RHO protein regulation of contraction in the human uterus

J Lartey and A López Bernal

Medical School, Institute of Cellular Medicine, University of Newcastle, 3rd Floor, William Leech Building, Framlington Place, Newcastle upon Tyne NE2 4HH, UK and 1Division of Obstetrics and Gynaecology, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Clinical Sciences South Bristol, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, UK

Correspondence should be addressed to J Lartey; Email: jon.lartey@ncl.ac.uk

Abstract

The state of contraction in smooth muscle cells of the human uterus is dependent on the interaction of activated forms of actin and myosin. Ras homology (RHO) proteins are small monomeric GTP-binding proteins that regulate actin polymerisation and myosin phosphorylation in smooth muscle cells. Their action is determined by their level of expression, GTP-bound state, intracellular localisation and phosphorylated status. Agonist activated RHO proteins bind to effector kinases such as RHO kinase (ROCK) and diaphanous proteins (DIAPH) to regulate smooth muscle contraction by two mechanisms: ROCK activates smooth muscle myosin either by direct phosphorylation at Ser19/Thr18 or through inhibition of myosin phosphatase which is a trimeric protein regulated by ROCK and by other protein kinases. Actin-polymerising proteins such as DIAPH homolog 1 increase filamentous actin assembly to enhance acto-myosin cross bridge formation and contraction. This review explores recent advances in RHO protein signalling in human myometrium and proposes areas of further research to investigate the involvement of these proteins in the regulation of uterine contractility in pregnancy and labour.

Introduction

The purpose of this paper is to provide an overview of the role of small GTP-binding proteins in myometrial function. The past decade has seen a rapid expansion in our knowledge of Ras homology (RHO)-related proteins in many areas of biology including angiogenesis, vascular contractility, cytoskeletal dynamics, endosomal trafficking, neuronal growth, stem cell proliferation, chemotaxis and inflammation, and there are excellent reviews covering these areas (Bishop & Hall 2000, Somlyo & Somlyo 2000, Heasman & Ridley 2008). Moreover, RHO GTPases are promising cellular targets for new anticancer drugs and other therapeutic agents for the management of asthma (Kume 2008), hypertension (Oka et al. 2008, Ponnuchamy & Khalil 2009), and CNS disorders, from stroke to Alzheimer’s disease (Kubo & Yamashita 2007, Shin et al. 2008).

The fundamental role of RHO proteins in multiple cell signalling pathways is well established and this is an area of rewarding research opportunities; however, it remains relatively unexplored in reproductive tissues. In this review, we have focused on RHO proteins that have been described in uterine tissues, but in some instances the experimental data are scarce and their possible role can only be described by analogy with evidence obtained in other systems. Our aim is to explain the relevance of RHO proteins in the control of myometrial smooth muscle contractility, especially in relation to the onset of labour. Although this approach can only provide a narrow perspective in the ever expanding field of small GTPases, we believe it is a framework of current understanding and a stimulus for research on this multifunctional family of proteins for scientists and clinicians interested in reproduction.

Premature birth is the most important cause of perinatal death and long term morbidity in the world today. Although an increasing proportion of premature births are induced secondary to pre-existing fetal and maternal conditions, over half of these deliveries are due to the spontaneous onset of uterine contractions (spontaneous preterm labour). So far, our ability to successfully treat women whose pregnancies are complicated by preterm labour is hampered by a lack of knowledge of the endocrine and biochemical factors that initiate human parturition.

The human uterus is a smooth muscle organ which undergoes considerable distension during gestation without expelling its contents. It is not clear what regulates this state of relative quiescence.
During labour, it contracts in a regular and coordinated fashion to forcibly expel the fetus. Contraction in smooth muscle tissues is regulated by two key enzymes: calcium–calmodulin-dependent myosin light chain kinase (MYLK) which phosphorylates the regulatory 20 kDa myosin light chain (MYL) to generate increases in tension and contraction, and a trimeric protein phosphatase called myosin phosphatase (MLCP) which induces a state of relaxation through dephosphorylation of activated MYL (Somlyo & Somlyo 1994, Word 1995). Therefore, the force of contraction and MYL phosphorylation in the human uterus during labour is determined by the equilibrium between MYLK and MLCP. The mechanisms that regulate these two enzymes in the human uterus remain poorly understood.

The RHO family

RHO proteins are small monomeric GTP-binding proteins that regulate acto-myosin interactions in smooth muscle cells. Their function is primarily determined by their level of expression, state of phosphorylation, subcellular localisation and binding of GTP. At a constant level of intracellular calcium ([Ca^{2+}]_i), activated GTP-bound RHOA binds to RHO kinase (ROCK) which inhibits the regulatory binding subunit of myosin phosphatase and prevents dephosphorylation of MYL. The increase in MYL phosphorylation and contraction at a constant [Ca^{2+}]_i is termed ‘Ca^{2+} sensitisation’ (Somlyo & Somlyo 1994). The purpose of this review is to examine the mechanisms that determine RHO protein function and outline how these proteins regulate uterine contractility during pregnancy and in labour.

RHO GTPs belong to the RAS super family of GTP binding proteins. They function as molecular switches which regulate a wide variety of cellular functions ranging from membrane trafficking, gene transcription, cell growth, actin polymerisation, stress fiber formation and smooth muscle contraction.

The mammalian RHO family is made-up of at least ten different proteins namely RHO gene family member A (RHOA); RHO gene family member B (RHOB); RHO gene family member C (RHOC); RHO family GTPase 1–3 (RND1–3); RAS-related protein RAC1–2; cell division cycle 42 GTP-binding protein (CDC42); RHO gene family member D (RHOD); RHO gene family member F in filopodia (RIF) also known as RHOF; RHO gene family member Q (RHOQ) also known as TC10 and ADP-ribosylation factors (ARF1 and ARF6). Human RHOA, RHOB, RHOC and RHOD genes are located on chromosomes 3p21.3, 2p24, 1p13.1 and 11q14.3 respectively. RAC1, CDC42 and their close RHO family relation RHOG are found at 1p22, 1p36.1 and 11p15.5–p15.4 respectively. A dendritic tree representation of the human family of RHO GTP binding proteins is outlined in Fig. 1.

The Structure and regulation of RHO proteins

These small monomeric GTP-binding proteins usually have between 190 and 250 residues and measure ~20–30 kDa in size. All RHO proteins have conserved residues at Gly14, Thr19, Phe30 and Gln63 which are required for GTP binding and hydrolysis. Their core structure includes an effector domain; four separate nucleotide binding regions which span the length of the core structure; a hypervariable region and a CAAX box motif, see Fig. 2. The effector domain changes conformation between the GTP- and GDP-bound states.

RHO proteins have intrinsically low levels of GTPase activity and cycle between an active GTP-bound state and an inactive GDP-bound state. RHO activity is determined by three classes of regulators: guanine nucleotide exchange factors (ARHGEFs); GTPase activating proteins (ARHGAPs) and guanine nucleotide dissociation inhibitors (ARHGDIAs). ARHGEFs activate the exchange of GTP for GDP (Van Aelst & D’Souza-Schorey 1997). ARHGDIAs reduce the levels of active GTP-bound RHO by accelerating the rate of GTP hydrolysis to yield GDP (Moon & Zheng 2003). ARHGDIAs extract prenylated RHO proteins from membranes and sequester them in the cytosol by shielding their prenyl groups (Michaelson et al. 2001). The effect of these regulatory proteins on the GTPase cycle is outlined in Fig. 3.

The complex interaction between these regulatory proteins imply that ARHGDI levels relative to ARHGAP and ARHGEF levels may determine RHO activity in uterine smooth muscle tissue during gestation. To this end, we used immunoblotting to quantify myometrial...
by stimulating the activity of RAC–GEF to increase RAC-dependent cell migration (Katoh et al. 2006). RHOB and RHOD are RHO family members which regulate receptor trafficking by controlling the motility of early endosomes (Murphy et al. 1996, Gampel et al. 1999). Expression of activated RHOD mutants attenuates lysophosphatidic acid (LPA)-induced stress fiber formation in 3T3 fibroblasts and baby hamster kidney cells (Murphy et al. 1996, Matsumoto et al. 1997). This appears to be regulated through the inhibition of RHOA; however, the exact mechanism is not known.

**RND proteins**

RND proteins are a subfamily of RAS small GTP-binding proteins made-up of three members RND1, formerly known as RHOD; RND2, also known as RHON or RHO7; and RND3, previously known as RH08. The name RND ‘round’ is due to their inhibition of RHOA stress fiber formation and induction of ‘rounded’ cell phenotype in Swiss 3T3 fibroblasts. The RND1 gene located in chromosome 12q12–q13, was first identified by screening a human fetal cDNA library with bovine RND1 as bait (Nobes et al. 1998). The human RND2 gene is located on chromosome 17q21 and it encodes a protein with 227 amino acids.

RND3 was first identified by Foster et al. (1996) by using rat ARHGAP5 as bait in a yeast two hybrid assay. Other investigators subsequently described an RND3 protein which had 15 extra residues at its N-terminal end compared to the protein described by Foster et al. (Nobes et al. 1998). Human RND3 is located on chromosome 2q23.3. RND proteins have three guanine-binding motifs, two loops and three major residues that coordinate magnesium binding in the GTP state. They have an effector region in common with other RHO family members but they possess key structural differences within the catalytic domain which distinguish them from other RHO proteins. Unlike RHO proteins, RND proteins almost have no GTPase activity and exist in a constitutively active GTP-bound state (Foster et al. 1996, Nobes et al. 1998, Wennerberg et al. 2003). RND proteins have a CAAX box motif and a terminal methionine which implies that they undergo farnesylation.

Other new GTPase deficient, constitutively active, proteins including GTP-binding protein overexpressed in skeletal muscle (GEM) and Ras-related associated with diabetes (RAD) have now been located on chromosomes 16q22 and 8q13–q21 respectively, Fig. 1.

**Regulation of RND protein activity**

RND protein activity is determined by the levels of protein expression, prenylation, subcellular localisation and state of phosphorylation. RND proteins bind to ROCK1 and inhibit RHOA downstream effects...
possibly by preventing RHOA binding to ROCK1 (Riento et al. 2003). Thus, the relative levels of RHOA and RND3 may determine the overall RHO activity and function.

RND2 is usually cytosolic and preferentially localises to early endosomes where it binds to vacuolar protein sorting 4A (VPS4A) to regulate endosomal trafficking, whereas RND3 also localises to perinuclear structures like the Golgi (Tanaka et al. 2002). After farnesylation, RND proteins are equally distributed between plasma membrane and the cytosol (Riento et al. 2003).

It is thought that RND3 exists in two conformations: a phosphorylated cytosolic-bound form with an extended half-life, which is associated with increased disruption of RHOA actin filaments; and an unphosphorylated form which is found in both cytosolic and membranous compartments (Riento et al. 2003, 2005). ROCK-mediated phosphorylation prolongs the half-life of RND3 and translocates the protein from the membrane to the cytosol where it disrupts RHOA stress fibers (Riento et al. 2005). The resultant RND phosphorylation requires an activated RHOA/ROCK and a protein kinase C (PRKC) pathway (Riento et al. 2005). Platelet-derived growth factor (PDGF) stimulation activates ROCK through a PRKC-dependent mechanism to cause phosphorylation of RND3 at Ser7 and Ser11. RND3 phosphorylation is inhibited by both ROCK and PRKC inhibitors (Riento et al. 2005). PRK-induced
phosphorylation increases RND activity by prolonging the half-life and stability of the phosphorylated protein and in some cases C-terminal phosphorylation causes membrane translocation (Berzet et al. 2005).

**RHO and RND effector interactions**

Structural differences can determine RHO and RND effector binding. RHO proteins have an ‘insert domain’ (residues 126–136) which forms a surface loop near the GTP binding site required for GTPase activity. Deletion of the RHOA insert domain makes the protein unstable (Zong et al. 1999). Sequence differences within the insert loop region between RHOA, RHOB and RHOC enables them to selectively bind to effectors like RHO-associated protein kinases (ROCK1, ROCK2). Protein kinase N1 (PKN1) and the human form protein diaphanous homolog 1 (DIAPH1; Wheeler & Ridley 2004). Other factors that determine effector specificity include: i) conformation changes in switch 1 (residues 27–40) and switch 2 (residues 59–78) domains between GTP and GDP-bound states; and ii) coordination of the interaction between the insert loop and the effector domain (residues 26–45). These lead to precise changes in effector region required for binding to specific downstream targets (Freeman et al. 1996, Aspenstrom et al. 2004).

ROCKs have four functional domains: an N-terminal kinase domain, a central coiled-coil domain which contains a RHO-binding domain (RBD) and a C-terminal pleckstrin homology (PH) domain, Fig. 4. The kinase domain binds to the PH and RBD domains to form an autoinhibitory closed inactive loop. Binding of active GTP-bound RHOA to the RBD leads to autophosphorylation and disruption of the autoinhibitory interaction between the C-terminal PH and RBD domains and the N-terminal kinase domain. The activated kinase adopts an open conformation that enables it to bind downstream targets.

ROCK and RND proteins bind to different regions on ROCK1. RND proteins binding to the N-terminal RND binding domain made-up of the first 420 amino terminal amino acids including the kinase domain while RHO proteins bind to the C-terminal RBD sited at the end of the central coiled-coil domain (Ishizaki et al. 1996). RND proteins optimally bind to RHOA-activated ROCK, suggesting that prior RHOA action is a prerequisite for RND inhibition. The mechanism of RND inhibition of RHOA may be threefold: direct inhibition of the RHOA effector ROCK1 as outlined above, stimulation of ARHGAPs or inhibition of ARHGEFs. Currently, there is no evidence that RND proteins are regulated through sequestration by ARHGDIs.

Activated ROCK binds to a number of downstream targets including myosin phosphatase RHO interacting protein (Surks et al. 2003, 2005), zip kinase also known as death-associated protein kinase 3 (DAPK3; Hagerty et al. 2007), integrin linked kinase (ILK; Kiss et al. 2002, Muranyi et al. 2002) and the PRKC inhibitory phosphoprotein CPI-17 or PPP1R14A (Kitazawa et al. 2000, 2003, Eto et al. 2001) to regulate MYL phosphorylation.

ROCKs regulate actin myofilament assembly by binding to actin nucleators and polymerizers such as DIAPH1, LIM domain kinase 1 and ERM (ezrin, radixin and moesin) proteins. Dysregulation of these actin-binding proteins can result in different disorders. For instance, mutations in DIAPH1 gene have been implicated in non-syndromic deafness (Lynch et al. 1997) and premature ovarian failure (Bione et al. 1998). We have recently characterised DIAPH1, DIAPH2 and DAPK3 expression in the human uterus during pregnancy and noted interesting changes in expression during labour (Lartey et al. 2007, Lartey & Lopez Bernal 2009a). It is not certain how these proteins regulate uterine smooth muscle MYL phosphorylation and contraction and their function needs to be investigated.

The two ROCK isoforms have their own distinct functions in the human placenta and in the developing fetus. ROCK2 deficient (ROCK2 −/−) mice usually die in utero due to placental dysfunction and intrauterine growth restriction caused by thrombus formation in the placenta. ROCK1 −/− mice have a different phenotype and usually survive pregnancy and labour to die post-natally from filamentous actin accumulation leading to impairment in the closure of the umbilical vein (Rikitaite et al. 2005). Analysis of ROCK2 null mice suggests that ROCK1 does not compensate for the loss of ROCK2 function (Thumkeo et al. 2003).
GPCR activation of RHOA/ROCK proteins in smooth muscle cells

GPCR-dependent activation of RHOA is primarily mediated through the Gα12/13 heterotrimeric proteins. The ‘active’ heterotrimeric Gα12/13 protein complex binds to ARHGEFs through a regulator of G-protein signalling (RGS) domain and sets off a downstream cascade by catalysing nucleotide exchange on ARHGEF1 and ARHGEF12. Thus, ARHGEFs with RGS domains function both as GTPase activating proteins and as effectors for Gα12/13 (Suzuki et al. 2009). Ligand-bound GPCR–Gα13–ARHGEF1 complex has a number of downstream actions: i) to stimulate GTP exchange of RHOA, leading to ROCK1 activation; and ii) activation of phospholipase D which cleaves phosphatidylcholine to yield phosphatidic acid and diacylglycerol. The latter stimulates the PRKCB-dependent phosphoprotein PPP1R14A which inhibits the PPP1c catalytic subunit of MLCP. Therefore, GPCR receptor activation results in ROCK and PRKCB-mediated inhibition of MLCP to cause increases in MYL phosphorylation and contraction at a constant [Ca^{2+}]	extsubscript{i}, see Fig. 5.

In addition to inhibitory phosphorylation of the myosin binding subunit, ROCKs directly phosphorylate MYL at Ser19/Thr18 (Amano et al. 1996). There is now evidence that individual pathways can cooperatively enhance MYL phosphorylation.

**RHO and RND protein regulation of uterine contractility**

The force of contraction and MYL phosphorylation in uterine smooth muscle cells depends on the resultant equilibrium between MLCK and MLCP. Agonist activation of the RHOA and subsequently ROCK1 causes inhibitory phosphorylation of the myosin-binding subunit of MLCP (PPP1R12A). This produces a greater level of MYL phosphorylation and tension at a given Ca^{2+}-dependent MLCK activity, resulting in Ca^{2+} sensitisation, see Fig. 5.

**Figure 5** RHO proteins, myosin equilibrium and Ca^{2+} sensitisation. Ligand-bound GPCRs regulated smooth muscle phosphorylation by three main pathways. Activated phospholipase C cleaves phosphatidyl inositol phosphates to yield inositol triphosphate (IP3) and diacylglycerol (DAG). Phospholipase D can also be activated to generate DAG from phosphatidylcholine (Ha et al. 1994, Malcolm et al. 1994, Exton 1997, Gong et al. 1997, Zhou et al. 2003). IP3 promotes intracellular Ca^{2+} release which stimulates Ca^{2+}-dependent calmodulin to activate myosin light chain kinase (MYLK). Phosphorylation of the regulatory myosin light chains (MYL) by MYLK causes contraction. Diacylglycerol (DAG) stimulates the PRKCB-dependent phosphoprotein PPP1R14A which inhibits the PPP1c catalytic subunit of MLCP to prevent dephosphorylation of activated MYL. The effect of PRKC can be mimicked by other protein kinases such as PKN1 (Hamaguchi et al. 2000). ARHGEF activated RHOA binds to ROCK and allows ROCK inhibitory phosphorylation of Thr696 on the myosin binding subunit PPP1R12A of MLCP. This attenuates the phosphatase activity and leads to higher levels of activated MYL. Agonists that increase Ca^{2+}–calmodulin dependent contraction can also increase PRKCB–PPP1R14A and RHOA–ROCK mediated MYL phosphorylation in a [Ca^{2+}]	extsubscript{i}-independent manner to cause further increases in contraction known as Ca^{2+} sensitisation. RND and RHOD proteins inhibit RHOA–ROCK activity. The exact mechanism of RHOD inhibition is not known.
During pregnancy, the myometrial contractile apparatus undergoes several changes that enable it to adapt to the physiological alterations in size, stretch and tension due to the growing fetus. These changes during gestation prime the myometrium for the increase in contractile activity required for normal labour. Although myometrial levels of actin, myosin, calponin and the steady state levels of MYL phosphorylation do not alter during pregnancy, tissue strips from pregnant women generate greater levels of tension at any given level of MYL phosphorylation in comparison with strips from non-pregnant women (Word et al. 1993, Riley et al. 2005). Pregnant myometrium is more sensitive to the effects of calcium and agonists like OXT than non-pregnant myometrium. In the rat, pregnant myometrium develops greater levels of tension than non-pregnant myometrium at a given level of MYL phosphorylation after exposure to a range of stimulants including OXT, prostaglandins (PGE2 and PGF2α), carbachol or high K+ (Kim et al. 1998). The mechanism of the increased response to contractile agonists in pregnancy is not completely understood, but it is likely to involve Ca2+ sensitization and be multi-factorial in origin. Moreover, other contractile proteins like caldesmon are elevated in pregnant compared to non-pregnant rat myometrium and may mediate this heightened contractile activity (Li et al. 2005). However, treating permeabilised rat myometrial strips with Ca2+ and calyculin-A (a phosphatase inhibitor) produced similar increases in force in non-pregnant and pregnant myometrial strips (Kim et al. 1998). This suggests that the increase in force generated in myometrial smooth muscle during pregnancy is due to inhibition of a phosphatase presumably myosin phosphatase.

The first physiological experiments which demonstrated the effect of RND protein on contraction in smooth muscle tissues were performed in permeabilised rat ileal muscle strips and showed that co-transfection of RHOA and RND1 reverses RHOA mediated Ca2+ sensitization (Loirand et al. 1999). Treating rat ileal strips with progesterone and oestrogen increased their RND1 membrane-bound content. These changes in RND1 expression led to a reduction in agonist-induced Ca2+ sensitization. The action of RND1 was independent of [Ca2+], and did not affect the calcium-force response curve or the force of contraction generated by the myosin phosphatase inhibitor calyculin-A. Subsequently, other investigators suggested that the increase in steroid hormones during gestation causes a time-dependent increase in Rnd1 mRNA expression in rat uterus (Kim et al. 2003). They noted that Rnd1 mRNA levels fell to pre-pregnancy levels at day 1 post partum. In line with previous observations they suggested that the increase in Rnd1 mRNA expression may function to inhibit RHOA Ca2+ sensitization and maintain uterine quiescence required during gestation. Pacaud’s group used cDNA micro array, real-time PCR and immunoblotting to show that RND3 mRNA and protein expression was upregulated in mid-pregnant rabbit myometrium and suggested that the mid-pregnant increase in RND3 expression was associated with inhibition of RHOA mediated Ca2+ sensitization (Cario-Toumaniantz et al. 2003).

Relevance to human myometrium

Our data in humans demonstrate that there is an increase in myometrial RND2 and RND3 mRNA levels during pregnancy, see Fig. 6. The expression of other rho family members, namely RHOA and RHOB, were also increased. We also noted a dramatic increase in RND2 and RND3 protein expression in the pregnant groups. In contrast, RHOA, RHOB and RND1 protein levels did not alter with pregnancy or labour.

![Figure 6: RHO and RND mRNA (A) and protein (B) expression in human myometrium. RHOA, RHOB, RND2 and RND3 mRNA expression in myometrial samples from non-pregnant (NP) and pregnant not in labour (P) women were measured by quantitative real-time PCR (Lartey et al. 2007). The amounts of RHO and RND mRNA were normalised to the amount of RNA polymerase II (RPII) endogenous control gene mRNA expression within each sample (n = 5 for each). Whole tissue homogenates from the samples above were immunoblotted with RHO and RND antibodies. All the samples were analysed in duplicate and normalised to individual tubulin bands and plotted in a logarithmic scale within allotted patient groups. One-way ANOVA was used to assess differences between the means of the three groups. Myometrial RHO and RND mRNA expressions were elevated in pregnant relative to non-pregnant groups. This is sharply contrasted by the immunoblotting analysis which only demonstrated significant pregnancy related increases in RND2 and RND3 protein expressions. RHOA, RHOB and RND1 levels were invariant with pregnancy. The histograms are means and the vertical bars represent S.E.M. (*P<0.01), Student’s t-test.](http://www.reproduction-online.org)
RHO and RND function in human myometrium may be determined by their levels of expression, state of phosphorylation, intracellular localisation and the activity levels of effector molecules like ROCK1 (Bishop & Hall 2000). The overarching hypothesis is that an increase in RHOA and ROCK1 expression may account for the increased contractile activity at the end of gestation and during labour (Niño et al. 1997, Moore et al. 2000, Moran et al. 2002, Tahara et al. 2002).

The potential regulatory role for RHO proteins in uterine smooth muscle contraction was first highlighted by demonstrating an increase in Rock1 and Rock2 mRNA expression in rat myometrium during pregnancy (Niño et al. 1997). Other investigations subsequently reported ROCK1 and ROCK2 mRNA and protein expression in pregnant and non-pregnant human myometrium (Moore et al. 2000, Moran et al. 2002, Friel et al. 2005, Riley et al. 2005). Myometrial RHOA, ROCK1 and ROCK2 levels in women are invariant among non-pregnant, pregnant not in labour, spontaneous labour and spontaneous preterm labour groups (Lartey et al. 2006d).

Myometrial ROCK activity was elevated after treatment with the caspase-3 inhibitor Z-DEVD-FMK which prevents cleavage of ‘active’ p160ROCK1 to the ‘inactive’ p130ROCK1 (Moore et al. 2002). Chronic thromboxane receptor stimulation with U46619 increased p160ROCK1 expression and these changes were reversed by pretreatment with SQ29548, a thromboxane receptor antagonist (Moore et al. 2002). Agonist activation of RHOA enhances ROCK activity by increasing the stability of the p160ROCK1 (Moore & López Bernal 2003).

There is evidence of other RHO effectors in human myometrium. A constitutively active p34 protein fragment of the RHO effector p21 protein activated kinase 2 (PAK2) is elevated in human myometrium during pregnancy (Moore et al. 2000). The expression of PKN (previously known as PRK-like protein; Mukai & Ono 1994) and its downstream target PPP1R14A are both elevated in human myometrium during pregnancy (Ozaki et al. 2003, Lartey et al. 2007). The human formin protein DIAPH1 expression is upregulated in spontaneous labour whole tissue homogenates (Lartey et al. 2007).

**Tonic versus phasic contractions**

Smooth muscle tissues like the uterus and the gut contract in two ways: ‘phasic’ contractions which are transient, and ‘tonic’ more sustained contractions (Murthy et al. 2003). During labour the uterus contracts in a predominantly phasic manner but also requires periods of sustained tension (i.e. tonic contraction) in between the phasic contractions which occur approximately every 2 min. In vitro experiments with human uterine muscle strips showed that ROCK inhibition with Y27632 was maximal during tonic rather than phasic agonist- and K⁺-stimulated contractions (Kupittayanant et al. 2001). This has led some workers to conclude that RHOA-induced Ca²⁺-independent contractions primarily regulate the strength of tonic contractions and may not play a significant role in augmenting the phasic activity of myometrial tissue in labour (Kupittayanant et al. 2001).

**Coupling of RHO activation, membrane translocation and myometrial contraction**

RHO proteins shuttle between an active GTP membrane-bound form and an inactive GDP cytosol and presumably ARHGDI sequestered form. Several investigators have now linked RHO-mediated increases in tension and MYL phosphorylation to changes in membrane-bound levels and GTP-bound state of RHO.

Exposure of myometrial tissue to stimulatory GPCR agonists, e.g. OXT, carbachol, activates RHOA and ROCK to produce Ca²⁺-independent increases in tension (Taggart et al. 1999, Kupittayanant et al. 2001, Tahara et al. 2002, 2005, Woodcock et al. 2004). LPA is a classical RHO agonist that increases MYL phosphorylation in myometrial tissue strips (Moore et al. 2000). Recent experiments demonstrating that LPA increases GTP-RHOA levels in treated human myometrial smooth cells suggest that LPA-induced MYL phosphorylation may in part be due to RHOA activation (Lartey et al. 2007). These experiments highlighted the potential role of agonist-induced RHO activation and MYL phosphorylation in human uterine smooth muscle contraction.

One of the key experiments linking GPCR receptor stimulation to protein translocation in uterine smooth muscle cells demonstrated that carbachol-induced Ca²⁺ sensitisation resulted in PRKCB, RHOA and ROCK translocation to the plasma membrane (Taggart et al. 1999). Carbachol stimulated increases in the force of contraction and MYL phosphorylation at a constant [Ca²⁺], were reversed by the ROCK inhibitor Y27632. The same authors showed that contractile agonists like carbachol and OXT cause membrane translocation of RHOA in rat myometrial cells (Oh et al. 2003).

Our experiments have demonstrated increases in RHOA, RHOG, RND2 and RND3 membrane-bound proteins in pregnant myometrium relative to non-pregnant myometrium (Lartey et al. 2006c). This suggests that the changes in myometrial cell shape, size and function during pregnancy are reflected by alterations in RHO protein activation. We observed increases in RHOA membrane translocation in human myometrial tissue strips after OXT and carbachol treatment under both tension free and isometric conditions (Lartey & López Bernal 2008a, 2008b, 2008c). Curiously, both OXT and carbachol also produced marked increases in RND1 and RND2 membrane translocation (Lartey & López Bernal 2008a).
The RND2 results are quite remarkable as this protein was only confined to the cytolic fraction of unstimulated tissue strips in all pregnant samples used in our study. At first glance, these findings are contrary to the concurrent increases in RHOA translocation and presumed activation observed in the stimulated uterine strips. However, it is noted that RND2 can function both as a RHO antagonist and agonist (Tanaka et al. 2006a). RND2 is usually cytolic but is recruited to a membrane compartment presumed to be early endosomes by an effector protein called VPS4A. Therefore, the differences in RND membrane translocation caused by OXT may be as a consequence of its involvement in GPCR receptor/vesicular trafficking (Tanaka et al. 2002). More work is required to understand RHO and RND functions in myometrial cells during pregnancy and in labour. A summary of RND interacting proteins and their mechanisms of actions are presented in Table 1.

There is evidence that agonist stimulation of other GPCRs like the corticotrophin-releasing hormone (CRH) type 2 receptor also results in MYL phosphorylation and contraction in human myometrial smooth muscle cells. Urocortin (a 38 kDa CRH neuropeptide) stimulates myometrial cell CRHR2 to increase MYL phosphorylation via a variety of mechanisms which include RHOA activation and RHO membrane translocation. This was confirmed by RHO inhibitors like C3 exotoxin and Y27623 which reversed MYL phosphorylation and RHOA translocation. In addition, urocortin mediated MYL phosphorylation was abolished by the PRKCB inhibitor bisindolylmaleimide and the MAPK1 inhibitor U0126 suggesting that these additional pathways also regulate myometrial cell MYL phosphorylation and contraction (Karteris et al. 2004).

Physiological uterotones such as OXT and PGF2α enhance myometrial sensitivity to Ca2+ through a RHOA–ROCK-dependent mechanism (Woodcock et al. 2004, 2006). We have mapped the effects of OXT and carbachol on RHO protein targets downstream of ROCK1. OXT-induced tension and contraction in human myometrial strips under isometric conditions are associated with concurrent increases in PPP1R12A-Thr696 and MYL-Ser19/Thr18 phosphorylation (Lartey associated with concurrent increases in PPP1R12A-human myometrial strips under isometric conditions are ROCK1. OXT-induced tension and contraction in carbachol on RHO protein targets downstream of and abnormal labour

Involvement of RHO proteins in uterine quiescence and abnormal labour

We examined a panel of over ten RHO–GTP-binding proteins to determine if changes in RHO protein expression were associated with the contractile activity in spontaneous labour and preterm labour. RND2 and RND3 levels were markedly upregulated relative to RHOA levels during gestation (Fig. 7). This increase in RND protein expression correlated with a reduction of ROCK1 inhibitory phosphorylation of PPP1R12A subunit in the pregnant tissues examined (Lartey et al. 2006d). These findings are consistent with the effects of RND proteins in rat and rabbit myometrium and imply that, in humans, these proteins may regulate the relative quiescence of the uterus during gestation in order to allow the fetus to reach an appropriate maturity prior to normal labour. The lack of changes in RND to RHOA ratios in labouring myometrium suggests that other mechanisms may be important at this interface. For instance, downregulation in the expression of RHOD, a known RHOA inhibitor, may account for the threefold increase in GTP-bound RHOA levels in labour, see Fig. 7 (Lartey et al. 2006b, 2007). RHOD inhibits other RHO–GTP-binding proteins. For example, it binds to the cytoplasmic RBD domain of the semaphorin receptors Plexin-A1 and Plexin-B1, preventing their dimerisation and activation by RND1 possibly through competitive binding (Zanata et al. 2002, Tong et al. 2007). The myometrial RHOA–RND–RHOD interactions in human labour require further investigation.

RHB and RHOD are endosomal GTP-binding proteins which regulate actin vesicular dynamics and GPCR trafficking (Murphy et al. 1996) and are expressed in pregnant myometrial tissue, see Fig. 8. We are currently investigating their role in human chorionic gonadotropin/LH and OXT receptor desensitisation.

We have noted some interesting changes in RHOA and RND protein function during abnormal dysfunctional labour, defined as spontaneous labour with ‘secondary arrest’ not treated with OXT. RHOA membrane levels increased during gestation and in normal labour at term but were reduced in the dysfunctional labour group (J Larney & A Lopez Bernal, unpublished observations). This represents attenuation in RHOA activation in dysfunctional labour relative to spontaneous labour. OXT stimulation increases both RHOA and RND2 membrane translocation therefore it plausible to suggest that the use of OXT will correct the abnormal RHO function in this subgroup.

ARF–GTP-binding proteins regulate GPCR receptor desensitization (Mukherjee et al. 2000). ARF–GEFs catalyse the nucleotide exchange on the ARF–GDP-bound GPCR complex allowing the receptor to internalize (Salvador et al. 2001). Recent experiments from our laboratory have found changes in ARF6 and PSCD3 (an ARF6 GEF) mRNA and protein expression in preterm myometrium from pregnancies complicated by pre-eclampsia and intrauterine growth restriction, suggesting for the first time that different mechanisms may regulate receptor trafficking during normal and complicated pregnancies (Lartey & Lopez Bernal 2008b).
Table 1 Known RND2 interacting proteins.

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<tr>
<th>Kinase</th>
<th>Effects and function</th>
<th>References</th>
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<td>Serine/threonine kinases</td>
<td>RND proteins inhibit RHOA activity through competitive inhibition of ROCK1. RND3 and RHOA bind to different regions on ROCK1. RND proteins bind to the N-terminal domain (1–76) while RHO proteins bind to the C-terminal region (934–1015) of ROCK1. The minimum region required for RND inhibition (1–420) includes the RND-binding domain, a kinase domain (76–338) and a further region (338–420). RND1 inhibition of ROCK1 may be dependent on prior activation by GTP–RHOA. RND3 binds optimally to caspase activated or truncated versions of activated ROCK1</td>
<td>Fujisawa et al. (1996) and Riento et al. (2003, 2005)</td>
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<td>ARHGAPs and ARHGEFs</td>
<td>ARHGAP5 (or p190RHOGAP)</td>
<td>Foster et al. (1996), Hansen et al. (2000) and Wennerberg et al. (2003)</td>
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<td>RND3 was initially identified as a binding partner to ARHGAP5, which has an N-terminal GTP-binding domain, a middle RND-binding region (382–1229) and C-terminal RHOGAP domain. The RND3–RHOGAP-binding motif include residues 16–93 which contain the phosphate-binding loop, switch 1 and 2 regions. RND1 and RND3 enhance GTPase activity by targeting ARHGAP5 to the plasma membrane where it reduces GTP–RHOA levels and RHOA stress fiber formation. Conversely, ROCK1 phosphorylates ARHGAP5 at Ser150 to reverse RND1 binding and attenuate GTPase activity</td>
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<td>RND2 binds to plexin-D1 and inhibits it functions</td>
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<td>UBX domain protein 11 (UBXN11) or Socius</td>
<td>Catoh et al. (2002)</td>
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<td>UBXN11 was the first protein to be identified that specifically interacts with RND proteins. Transfection of activated RND3 proteins into COS7 cells targets UBXN11 in the plasma membrane to cause actin stress fiber formation and cell rounding. No downstream effects were observed when RND2 and RND3 were co-transfected with UBXN11, suggesting that it is a RND1-specific effector mediating RHOA/ROCK inhibition</td>
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<td>Growth factor receptor-bound protein 7 (GRB7)</td>
<td>Vayssiere et al. (2000)</td>
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<td>GRB7 is a member of the GRB1/10/14 family which contains a Src homology 2 (SH2) domain, a central pleckstrin homology domain and an N-terminal proline rich SH3 binding motif. The GRB7–RND1 switch 2 and formin binding region binds to the SH2 domain in GRB7 and the resultant complex is translocated to the membrane. Therefore, GRB7 acts as an adaptor protein which targets RND1 to the plasma membrane where it stimulates GTPase activity towards GTP RHOA</td>
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<td>Rapostilin (Apostle of RND2)</td>
<td>Jalink et al. (1994), Chan et al. (1996) and Fujita et al. (2002)</td>
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<td>Rapostilin is a RND2-specific effector which is homologous to a human formin binding protein and a RHO–CDC42 effector protein called CIP4. It has two functional domains: an N-terminal CIP4 domain and a SH3 domain at the C-terminal. GTP-bound RND2 binds to Rapostilin SH3 domain to reverse RHOA induced changes in cell morphology and function</td>
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<td>Plexins – A1, B1 and D1</td>
<td>Rohm et al. (2000), Zanata et al. (2003, 2006) and Uesugi et al. (2009)</td>
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<td>RND1 binds to plexin-A1 increasing its GTPase activity which opposes RHOA to cause cell contraction in COS7 cells. Active RND1 binds to plexin-A1 and increases its GAP activity to cause actin rearrangements. RND2 binds to plexin-D1 and inhibits it functions</td>
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<td>Pragmin (Pragma of RND2)</td>
<td>Tanaka et al. (2006a)</td>
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<td>RND GTG-binding proteins mediate almost all of their intracellular effects by inhibition of the RHOA–ROCK1 pathway. However, in some instances RND proteins can act as activators of RHOA. Activated RND2 binds to pragmin which activates ROCK1 to cause cell contraction</td>
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<td>Fibroblasts growth factor receptor associated substrate 2-α and β (FRS2α and FRS2β)</td>
<td>Harada et al. (2005)</td>
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<td>RND protein function may also be regulated through sequestration by specific binding proteins. RND1 normally binds to FRS2α and FRS2β which are docking proteins of fibroblast growth factor (FGF) receptors. FRS2β bound RND1 has no effect on RHOA</td>
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<td>Male germ cell RAC GTPase activating protein (MgcRACGAP) or RACGAP1</td>
<td>Naud et al. (2003)</td>
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<td>RACGAP1 is a human ARHGAP which is highly expressed in male germ cells in spermatocytes and spermatids. RND2 and RND3 bind to the GAP domain of RACGAP1 and mediate RHOA inhibition by increasing GTPase activity</td>
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<td>Vacuolar protein sorting 4A (VPS4A)</td>
<td>Tanaka et al. (2002)</td>
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<td>VPS4A belongs to a family of ATPases which couple the chemical energy obtained from ATP hydrolysis to the mechanical force of myosin. VPS4A recruits RND2 to early endosomes where it regulates endosomal sorting during vesicular trafficking</td>
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<td>GEM interacting protein (GMIP)</td>
<td>Aresta et al. (2002)</td>
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<td>GMIP is an ARHGAP with a cysteine-rich domain and a RHOGAP domain in its C-terminal half. It inhibits RHOA by accelerating RHOA GTPase activity and downregulates many processes dependent of the RHO pathway</td>
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Novel RHO effector mechanisms during pregnancy and in labour

We have outlined the complex interaction of kinases downstream of the RHOA–ROCK complex in regulating smooth muscle MYL phosphorylation. To date, six RHO effectors, ROCK1, ROCK2, DIAPH1, DIAPH2, PAK2 and PKN1 have been identified in human myometrium (Lartey et al. 2007). ROCKs are the best characterized RHO family proteins in smooth muscle cells. Although ROCK levels are not altered with pregnancy or labour, GTP–RHOA levels are elevated in labour and it appears that the changes upstream of ROCK control MYL phosphorylation and contraction in labour. Preliminary investigations have revealed increases in ROCK1 and ROCK2 nuclear localization in myometrial and interstitial uterine cells myometrial tissue sections during labour (Lartey & Lopez Bernal 2009b). There are previous reports of nuclear localisation of ROCK proteins in other tissues and cells lines. ROCK2 localises to the nucleus in HeLa cells, where it phosphorylates p300 acetyltransferase, increasing histone acetyltransferase activity and gene transcription (Tanaka et al. 2006b). ROCK1 co-localizes with centromeres (Chevrier et al. 2002) and ROCK2 is found in the cleavage furrow in late mitosis (Inada et al. 1999). Progesterone and oestrogen regulate the nuclear localisation of some kinases including cyclin-dependent kinases (cdc4; Tong & Pollard 1999) and adaptor molecules like CT10 regulator of kinase in uterine smooth muscle and epithelial cells (Nautiyal et al. 2004). ROCK also induces nuclear translocation of MAPK3/1 (ERK1/2) in smooth muscle cells (Liu et al. 2004). ROCK2 regulates PDGF-induced nuclear localization of serum response factor during differentiation of proepicardial cells into coronary smooth muscle cells (Lu et al. 2001). Growth factors can induce ROCK nuclear translocation. Transforming growth factor-β translocates ROCK2 into the nucleus where it induces CDC24 phosphatase to regulate cell cycle progression (Bhowmick et al. 2003). Nuclear localised ROCK2 has kinase activity against PPP1R12A and sensitivity to Y27632 (Narumiya et al. 2000). The nuclear localisation of both proteins during pregnancy suggests a role in myometrial cell division and regulation of gene expression. However, more investigation is required to determine whether this change in ROCK protein function has any effect of PPP1R12A activity and myofilament sensitivity to physiological uterotones like OXT and PGE\(_2\) in labour.

PKN1, the first RHOA effector to be discovered (Mukai et al. 1994, Palmer et al. 1994) was also present in our myometrial samples. Its expression was upregulated in all the pregnant groups compared to non-pregnant groups. PKN1 expression was mirrored by a similar increase in one of its downstream targets, namely phosphoThr38–PPP1R14A. This represents another mechanism of Ca\(^{2+}\) sensitization in pregnant myometrial tissue. Thus, the PKN1–PPP1R14A and the RHOA–ROCK–RND pathways may converge on MLCP at PPP1c and PPP1R12A sites respectively and crucially can function independently of each other, see Fig. 5.

We have found that the expression of other RHO effectors such as DIAPH1 and DIAPH2 (Watanabe et al. 1997, 1999) is altered with human labour (Lartey et al. 2007). DIAPH1 and DIAPH2 are human formin proteins involved in actin reorganization and have been linked to RHOB and RHOD formation of endosomal vesicles (Murphy et al. 1996, Gasman et al. 2003). The role of DIAPH family proteins in uterine myofilament remodelling during gestation and in priming the contractile apparatus for labour is not well understood and is an area of considerable research interest.

Protein kinases other than ROCK can inhibit MLCP activity by phosphorylating PPP1R12A at Thr696. The candidates kinases include PAK (Takizawa et al. 2002), distrophia-mytonic protein kinase (Murai et al. 2001), ILK (Murai et al. 2002), and DAPK3 (MacDonald et al. 2001a). We used immunoblotting
and immunohistochemistry to characterize DAPK3 expression in our human tissue samples. Our preliminary findings indicate that DAPK3 may undergo differential signalling and activation during labour (J Lartey & A López Bernal, unpublished observations). DAPK3 may cause Ca\(^{2+}\)-independent contraction by either phosphorylation of the PPP1R12A subunit of myosin phosphatase at Thr696 (MacDonald et al. 2001a, Niiro & Ikebe 2001, Borman et al. 2002), activation of PPP1R14A by phosphorylation at Thr38 (MacDonald et al. 2001b) or direct phosphorylation of MYL at Ser19 and Thr18 (Murata-Hori et al. 1999).

Pharmacological regulation of the RHO pathway

**ROCK inhibition by pyridine derivatives**

Physiological uterotones such as PGF\(_{2\alpha}\) and OXT activate GPCRs to promote Ca\(^{2+}\) release and entry into the cells, promoting Ca\(^{2+}\)-calmodulin-dependent MYLK activation. Moreover, these agonists generate increases in tension at a constant activity of intracellular Ca\(^{2+}\). We have shown that myometrial GTP–RHOA and ROCK activity are elevated during spontaneous preterm labour (Lartey et al. 2007). Thus, it is conceivable that inhibition of ROCK activity may lead to attenuation of the tension that may be associated with spontaneous preterm labour. Several compounds have been manufactured to inhibit ROCK function. The most widely used N-(4-pyridyl)-4-(1-aminoethyl) cyclohexane carboxamide Y27632 is a highly potent, cell-permeable pyridine derivative which acts as an ATP-competitive inhibitor with equal potency against ROCK1 and ROCK2 (Uehata et al. 1997). Y27632 inhibits agonist-induced phosphorylation of myosin phosphatase and MYL to disrupt RHOA mediated stress fibers and cause smooth muscle relaxation (Uehata et al. 1997). However, Y27632 and other similar compounds like Y32885 inhibit other related kinases like PRK1 and PRK2 at concentrations required for ROCK inhibition (Davies et al. 2000). This multi kinase inhibition is also observed with another ROCK inhibitor compound HA 1077 (AT877 or fasudil hydrochloride; Amano et al. 1999, Niggli 1999). Therefore, the potential of ROCK inhibitors to relax the uterus may be limited due to their lack of kinase specificity. Further research is required on the effect of ROCK inhibitors on myometrial tissue during agonist-induced and spontaneous contractions in normal and preterm labour. Furthermore, we need to determine the transcription factors and ERE/PREs that control the remarkable increases in RHO and RND protein expressions and activity during pregnancy and labour. We also need to elucidate whether oestrogen stimulation or a functional progesterone withdrawal, or both, can produce changes in uterine smooth muscle Ca\(^{2+}\) sensitization in women as demonstrated in some animal species as there can be quite marked changes in RHO protein expression and function among species. Moreover, steroids may induce changes in the activity of signalling enzymes that result in post-translational modification or phosphorylation of the proteins to precipitate labour. There are predicted putative oestrogen and progesterone response elements of human RND genes, see Fig. 9.

**Farnesyltransferase and geranylgeranyltransferase regulation of RHO protein function**

Farnesyltransferases and geranylgeranyltransferases catalyse the irreversible addition of farnesyl for RAS-related associated with diabetes or RND proteins; or geranylgeranyl for RHO, RAS, RAC, RAP and CDC42. Pharmacological inhibition by farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase inhibitors (GGTIs) prevent isoprenoid modification and block...
the lipid-mediated association of RAS and RHO proteins with the plasma membrane (Sebti & Hamilton 2000). Other small molecule inhibitors such as farnesyl thiosalicylic acid compete with the isoprenoid attachment of small GTPases to membrane binding sites (Smalley & Eisen 2003, Goldberg & Kloog 2006). However, these RHO inhibitors can have other diverse effects on RHO protein function. For instance, FTIs and GGTIs stimulate RHOB transcription leading to increased RHOB expression. FTIs activate transcription by a number of mechanisms including histone deacetylase 1 dissociation, histone acetyltransferase association and histone acetylation of the RHOB promoter (Delarue et al. 2007). The upregulation of RND protein expression during gestation may function to inhibit RHOA activity thereby maintaining uterine quiescence. Therefore, the use of RND proteins inhibitors (FTIs) at appropriate gestations may induce labour in conditions such as pre-eclampsia and intrauterine growth restriction which necessitate delivery of the fetus.

**RHO proteins and uteroplacental vascular disease**

RHO proteins have been implicated in a range of vascular smooth muscle disorders including hypertension, arteriosclerosis and also directly in ischaemic myocardial damage. ROCK inhibitors have been used in animal models of cardiovascular diseases with varying degrees of success. The ROCK inhibitor Y27632 reduces U46619 induced contraction in myometrial and placental arteries in normal term pregnancies (Wareing et al. 2005). We have quantified the expression of RHO proteins during normal and abnormal labour (Lartey et al. 2006). Analysis of paired placenta and myometrium from individual samples shows that placentation RHO protein levels were up to threefold higher than corresponding myometrial levels, see Fig. 10. RND3 proteins were differentially expressed in primary, secondary and tertiary human placental trophoblasts (Lartey et al. 2006a). RND3 regulation

![Figure 10](https://example.com/fig10.png) **Figure 10** RHO GTPase expression in human myometrial and placental tissue samples. Paired myometrial (MYO) and placental (PLAC) tissue homogenates from five different spontaneous labour donors were immunoblotted with RHOA, RHOB, RHOD and ARF6 antibodies and visualized with ECL. Individual RHO protein bands were normalised to their own tubulin bands. The normalised RHO densitometry values were plotted on a logarithmic scale within allotted groups (*P<0.05, **P<0.001).
of normal placental development and its role in uterovascular diseases and placental insufficiency need to be explored. Interestingly, although RND1, RND2 and RND3 mRNAs were expressed in the placentas samples examined, we did not find any evidence of RND1 or RND2 proteins. This suggests differences in transcriptional or translational regulation of RND proteins between the human placenta and myometrium. Moreover, the human placenta is a rich source of ARF6 (Fig. 10) and its role in the regulation of placental GPCR function needs to be explored.

Conclusion

The management of preterm labour is hampered by our poor understanding of the mechanism of parturition in women and the lack of uterine-specific drugs to control myometrial contractility. In order to optimize the tocolytic therapy in preterm labour, we need to use agents that are effective between 23 and 30 weeks gestation, when neonatal risk is high. Furthermore, these agents must be effective for a defined period of time and ideally be given orally. RHO and ROCK inhibitors may fulfil the requirements above and urgent work is required to assess their efficacy in inhibiting spontaneous and agonist-induced contractions in humans. Nevertheless, tocolytic therapy is only symptomatic and better approaches are required for the prevention and management of preterm labour. These breakthroughs will come from further research into the physiological and pathophysiological mechanisms of parturition. The role of RHO proteins in uterine function is complex and increased investigation of their function will complement other approaches towards a better understanding of the bio-endocrine pathways leading to labour. This is the only way forward to obtaining the best outcome for the mothers and their babies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

Academic Clinical Fellowship, Northern Deanery, Newcastle upon Tyne, UK and Wellbeing of Women (RCOG) Research Training Fellowship.

Acknowledgements

J Lartey and A L Bernal are members of the European Preterm Labour Group http://www.europarturition.eu.

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Received 23 April 2009
First decision 2 June 2009
Accepted 8 July 2009