also express transcripts encoding RXFP2, the receptor for the closely related hormone, INSL3. Although able to respond to relaxin at high concentrations, this receptor does not appear to function by contributing to the cAMP production in human myometrial cells, nor does INSL3 act as a functional agonist or antagonist of relaxin action. In conclusion, the inability of relaxin to inhibit contractility in human myometrial cells would appear to be due to events downstream of simple cAMP generation.

Keywords: relaxin; human myometrium; INSL3; uterine quiescence; menstrual cycle

Introduction

The heterodimeric peptide hormone relaxin has long been recognized as a key factor in regulating uterine function during pregnancy (Sherwood, 1994; Ivell and Einspanier, 2002). Based on several animal models, relaxin has been shown to assist in the reorganization of connective tissue in the uterus and cervix to accommodate the growing fetus and birth (Bryant-Greenwood and Schwabe, 1994). Relaxin also contributes to the growth of the endometrium in the process of decidualization, and to its vascularization, probably through the local induction by relaxin of VEGF (Unemori et al., 1999; Palejwala et al., 2002). Finally, relaxin has been shown in rats and pigs to induce myometrial quiescence during pregnancy, inhibiting both oxytocin-induced and spontaneous contractions (Downing and Hollingsworth, 1993). In addition, relaxin has been shown to have marked cardiovascular properties in non-reproductive tissues causing local neoangiogenesis and vasodilation, probably by the release of NO, and has markedly ameliorative effects on fibrotic tissue (Nistri et al., 2007; Samuel et al., 2007). All these properties have been linked to the recent evolution of this hormone in mammals as a ‘neohormone’ to address specifically mammalian functions (Ivell and Bathgate, 2006).

One of the more problematic aspects of relaxin physiology is that while many effects can be easily shown in rodents, pigs or other species, responsiveness in human tissues is sometimes difficult to demonstrate. This in turn suggests that relaxin may not be of great value for pharmaceutical development, thus hindering further research. Part of this situation has been exacerbated by a misunderstanding of circulating relaxin concentrations. Whereas in many mammals, such as pigs or rodents, relaxin is a major circulating hormone of the third trimester of pregnancy with peripheral concentrations in the region of 100 ng/ml, in humans peripheral relaxin peaks in the first trimester, including the time of implantation, and has maximal circulating concentrations of \( \leq 1 \text{ ng/ml} \) (~0.16 nM). Although this amount is sufficient to cause modest activation of the specific relaxin receptor, RXFP1 (formerly LGR7; Bathgate et al., 2006), in the human, relaxin is probably best envisaged not as an endocrine factor, but more as a locally acting paracrine factor (Ivell and Einspanier, 2002). Besides the corpus luteum of both the cycle and pregnancy (Bryant-Greenwood and Schwabe, 1994; Sherwood, 1994), in humans and primates relaxin is also made locally within the endometrium and placenta (Bryant-Greenwood and Schwabe, 1994; Ivell and Einspanier, 2002), and thus all uterine tissues can still be subject to effective concentrations of the hormone. We have recently begun assessing the role of relaxin on individual tissue components of the human uterus and have shown a very marked impact on the decidualization process undergone by human endometrial stromal cells in primary culture, primarily by a sustained activation of intracellular cAMP generation, following stimulation of the G-protein coupled relaxin receptor, RXFP1 (Lane et al., 1994; Bartsch et al., 2001, 2004).

In the rat and the pig, relaxin at physiological doses is able to induce complete quiescence of the myometrium both in vivo and in vitro using myometrial strips (MacLennan et al., 1986; Downing and Hollingsworth, 1993). In the conventional paradigm, myometrial strips are subjected to an oxytocin-induced or spontaneous contractile stimulus in a physiological bath (Sanborn et al., 1980; MacLennan...
et al., 1986). For the human cell-line PMI-1-41, it has been suggested that this quiescence is due to a relaxin-induced increase in intracellular cAMP, in turn causing a protein-kinase A (PKA)-dependent recruitment of signalling molecules, essential for contractility, into an inhibitory complex (Dodge et al., 1999). Although relaxin is effective on myometrial smooth muscle cells for rats and pigs, it has not been possible to replicate such quiescent effects for intact human tissues (MacLennan et al., 1986; Peterson et al., 1991; Downing and Hollingsworth, 1993). Nevertheless, it has been shown that relaxin can cause a small increase in cAMP content in intact human myometrial tissue, and in a human-derived myometrial cell-line (Dodge et al., 1999; Kuznetsova et al., 1999), and more recently it has been shown to induce phosphorylation of phospholipase CB3 (Zhong et al., 2008) in human myometrial cells from term uteri. In the present study, we have investigated the action of relaxin on primary cultures of human myometrial smooth muscle cells from the non-pregnant cycle, thereby avoiding the difficulties of interpretation afforded by using an immortalized cell-line, or a complex tissue comprising many different cell types. Furthermore, by using cells from the menstrual cycle, we are focussing on a time point when the induction of uterine quiescence would make sense to encourage proper blastocyst implantation and the establishment of pregnancy. This is a time when circulating levels of relaxin are maximal in women (Bryant-Greenwood and Schwabe, 1994; Sherwood, 1994).

Materials and Methods

Cell culture and drug treatment

Human uterine tissue from anonymized biopsy samples were obtained from pre-menopausal and cycling women (35–50 years old) undergoing hysterectomy for benign gynaecological disorders at the Women’s and Children’s Hospital, South Australia. All patients samples were collected in accord with the Helsinki declaration and authorization of the local Ethics Committee. Primary human myometrial smooth muscle cells were prepared as described by Fahrenstich et al. (2003). Briefly, the tissue samples were collected, transported and stored for a maximum of 12 h until further processing at 4 °C in DMEM without phenol red (Invitrogen, Mount Waverley, Victoria, Australia); Ham’s F-12 (Sigma–Aldrich, Castle Hill, Australia) (1:1), and 100 IU/ml Penicillin and 100 µg/ml Streptomycin (Gibco cell culture, Invitrogen). Myometrial tissue was separated, cut into small pieces (5 mm³) and left in a Petri dish for 13 h at 37 C/5% CO₂ for digestion in fresh medium, now also including 128.4 U/ml Collagenase Type II (Worthington, Lakewood, NJ, USA) and 100 U/ml deoxyribonuclease type I (Sigma–Aldrich). The digested suspension was diluted four times with myometrial culture medium (MCM) [DMEM without phenol red; Ham’s F-12 (1:1), 2 mM L-glutamine (Invitrogen), 1% antibiotic-antimycotic fluid (Gibco cell culture, Invitrogen), and 10% FCS (Invitrogen)]. After removing the larger pieces by unit sedimentation, the supernatant was centrifuged at 1200 rpm (270 g) for 3 min to yield a pellet of myometrial cells that was resuspended and cultured at 37 C/5% CO₂ in MCM. Only cells from the second passage were used for experiments.

The human monocyte cell-line THP1 cells and the primary human endometrial stromal cells prepared from the hysterectomy samples were cultured exactly as described by us recently in Anand-Ivell et al. (2007). HEK293T cells expressing human RXFP2 were a kind gift from Dr Aaron Hsueh, Stanford University, CA. Cell cultures were maintained as described earlier (Anand-Ivell et al., 2007) in 1:1 DMEM:Ham’s F-12 supplemented with 2 mM L-glutamine, 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen).

Depending on the experimental paradigm, the cells were seeded overnight: either 50 000 cells in a 48-well plate (to check the cAMP response) or 750 000 cells in a six-well plate in MCM (to prepare total RNA). After changing the initial medium, whenever IBMX (Sigma, Australia) or rolipram (Calbiochem, Kilsyth, Australia) were used, the cells were pre-incubated for 20 min before stimulation with 100 ng/ml relaxin (>85% pure porcine relaxin; courtesy Dr O.D. Sherwood, Urbana-Champaign, IL, USA), 0.05–100 nM INSIL3 (courtesy Drs C. Schwabe and E. Bullesbach, Charleston, SC, USA), 100 ng/ml insulin (Invitrogen), 10 ng/ml IGF1 (Roche Diagnostics, Castle Hill, Australia), 50 ng/ml hCG or 100 nM isoproterenol (IPT; Calbiochem). Where indicated, the cells were additionally pre-incubated for 20 min with phosphodiesterase (PDE) inhibitors (1 mM IBMX or 100 µM rolipram) followed by another 30 min incubation with either of the following inhibitors: 10 µM H89, 50 nM Jak inhibitor, 10 µM PFP2, 10 µM AG879, 100 µM AG527, 50 µM LY294002, 5 µM forskolin (all from calbiochem) or 10 µM U0126 (Cell Signaling Technology Inc., Denver, MA, USA) prior to stimulation by relaxin or other effectors. Appropriate controls were run simultaneously using the respective vehicle (cell culture medium or DMSO; when used the latter never exceeded 0.5%).

cAMP determination

Total cAMP-content (cells plus media) was measured by time-resolved fluorescent immunoassay (TRFIA) using a Victor3™ 1420 multilabel counter (Perkin Elmer, Fremont, CA, USA) following the procedure described previously (Anand-Ivell et al., 2007). The cross-reactivity of the CAMP-specific TRFIA with a range of structurally analogous compounds like cGMP was <0.003%.

RT–PCR analysis

Total RNA was prepared from all tissues and cells using the Trizol reagent (Invitrogen). DNA contamination was avoided by treating the total RNA with Turbo DNase (Ambion, Applied Biosystems, Scoresby, Australia) and the integrity of the rRNA was checked by agarose gel electrophoresis. A 2:1 ratio of sharp and clear ethidium bromide stained 28S/18S rRNA bands was observed for all samples. Total RNA was subjected to single-stranded cDNA preparation using Superscript II reverse transcriptase (Invitrogen) primed from oligo(dT), exactly according to the manufacturer’s instructions. Semi-quantitative RT–PCR was carried out to characterize both RXFP1 and RXFP2 transcripts and possible alternatively spliced variants using different sets of oligonucleotide primers as listed in Table I. These were devised using Primer3 software from the cDNA sequences available in GenBank (NM_021634, NM_130806), or had been previously used by Muda et al. (2005) as indicated. The positions of the different primers and their products are shown in Figs 4 and 5. As controls for RNA equivalence, RT–PCR was also carried out for the constitutively expressed transcripts either for ribosomal protein S27a (Anand-Ivell et al., 2006) or the housekeeping enzyme GAPDH, with care being taken to use a moderately low cycle number so as to maintain amplification within the linear phase. All PCRs were repeated at least three times with cells prepared from different individuals or different batches (for HEK293T expressing RXFP2); typical results are illustrated. All PCR products were subjected to DNA sequencing to confirm identity. Each reaction mixture of 20 µl contained 1 µl of the cDNA, 1 µM of each primer and 1 x of premixed TaqTM (TaKaRa ExTaqTM; Takara Bio, Japan).

The following cycling conditions were used to run the PCRs with an initial denaturation step at 95 °C for 3 min and final elongation step at 72 °C for 10 min followed by cooling at 10 °C;

S27a (79/80): 19 cycles of denaturation at 95 °C, annealing at 64 °C and elongation at 72 °C, all of 30 s duration; GAPDH (150/151): 19 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min; touch-down protocols for RXFP1 primer pairs 180/197, 191/197, 180/176, 191/176, 180/178, 191/177, 191/178: denaturation at 95 °C for 1 min, annealing 54–50 °C (decrease 2°C per two cycles each) and 48°C (40 cycles) for 1 min, elongation at 72°C for 2 min; RXFP1 primer pairs 180/177, 192/193: denaturation at 95 °C for 1 min, annealing 56–50°C (decrease 2°C per two cycles each) and 48°C (40 cycles) for 1 min, elongation at 72°C for 2 min; RXFP2 primer pair 194/195: denaturation at 95°C for 1 min, annealing 54–48°C (decrease 2°C per two cycles each) and 46°C (40 cycles) for 1 min, elongation at 72°C for 2 min; RXFP2 primer pairs 235/236, 237/238 and 239/240: denaturation at 95°C for 30 s, annealing from 67 to 63 °C (one cycle each at 1°C intervals) and 62°C (40 cycles) for 45 s, elongation at 72°C for 1 min; and RXFP2 primer pair Muda3/R17: denaturation at 95°C for 1 min, annealing 65–57°C (decrease 2°C per two cycles each) and 55°C (35 cycles) for 1 min, elongation at 72°C for 2 min. When using the primers for RXFP2 (Muda 1–4) recommended by Muda et al. (2005), a nested PCR approach was adopted exactly as described by these authors. All PCR products were analysed by gel electrophoresis on 1.5% agarose gels with 1% ethidium
Table 1. List of oligonucleotides used to prime PCRs.

<table>
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<tr>
<th>Transcript</th>
<th>No.</th>
<th>Orientation</th>
<th>Sequence (5’–3’)</th>
<th>Location</th>
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<td>176</td>
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<td>gcctaaataaacttagtggccatc</td>
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<td>Forward</td>
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<td>NM_002954 (128–153)</td>
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<td>Reverse</td>
<td>cgtctgacaccacctctgcc</td>
<td>NM_002954 (894–876)</td>
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</table>

*The sequence of the primer Muda4 was based on the originally submitted sequence (AF403384) of Muda et al. (2005) and differs from the Unigene sequence NM_130806 (equivalent location: 1045–1029) by a single nucleotide.

bromide (Sigma), followed by fluorescence digitization using a Bio-Rad GelDoc XR system (Bio-Rad, Gladesville, Australia).

Reproducibility and statistics

All experiments were repeated at least three times. In general, typical experiments are shown, indicating absolute levels of cAMP production, with error bars denoting within-experiment variation. Where indicated in the text, replicate experiments are together collated as fold increase over basal, with standard deviation then representing between-experiment variation. Where probability (significance) values are reported, these always refer to such between-experiment variation. Significance was tested by one-way ANOVA followed by a post hoc Neuman–Keuls assessment.

Results

Relaxin induces cAMP in primary cultures of human myometrial cells

In the absence of any other effector, relaxin is able to induce a modest increase in intracellular cAMP (Fig. 1; 1.57 ± 0.75-fold over basal, n = 14 independent experiments). Relaxin-stimulated cAMP production is relatively low (0.6 ± 0.8 pmol/100 000 cells/20 min, n = 14) compared with primary endometrial stromal cells (1–2 pmol/100 000 cells/20 min; unpublished) or the monocye cell-line THP1 (~12 pmol/100 000 cells/20 min; Bartsch et al., 2001; Anand-Ivell et al., 2007), probably reflecting the known high levels of PDEs in human myometrial cells (Mehats et al., 1999; Bartsch et al., 2004). The general PDE inhibitor, IBMX, was therefore added in most subsequent experiments, increasing both the fold-stimulation by relaxin (2.06 ± 1.91-fold over basal, n = 19 independent experiments), as well as the net relaxin-stimulated cAMP production (4.0 ± 4.2 pmol/100 000 cells/20 min, n = 19). In some experiments, the more specific PDE4 inhibitor, rolipram, was used (Fig. 1), and the results show that this class of PDE is a major component of the resident enzymatic activity.

It should be noted that in these primary myometrial cell cultures, the β-adrenergic agonist, isoproterenol, had only a very marginal effect (Fig. 1A), and hCG was completely without effect.

Addition of the adenylyl cyclase allosteric activator, forskolin, also caused a marked increase in the effect of relaxin (Figs 1 and 2), whereas the inhibitor of PKA, H89, had only a limited effect, unless IBMX was present (not shown), where the effect was more marked. The specific tyrosine kinase inhibitors, AG527 and AG879, which we had shown previously to have a dramatic inhibitory effect on cAMP generation in human endometrial stromal or THP1 cells (Bartsch et al., 2001; Anand-Ivell et al., 2007) appeared to have only a marginal effect on primary myometrial cells (not shown), unless cAMP generation was augmented by the addition of IBMX (Fig. 2A and B) and/or forskolin (not shown). It should be noted here that whereas AG527 was invariably and significantly inhibitory, this was not always the case for AG879, where in half of the cultures tested, using different batches of cells, no effect of this tyrophostin was detected (negative results not shown). Addition of the specific PI3-kinase inhibitor LY294002, whether in the presence or absence of IBMX, had no significant effect, at least in the experiments where cAMP production was modest (Fig. 2C). On the other hand, addition of this compound together with forskolin indeed demonstrated a specific inhibitory action on relaxin-induced cAMP generation (Fig. 2D and E). There were some marked variations to this response dependent upon the batches of primary cells being used and their capacity to generate cAMP. Although for most batches forskolin had little effect on the basal expression of cAMP (e.g. Fig. 2D), in some batches there is a marked stimulation by this factor alone as well as in the added presence of relaxin (Fig. 2E). Altogether, in five experiments using independent batches of primary myometrial cells, simply testing the effect of forskolin alone on the basal cAMP production, three batches indicated <50% increase due to forskolin, whereas two batches showed a greater than 10-fold increase over basal. Interestingly, LY294002 was also able to inhibit the increase...
in cAMP attributable to forskolin action alone (Fig. 2E), suggesting that PI3-kinase involvement was not dependent upon the activation of RXFP1, but rather was independent of this and related only to the activity levels of adenylyl cyclase. Of other effectors tested, neither insulin nor IGF1 showed any significant influence on the relaxin-induced induction of cAMP (not shown). Nor did the pharmacological inhibitors of MAP-kinase (U0126), p38-Src (PP2) or Janus kinase (Jak-i) (data not shown).

Because relaxin action has been described in the monocyte cell-line, THP1, as involving at least two temporal phases of signalling, albeit with high variability, the time-course for the response of primary human myometrial cells to relaxin and different effectors was also assessed for independent batches of primary myometrial cells in the presence and absence of enhancers, like IBMX or forskolin (Fig. 3A and B). In the absence of either IBMX or forskolin (Fig. 3A), the production of relaxin follows a type A curve (according to the description of Nguyen et al., 2003), with an immediate response, peaking at 5 min followed by a lower but sustained cAMP production. In the illustrated experiment, the PI3-kinase inhibitor, LY294002, is able to inhibit both early and late phases of cAMP production. In the presence of IBMX (Fig. 3A) or forskolin (Fig. 3B), this first phase is variable and may be masked by the high level of adenylyl cyclase activity, or is simply absent, with the sustained cAMP production (Phase 2) being highest at ca. 20 min. Again LY294002 appears to inhibit both phases of cAMP production (Fig. 3B). It should be noted that this experiment represents the situation where maximum relaxin expression in the absence of other effectors is greater than 2-fold over background, with modest to high absolute levels of cAMP production, and thus agrees well with the other experimental data.

Expression of the relaxin receptors, RXFP1 and RXFP2, in human myometrial cells

Relaxin has been shown to be able to activate several different G-protein coupled receptors (Bathgate et al., 2006). Whereas the receptors GPCR135 and GPCR142 are restricted to the brain (Bathgate et al., 2006), the uterus in most species appears to express RXFP1, as well as RXFP2 which is the receptor for the closely related hormone INSL3. In humans, besides its conventional ligand, INSL3, RXFP2 is also known to respond to relaxin at higher concentrations (Bathgate et al., 2006). In order to determine the mechanism by which relaxin is probably acting in human myometrial cells, RNA from purified primary cells was examined for the expression of both RXFP1 and RXFP2 expressing transcripts. Because RXFP1 has been shown to be expressed in numerous splice variant forms, some at least of which are known to be non-functional, and possibly even antagonistic (Muda et al., 2005; Scott et al., 2005), it was important to verify that the RXFP1 transcripts identified in myometrial cells were indeed those encoding the full-length functional receptor. Exhaustive RT–PCR analysis using a large combination of oligonucleotide primers (Fig. 4) consistently showed a single product corresponding to the full-length RXFP1 receptor. Only with some combinations was a faint alternative band detected (Fig. 4, black arrowhead). Sequencing of this alternative PCR product showed that smaller amounts of a splice variant in which exon 2 is missing were also expressed. This variant has not been previously described, though a single EST (expressed sequence tag) corresponding to this transcript is reported from the hippocampus (accession number DC335325). Importantly, repeated sequencing of this PCR product confirm the observation from the EST DC335325 that there is a single G nucleotide insertion at the exon 1–exon 3 splice site, which restores the correct reading frame in exon 3.

Similar analysis for RXFP2 transcripts showed that this receptor was also represented at the RNA level (Fig. 5). Sequencing of all RT–PCR products obtained revealed that almost exclusively only the full-length variant was represented. A small minor band was detected, which corresponded to the variant L8.1 described by Muda et al. (2005) in which exon 11 is missing. All other products visualized represented false priming products of quite different cDNA.
The observation that full-length transcripts for both RXFP1 and RXFP2 were expressed in these cells begged the question whether human myometrial cells are also responsive to INSL3, which has been suggested to be a product of the placenta in some mammals (Hombach-Klonisch et al., 2001). At no concentration did human INSL3 have any effect on cAMP production in the primary myometrial cell cultures (Fig. 6A), either in the presence or absence of IBMX. Since INSL3 has also been suggested to activate Gi systems, as well as Gs (Bathgate et al., 2006), possible antagonism of relaxin-stimulated cAMP production was also tested (Fig. 6B), but without effect. The functionality of this batch of peptide had been tested using an RXFP2-expressing HEK-293T cell-line (Fig. 6C).

**Discussion**

Repeated studies using the classical muscle strip contractility assay show that the human myometrium appears to be relatively insensitive to relaxin as a quiescent agent, in contrast to the myometrium of other mammals.
species (MacLennan et al., 1986; Downing and Hollingsworth, 1993).

It is believed that relaxin in these species is serving both to maintain quiescence at around the time of implantation, as well as to inhibit the increasing potential for spontaneous and oxytocin-induced contractility in the preterm uterus. It is to be noted that those species where this latter effect can be most easily demonstrated are those with high circulating endocrine levels of relaxin in the third trimester.

In rats and pigs, where this effect has been best studied, relaxin acts to increase intracellular cAMP, thereby in a PKA-dependent fashion recruiting PI3-kinase to an AKAP complex, and thus preventing its availability to transduce signals from an activated oxytocin receptor (Dodge et al., 1999). In this way, relaxin and oxytocin form a yin-yang couple, with the latter only achieving labour-type contractility when its signalling capacity greatly overrides the relaxin effect.

Figure 3: Time course for the expression of cAMP in primary cultures of human myometrial cells stimulated by relaxin (100 ng/ml) in the presence (solid lines; illustrating three independent experiments A, B and C) or absence (broken lines) of IBMX (1 mM; A) or forskolin (5 μM; B). Symbols: (A) open squares, 50 μM LY294002 (LY) only; inverted triangles, 50 μM LY294002 plus relaxin (RLX); diamonds, relaxin only; filled triangles, squares and circles represent different batches (expts A–C) of primary cells stimulated with relaxin in the presence of IBMX. (B) inverted triangles, 50 μM LY294002 plus forskolin (F); squares, 50 μM LY294002 plus forskolin plus relaxin; diamonds, forskolin only; triangles, forskolin plus relaxin.

Figure 4: Semi-quantitative RT–PCR analysis for different regions of the specific transcripts of the human RXFP1 gene. The relative positions of the oligonucleotide primers used are indicated in the panel below illustrating the exonic structure of the gene, and their sequences are listed in Table I. Abbreviations: D, 1 kb (upper panels) or 100 bp (lower panels) DNA ladder; C, negative control; E, human endometrial stromal cells from the non-pregnant cycle; M, primary human myometrial cells; T, THP1 human monocyte cell-line. The black arrowhead indicates the sequenced splice variant missing exon 2.
This balance is also affected by steroid levels, expression of tight junctions, and by other effectors able to influence the balance of second messengers in the myometrial cells.

In the present study, we have shown for the first time that relaxin is indeed able to stimulate an increase in intracellular cAMP in purified primary human myometrial cells from the non-pregnant uterus, which is presumed to be similar to the uterus at the time of fertilization and implantation. This is approximately equivalent to the situation in the first trimester, when it is maximally expressed as a circulating hormone in the human (Bryant-Greenwood and Schwabe, 1994), though local expression in the uterus during decidualization may be higher (Ivell and Einspanier, 2002). This paracrine expression and action of relaxin may explain the ability of embryos to implant in women lacking functional ovaries and only substituted with steroids (Tarlatzis and Pados, 2000). Relaxin is probably acting in the myometrium through the RXFP1 receptor (Ivell et al., 2003). The failure of relaxin to induce quiescence in the human would appear therefore to be due to an uncoupling of PKA from the oxytocin signalling pathway, although the recent work of Zhong et al. (2008) suggests that indeed relaxin at least in the term human myometrium is able to influence part of this pathway in a PKA-dependent fashion. It is interesting to note that the β-adrenergic agonist, isoproterenol, was much less effective than relaxin in generating cAMP in our smooth muscle cultures (Fig. 1).
been used to induce uterine quiescence in a clinical setting (Berg et al., 1985).

The second important set of findings to result from this study relate to the characterization of the relaxin-signalling pathway itself. We have shown earlier that human endometrial stromal cells, and the human monocyte cell-line THP1, that naturally expressed RXFP1 receptors, appear to make use of other intracellular components in addition to the common Gs-coupling to adenylyl cyclase seen in transfected cell systems. First, relaxin-dependent cAMP generation appears to be entirely dependent on a tyrosine kinase activity which can be inhibited by a select group of specific tyrophostins (Bartsch et al., 2001; Anand-Ivell et al., 2007). A similar finding was observed here also for human myometrial cells, although the inhibitory impact appears not to be as great as in the other cell types. Secondly, it has been shown for THP1 cells that part of the cAMP signal resulting from relaxin stimulation is contributed through a PI3-kinase-dependent pathway, probably activated by Gβγ and involving PKCζ to allosterically activate adenylyl cyclase (Nguyen and Dessauer, 2005). This pathway can be blocked by application of the inhibitor LY294002 (Nguyen and Dessauer, 2005). We have shown for THP1 cells that this pathway is not always used particularly where cAMP levels are not artificially stimulated by other effectors (Anand-Ivell et al., 2007). In myometrial cells, LY294002 generally has no effect, whether or not a cAMP augmenting agent such as IBMX is applied, showing that under these conditions, the PI3-kinase-dependent pathway is not part of the relaxin signalling system. This is logical if one recalls that PI3-kinase is an essential component of the contractile signalling pathway. It is unlikely to be involved directly in the up-regulation of cAMP, though recently an alternative hypothesis has been put forward (Pertseva et al., 2006). Nevertheless, and very interesting, was the observation that when forskolin was used to increase the accumulation of relaxin-induced cAMP, then indeed LY204002 was effective (Fig. 2D and E). Forskolin acts as an allosteric agent directly on the adenylyl cyclase enzyme. It is believed that PI3-kinase, acting through the intermediary PKCζ, also induces a direct phosphorylation of adenylyl cyclase (Nguyen and Dessauer, 2005). It is tempting to speculate that for human myometrial cells LY294002 is in some way directly antagonistic to this specific action of forskolin on adenylyl cyclase. This is reinforced in those experiments where the particular batch of primary cells was shown to exhibit high basal forskolin-dependent production of cAMP, when no relaxin stimulation was involved (Fig. 2E). It should also be noted that the effect of LY294002 is in no way influenced by the addition of the PKA inhibitor, H89, in these cell cultures (data not shown), suggesting that the involvement of PI3-kinase, and possibly PKCζ, is not dependent on PKA action, but is independent of this.

It has been postulated for transfected cell systems that relaxin signalling may involve a two-phase process with temporally differential association of different G-proteins (Nguyen et al., 2003; Halls et al., 2006). Although there is no consistency in the time-course patterns of relaxin-dependent cAMP generation (Fig. 3), there appears to be an earlier acute phase (<10 min) and a later sustained phase (>10 min). However, both appear to be inhibited by LY294002, thus not supporting the notion of a temporal shift from a more Gi-coupled to a Gs-coupled system, at least in these primary myometrial cell cultures. Part of the difficulty in interpretation here is due to the considerable variability in relaxin responsiveness in these primary cell cultures. This may be due to a combination of low RXFP1 expression, together with a variability in the expression and localization of other components involved, for example, the adenylyl cyclase isoforms (Yuan and Lopez-Bernal, 2007).

Finally, RT–PCR analysis indicated that besides full-length RXFP1 receptor transcripts, also transcripts for an alternatively spliced version of this receptor were expressed, wherein the sequence for exon 2 is missing, which encodes part of the signal peptide and the first half of the LDLα region. It is possible that such splice variants, if expressed at the protein level, might act as modulators of receptor function (Scott et al., 2005), particularly if it can be shown that these participate in natural dimerization of the receptors at the cell surface. However, it is more likely that such aberrant transcripts are quickly degraded, since they lack a complete plasma membrane targeting signal. In over-expressing artificial systems, such variant receptors have been shown to be incapable of signalling, and might thus act as dominant negative elements if heterodimers can be formed. There is no information, however, whether such events occur or are relevant for naturally expressed receptors, where the numbers of receptors expressed per cell are orders of magnitude lower. Although transcripts for the full-length RXFP2 receptors were also detected, and for the human these are known to be able to respond to relaxin (Bathgate et al., 2006), as well as to their natural ligand INSL3, tests using pure human INSL3 showed that this system is not functional in human myometrial cells, and that INSL3, possibly of placental (Hombach-Klonisch et al., 2001) or ovarian (Bamberger et al., 1999) origin, does not contribute either agonistically or antagonistically to cAMP metabolism in these cells.

In conclusion, we have demonstrated that pure human primary myometrial cells from the non-pregnant uterus do indeed respond to relaxin by elevating intracellular cAMP, using similar intracellular signalling systems as in other human cells naturally expressing the relaxin receptor RXFP1. The failure of the human myometrium to respond to relaxin by suppression of spontaneous or oxytocin-induced contractility must therefore be due to the absence of the appropriate intracellular coupling system described for those species with high circulating relaxin in the third trimester.

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**Authors Role**

Ms Kee Heng carried out most of the experimental work and contributed to the writing of the final version. Prof Richard Ivell was largely responsible for the conception and design of the study, and assisted in the writing of the manuscript. Dr Prabhath Waagarachchi was responsible for all clinical aspects of this study. Dr Ravinder Anand-Ivell was Ms Heng’s main supervisor, carried out significant parts of the experimental work, was jointly responsible for the conception and design of the study, and significantly contributed to conceiving and writing the final manuscript.

**References**


Dodge KL, Carr DW, Sanborn BM. Protein kinase A anchoring to the myometrial plasma membrane is required for cyclic adenosine 3′,5′-monophosphate regulation of phosphatidylinositol turnover. Endocrinology 1999;140:5165–5170.


Ivell R, Bathgate RAD. Hypothesis: Neohormone systems as exciting targets for drug development. Trends Endocrinol Metab 2006;17:123.


Sanborn BM, Kuo HS, Weisbrod NW, Sherwood OD. The interaction of relaxin with the rat uterus. I. Effect on cyclic nucleotide levels and spontaneous contractile activity. Endocrinology 1980;106:1210–1215.

Scott DJ, Tregear GW, Bathgate RA. LGR7 truncate is a splice variant of the relaxin receptor LGR7 and is a relaxin antagonist in vitro. Ann NY Acad Sci 2005;1041:22–26.


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