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Modulation of RhoA–Rho kinase-mediated Ca^{2+} sensitization of rabbit myometrium during pregnancy – role of Rnd3

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During pregnancy, the uterus undergoes major functional and structural remodelling. It is well known that during the major part of pregnancy, the myometrium normally remains relatively quiescent but is able to generate powerful contractions at the time of parturition. However, the intracellular molecular events regulating myometrial contractility during pregnancy still remain poorly understood. We applied differential gene expression screening using cDNA array technology to probe myometrium samples from non-pregnant and mid-pregnant (15 days) rabbits. Among the differentially expressed genes, the farnesylated small G-protein of the Rho family, Rnd3, was found to be upregulated (3.6-fold) at mid-pregnancy. Upregulation of Rnd3 was confirmed at the protein level by a 3.4-fold increase in Rnd3 expression in mid-pregnant myometrium. Measurements of contractile properties of β-escin permeabilized smooth muscle strips revealed that the upregulation of Rnd3 correlated with an inhibition of RhoA–Rho kinase-mediated Ca^{2+} sensitization at mid-pregnancy. Treatment of muscle strips from mid-pregnant myometrium with the farnesyl-transferase inhibitor manumycin A (10 μM) led to the recovery of RhoA–Rho kinase-dependent Ca^{2+} sensitization. At late pregnancy (31 days), upregulation of RhoA and Rho kinase expression was associated with an increase in Ca^{2+} sensitivity of contractile proteins that was inhibited by the Rho kinase inhibitor Y-27632 (10 μM). These data thus demonstrate the time-dependent regulation of the RhoA–Rho kinase-mediated Ca^{2+} sensitization during the course of pregnancy. The depression of this mechanism at mid-pregnancy followed by its constitutive activation near term is associated with a co-ordinated modulation of Rnd3, RhoA and Rho kinase expression. The RhoA–Rho kinase signalling pathway and its regulators might thus represent potential targets for the development of new treatments for pre-term labour.

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During pregnancy, the uterus undergoes major functional and structural remodelling. For the major part of pregnancy, the uterus gradually increases in size and the myometrium normally remains relatively quiescent. On the contrary, at the time of parturition, the myometrium has to generate co-ordinated and powerful contractions. Several extracellular signals including hormones, neurotransmitters and mechanical factors, as well as a change in the expression levels of membrane receptors, trimeric G-proteins or ion channels have been proposed to contribute to the switch to contractile activity at term (Boyle *et al.* 1987; Inoue & Sperelakis, 1991; Europe-Finner *et al.* 1993; Lopez-Barnal *et al.* 1995). However, the intracellular molecular events regulating myometrial contractility during pregnancy still remain poorly understood.

The major regulatory mechanism of smooth muscle contraction is phosphorylation and/or dephosphorylation of the 20 kDa myosin light chain (MLC) (Somlyo & Somlyo, 1994; Somlyo, 1997). MLC is phosphorylated by the Ca^{2+}–calmodulin-activated myosin light chain kinase (MLCK) and dephosphorylated by the Ca^{2+}-independent myosin light chain phosphatase (MLCP). Thus, a rise in cytosolic Ca^{2+} concentration produces smooth muscle contraction by activation of MLCK and consequent phosphorylation of MLC. However, it is now well established that MLC phosphorylation and tension can be induced independently of changes in cytosolic Ca^{2+} concentration (Somlyo & Somlyo, 1994; Somlyo, 1997). Numerous agonists that bind to G-protein-coupled receptors produce contraction by increasing both the cytosolic Ca^{2+} concentration and the Ca^{2+} sensitivity of the contractile apparatus. The increased sensitivity of smooth muscle towards Ca^{2+} results from inhibition of MLCP activity leading to increased MLC phosphorylation and tension at a constant Ca^{2+} concentration. This Ca^{2+}-sensitizing effect is ascribed to the activation of the small G-protein RhoA that activates Rho kinase, which in turn,

Table 1. Nucleotide sequence of the PCR primers used to assay gene expression by real-time quantitative PCR

Gene	Forward primer	Reverse primer
β-Actin	5′ GGCGTACAGGTCTTGCGG 3′	5′ CTCTTCCAGCCGTCCTTCC 3′
Rnd1	5′ GCCTGCTTCTGGTGGGAC 3′	5′ AATTACACAGCCTGTTTGGAGAC 3′
Rnd2	5′ TCCTTGCCACTTCTTGAGAACAC 3′	5′ ATGTCCCCACCGTGTTTGAG 3′
Rnd3	5′ CAGAAGTGTCCCATAGGCTCAA 3′	5′ CCCCGAGAATTATGTGCCC 3′
Rho-kinase I	5′ AATGCACCTCTACCAATCACTTTC 3′	5′ GCTGGATGGATTGGATGCTT 3′
Rho A	5′ CATTTTTCTGGGATGTTTTCTAAACT 3′	5′ GAGTTGGCTTTATGGGACACAG 3′

phosphorylates the regulatory subunit of MLCP and inhibits its activity (Gong *et al.* 1996; Otto *et al.* 1996; Fujihara *et al.* 1997; Uehata *et al.* 1997). RhoA–Rho kinase-dependent MLCP inhibition is not only involved in the RhoA-dependent Ca^{2+} sensitization in smooth muscle but also controls actomyosin-based cytoskeleton organization (Togashi *et al.* 1998; Hirshman & Emala, 1999), extracellular matrix assembly (Zhong *et al.* 1998) and its interaction with the cell membrane (Schoenwaelder & Burridge, 1999). Besides RhoA that regulates contraction through its activity, other members of the Rho protein family, namely Rnd proteins, negatively control smooth muscle contraction through regulation of their expression level (Loirand *et al.* 1999). Rnd proteins are constitutively active and selectively inhibit RhoA–Rho kinase-mediated Ca^{2+} sensitization (Nobes *et al.* 1998; Loirand *et al.* 1999).

It has recently been demonstrated in rat and human myometrium, that muscarinic or oxytocin receptor stimulation induces Rho kinase-mediated Ca^{2+} sensitization of contraction (Taggart *et al.* 1999; Lee *et al.* 2001; Kupittayanant *et al.* 2001; Moran *et al.* 2002; Tahara *et al.* 2002). Analysis of RhoA and Rho kinase expression at the mRNA or protein level revealed that upregulation of the enzyme at the end of pregnancy could be involved in the mechanism underlying the increased myometrium contractility at term (Niiro *et al.* 1997; Moore *et al.* 2000; Moran *et al.* 2002; Tahara *et al.* 2002). In addition, the extent of Ca^{2+} sensitization of uterine contractions induced by various agonists (prostaglandin, oxytocin) changes during pregnancy (Coleman *et al.* 2000; Tahara *et al.* 2002). Although these results suggest a role for Rho protein signalling in the myometrium, available data only focused on RhoA. Furthermore, most studies aimed to understand the mechanisms involved in the augmentation of uterine contractility at term, while data regarding myometrial quiescence during pregnancy remain limited.

In this study, the global gene expression of small G-proteins, their targets and regulators as well as genes involved in small G-protein-regulated functions has been compared in non-pregnant myometrium and at mid-pregnancy. We applied differential gene expression screening using cDNA array technology to probe myometrium samples. In order to determine the significance of the observed changes in expression, in relation to the contractile properties of myometrium, measurements of Ca^{2+} sensitization in permeabilized muscle strips have been performed.

METHODS

Tissues

All experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals. Non-pregnant and pregnant rabbits (mid-pregnancy, day 15; late pregnancy, days 30–31) were killed with sodium pentobarbital (100 mg kg^{-1} intravenously). The uterus was removed and placed in sterile physiological saline solution (PSS, mM; 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 10 Tris; pH adjusted to 7.4 with HCl).

Isolation of mRNA

Myometrial tissues isolated by dissection from rabbit uterus were immediately frozen in liquid nitrogen. RNA was extracted from the tissue using TRIzol reagent and samples were treated with RNase-free DNase. mRNAs from non-pregnant ($n = 5$) and pregnant myometria ($n = 5$) were isolated using OligotexTM mRNA Midi Kit (Qiagen) according to manufacturer's instructions.

Labelling of cDNA probes and hybridization to cDNA arrays

Atlas Cell interaction Arrays (Clontech) containing 265 genes involved in cell signalling and interaction were screened using ^{32}P-labelled cDNA probes. Briefly, for each sample, 1 μg mRNA was mixed with 1 μl 10 × CDS primer mix and converted into ^{32}P-cDNA labelling first strand cDNA by means of Moloney murine leukemia virus (MMLV) reverse transcriptase. Unincorporated ^{32}P-labelled nucleotides were removed by Nucleospin column. cDNA arrays were prehybridized for 30 min at 60 °C in 15 ml hybridization buffer (ExpressHyb, Clontech) with 0.5 mg sheared salmon spermDNA. The denatured labelled ^{32}P-cDNA product (4×10^6 c.p.m.) was added to the hybridization solution and incubated for 16 h at 60 °C. Arrays were washed according to manufacturer's protocol then exposed to phosphorimager. Results were analysed using Clontech software (Atlas Image 1.5).

Real-time quantitative PCR

Differential expression of selected genes was confirmed by gene-specific real-time quantitative PCR. Total RNA (1 μg) was used as the template for the reverse transcriptase (RT) reaction using MMLV, 2 μg random hexamers, 0.2 mM deoxyribonucleoside triphosphates (dNTPs) and 5 mM dithiothreitol (DTT; Life Technologies). Primers used for the PCR are listed in Table 1. All of the PCR primers were synthesized by Sigma-Genosys. Real-time quantitative PCR was performed in the iCycler iQ Detection System (Bio-Rad Laboratories) using Sybr green detection (Molecular Probes) and Titanium *Taq* DNA polymerase

(Clontech), according to the manufacturer's recommendations. The following temperature profile was used: 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted non-pregnant myometrium cDNA samples. Expression of target genes was normalized to β-actin levels. The delta Ct (δ Ct) (cycle threshold) method was used to calculate relative expression levels.

Western blotting

Myometrium samples were homogenized with a polytron in ice-cold lysis buffer containing PBS, pH 7.4, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium desoxycholate, 0.1 % SDS and protease inhibitor (Complete, 1 tablet per 50 ml). Nuclei and unlysed cells were removed by low speed centrifugation. Protein concentration was measured and adjusted, then Laemmli sample buffer was added and equal amounts of protein were loaded on SDS– polyacrylamide gel, which was then electrophoresed and transferred to nitrocellulose. The amount of protein was checked by staining with Ponceau Red and by reprobing the membrane with anti-β-actin antibody. Before immunoblotting, the membrane was blocked with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween 20, 5 % non-fat milk for 1 h at room temperature. The expression of Rnd3 protein was examined with anti-Rho8 antibody (Santa Cruz Biotechnology, Inc., CA, USA). The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and subsequent Enhanced ChemiLuminescence detection (Amersham Biosciences), then quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA).

Isometric tension measurement in skinned fibres

Small strips (approximately 200 μm wide and 4 mm long) of longitudinal muscle from myometrium were dissected and tied at each end with a single silk thread to the tips of two needles, one of which was connected to a force transducer (AE 801, SensoNor, Norway). Strips were placed in a well on a bubble plate filled with PSS (Horiuti, 1988) and stretched to about 1.3 times the resting length. The solution was rapidly changed by sliding the plate to an adjacent well. After measuring contraction evoked by high K^+ solution, the strips were incubated in normal relaxing solution ((mM): 85 KCl, 5 $MgCl_2$, 5 Na_2ATP, 5 creatine phosphate, 2 EGTA, and 20 Tris maleate, brought to pH 7.1 at 25 °C with KOH) for a few minutes, followed by treatment with β-escin (50–70 μM) in relaxing solution for 35 min at 25 °C as described previously (Loirand *et al.* 1999). The skinned muscle strip was then washed several times with fresh relaxing solution containing 10 mM EGTA. Calmodulin (1.5 μM) was present in the bathing solutions throughout the experiments. Tension developed by permeabilized muscle strips was measured in activating solutions, containing 10 mM EGTA and a specified amount of $CaCl_2$ to give a desired concentration of free Ca^{2+} (pCa = $-\log[Ca^{2+}]$) (Loirand *et al.* 1999). To evaluate the effect of farnesyl-transferase inhibitors, muscle strips were pretreated for 12 h in sterile PSS. For this experimental condition, results were compared with those obtained in control strips taken from adjacent pieces of tissue and handled under similar conditions, using similar buffers.

Measurement of RhoA activity.

RhoA activity was assessed in homogenized myometrium samples by a pull-down assay, using the Rho-binding domain (RBD) of the Rho effector protein Rhotekin as described previously (Ren *et al.* 1999). Precipitated GTP-bound RhoA and total RhoA were then analysed by Western blot using a mouse monoclonal anti-RhoA antibody (Santa Cruz).

Statistics

All results are expressed as means ± S.E.M. with n the sample size. Significance was tested by means of Student's t test. Probabilities less than 5 % ($P < 0.05$) were considered significant.

Chemicals and drugs

Calmodulin was purchased from Roche diagnostic (Meylan, France). The protein kinase C inhibitor GF109203x and the farnesyl-transferase inhibitor manumycin A were provided by Calbiochem (Merck Eurolab, Germany). The Rho kinase inhibitor Y-27632 was a gift from Pr Lesieur (Lille, France). All other reagents were purchased from Sigma (France).

RESULTS

Comparative gene expression profiling

Labelled complementary DNAs (cDNAs) prepared from non-pregnant and mid-pregnant rabbit myometrium were hybridized to cDNA arrays analysing the expression of 265 known genes. Spots of housekeeping genes consistently gave comparably positive signals attesting that equivalent amounts of cDNA had been used (n = 5; Fig. 1). Among the 265 spots, 145 gave detectable signals. Analysis of the median densitometric signal intensity revealed that expression of 36 (25 %) genes differed between non-pregnant and mid-pregnant myometrium by a factor of

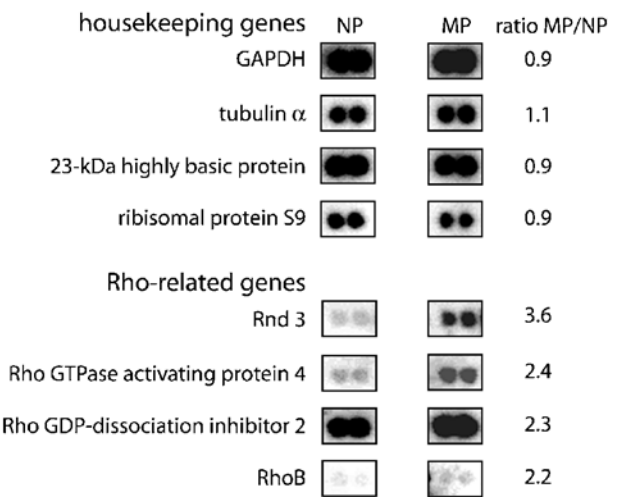

Figure 1. Differential gene expression in mid-pregnant myometrium using cDNA arrays

Typical results of two hybridization experiments with cDNA generated from non-pregnant (NP) and from mid-pregnant (MP) myometrium showing hybridization signals for housekeeping genes (top) and Rho-related genes (bottom).

Table 2. List of differentially expressed genes in mid-pregnant myometrium (MP) compared to non-pregnant myometrium (NP); genes are arranged in accordance with the function of their products

Gene name	Accession number	Ratio MP/NP
Cell adhesion		
Cadherin-6	D31784	3.5
CD27 ligand	L08096, S69339	4.7
Integrin α3	M59911	2.4
Integrin α5	X06256	4.9
Integrin α9	D25303, L24158	3.1
Integrin β3	J02703, M25108	3.5
Integrin β7	M62880	3.2
Cytoskeletal interaction		
Ezrin	X51521	2.0
Merlin	L11353, Z22664, X72657, L27133	2.0
Zyxin + zyxin 2	X94991, X95735	3.2
Zyxin-related protein ZRP-1	AF000974	2.0
Extracellular matrix		
Aggrecan 1	M55172	2.1
Collagen type IVα	X05610	2.1
Collagen type IIα1	X16468	2.4
Collagen type XVIα1	M92642	0.36
Extracellular matrix metalloproteinase inducer (emmprin)	L20471	2.1
Heparan sulfate proteoglycan	M85289	3.4
Laminin α4	X70904, X91171	2.1
Tenascin C	X78565	5.2
Versican, isoforms V1, V2 and V3	U16306, X15998, U26555, D32039	3.5
Vitronectin precursor	X03168	2.3
Extracellular matrix regulators		
MMP-9	J05070	2.8
MMP-17	X89576	2.1
TIMP-1	X03124	2.5
TIMP-2	J05593	2.9
P37NB	U32907	2.8
Protein C inhibitor	M68516, J02639	3.0
Monomeric G proteins and regulators		
Rho B	X06820	2.2
Rho GDP-dissociation inhibitor 2	L20688	2.3
Rho-GTPase-activating protein 4	X78817	2.4
Rnd 3	X95282	3.6
Secreted molecules		
Growth hormone-dependent insulin-like growth factor-binding protein	M31159, M35878	4.5
Insulin-like growth factor binding protein 6	M62402	2.0
Insulin-like growth factor binding protein 2	M35410	4.0
KISS-1/metastin	U43527	2.1
TNF-β	D12614	2.2

The change in expression was expressed as the ratio of radioactivity intensity of each spot in mid-pregnant (MP) myometrium to its intensity in non-pregnant myometrium (NP).

at least two, the majority being upregulated. Table 2 summarizes the differentially expressed genes, arranged according to their functions. Interestingly, the differentially expressed genes included genes related to small G-protein signalling such as RhoB, Rho-GDP dissociation inhibitor 2, Rho-GTPase activating protein 4 and Rnd3 (also known as Rho8 or RhoE) found to be upregulated (Fig. 1). The previously demonstrated inhibitory effect of Rnd proteins on RhoA-dependent processes (Nobes *et al* 1998; Loirand *et al.* 1999) suggested the potential role of the upregulation of Rnd3 in the control of myometrium contractility during pregnancy.

Upregulation of Rnd3 mRNA and protein expression

To confirm up-regulation of Rnd3 at mid-pregnancy, we performed reverse transcription and real-time PCR analysis of non-pregnant and mid-pregnant myometrium RNA with specific oligonucleotides. In addition, since changes in RhoA and Rho kinase expression during pregnancy have previously been reported in other animal species ((Niiro *et al.* 1997; Moore *et al.* 2000; Moran *et al.* 2002; Tahara *et al.* 2002), real-time PCR using Rnd3, RhoA and Rho kinase I primers was also performed with in non-pregnant, mid-pregnant and late-pregnant rabbit myometrium cDNA. Results displayed in Fig. 2*A* confirmed upregulation of Rnd3 mRNA at mid-pregnancy, and showed that this upregulation was maintained up to the end of the pregnancy. The absence of significant changes in RhoA and Rho kinase mRNA levels at mid-pregnancy was also confirmed, whereas an upregulation of both transcripts was detected at late pregnancy, in agreement with previous reports on rat and human (Niiro *et al.* 1997; Moore *et al.* 2000; Moran *et al.* 2002; Tahara *et al.* 2002). Western blot analysis revealed that the upregulation of Rnd3 mRNA correlated with the increase of Rnd3 protein expression both at mid- and late pregnancy (Fig. 2*B*). The following experiments were thus designed to examine whether the upregulation of Rnd3 in mid-pregnant and late-pregnant myometrium was associated with a change in RhoA–Rho kinase-mediated Ca^{2+} sensitization of the contraction.

Ca^{2+} sensitization is abolished at mid-pregnancy

The resting Ca^{2+} sensitivity of contractile proteins has been assessed by measuring the amplitude of the rise in tension produced by a cumulative increase of $[Ca^{2+}]$ (from pCa 8.5 to 4.5) in β-escin permeabilized myometrial muscle strips (Figs 3 and 4). Resting pCa–tension relationships were similar in non-pregnant and mid-pregnant myometrium, as illustrated by the close values of pCa that induced the

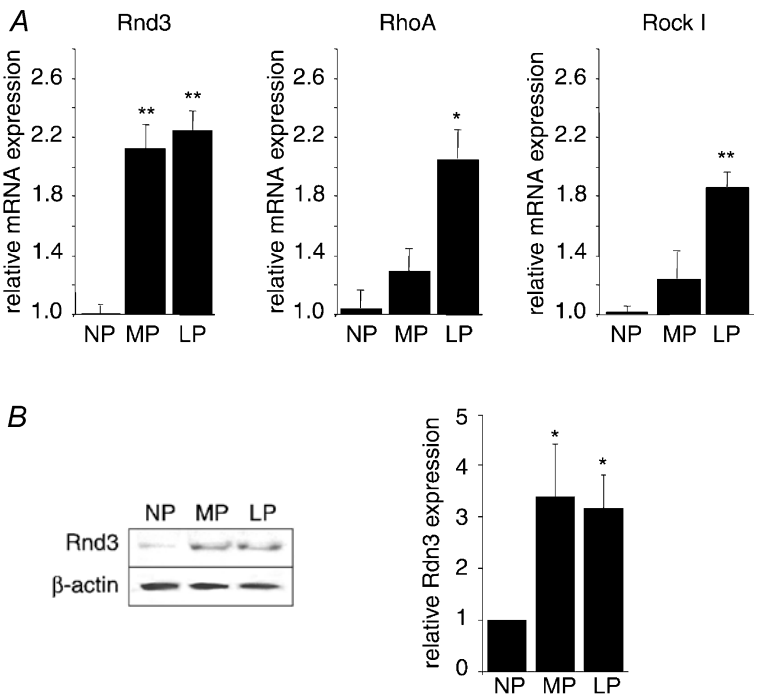

Figure 2. Upregulation of Rnd3

A, real-time RT-PCR assays for Rnd3, RhoA and Rock I mRNA in non-pregnant (NP), mid-pregnant (MP) and late-pregnant (LP) myometrium. Results were normalized to β-actin and expressed as percentages of Rnd3, RhoA or Rock I expression in non-pregnant myometrium (designated 100 %; $n = 3$; * $P < 0.01$; ** $P < 0.001$). *B*, representative Western blot and corresponding densitometric analysis showing the increase in Rnd3 expression in MP and LP myometrium. Results were normalized to β-actin and expressed relative to that in NP as 1 (* $P < 0.01$, $n = 3$).

Table 3. pCa_{50} values in the absence or presence of GTPγS and the maximal amplitude of the contraction induced at pCa 4.5 in non-pregnant, mid-pregnant and late-pregnant β escin-permeabilized myometrium muscle strips

	Resting pCa_{50}	pCa_{50} in the presence of 10 μM GTPγS	Maximal amplitude (pCa 4.5)(mg)
Non-pregnant	6.08 ± 0.06 (9)	6.55 ± 0.04 * (10)	105 ± 14 (19)
Mid-pregnant	5.94 ± 0.03 (6)	6.03 ± 0.03 † (6)	60 ± 7 † (15)
Late-pregnant	6.28 ± 0.04 † (11)	6.29 ± 0.04 † (11)	266 ± 38 † (24)

The number of samples is given in parentheses. * Significantly different from resting pCa_{50} at the same stage ($P < 0.0001$). † Significantly different from non-pregnant under similar conditions.

half-maximal tension (pCa_{50}) (Fig. 4*A* and *B* and Table 3). However, the mean amplitude of the maximal tension raised by pCa 4.5 was significantly smaller at mid-pregnancy compared with responses evoked in non-pregnant myometrium (Table 3). In contrast, the resting sensitivity towards Ca^{2+} was increased at late pregnancy with a significant increase in the pCa_{50} (Fig. 4*C* and Table 3). This was associated with a 2.5-fold increase in the amplitude of pCa 4.5-induced tension, compared with that recorded in non-pregnant myometrium (Table 3).

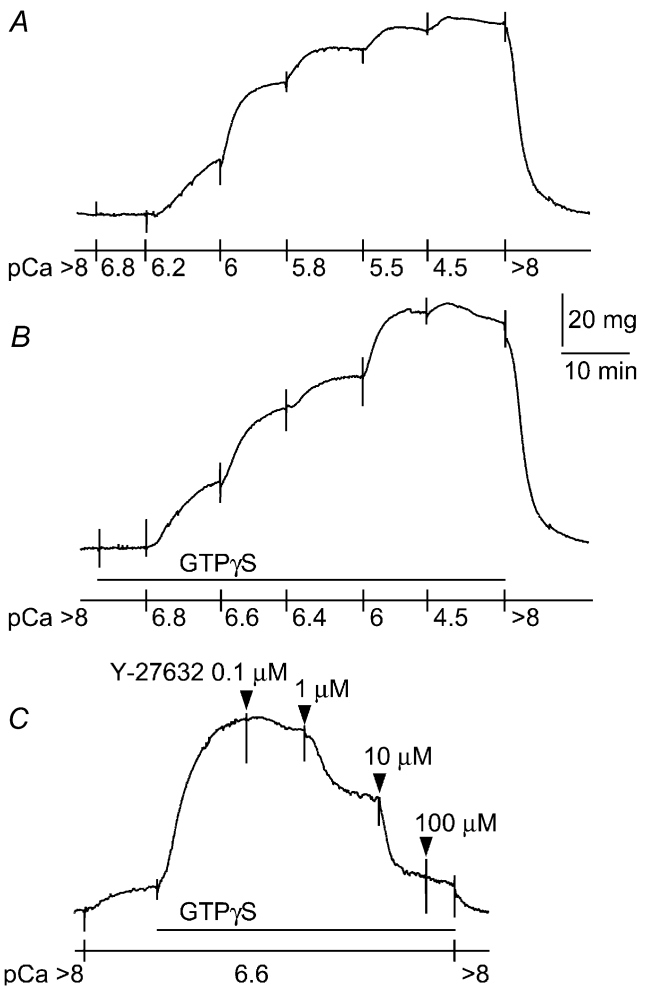

Figure 3. pCa–tension relationship and GTPγS-induced Ca^{2+} sensitization in non-pregnant myometrium

Typical traces illustrating the gradual rise in tension induced by cumulative increases of $[Ca^{2+}]$ in non-pregnant permeabilized myometrium in the absence (*A*) and presence of GTPγS (*B*). The GTPγS-induced Ca^{2+} sensitization was inhibited by the Rho kinase inhibitor Y-27632 in a concentration-dependent way (*C*).

Addition of GTPγS (10 μM) induced Ca^{2+} sensitization of the contractile apparatus, which appeared as a leftward shift of the pCa–tension relationship and a significant increase in the pCa_{50} value in non-pregnant myometrium (Figs 3*A* and *B* and 4*A* and Table 3). The Ca^{2+}-sensitizing effect of GTPγS was completely inhibited by the Rho kinase inhibitor Y-27632 (Uehata *et al.* 1997) with IC_{50} of 1 μM (Fig. 3*C*), but was not modified by the protein kinase C inhibitor GF109203x (5 μM) ($pEC_{50} = 6.03 \pm 0.03$ in control and 6.52 ± 0.02 in the presence of GTPγS, $n = 4$; $P > 0.5$). These results demonstrate the major role of the Rho–Rho kinase pathway in GTPγS-induced Ca^{2+} sensitization. GTPγS-induced leftward_shift of the pCa–tension relationship was not observed in mid-pregnant myometrium, indicating an inhibition of GTPγS-mediated Ca^{2+} sensitization (Fig. 4*B* and Table 3). In late-pregnant myometrium, the Ca^{2+} sensitivity, already high in resting conditions, was not further increased in the presence of GTPγS (Fig. 4*C* and Table 3).

Negative regulation of activating mechanisms and/or positive regulation of inhibitory mechanisms could account for the inhibition of RhoA–Rho kinase-mediated Ca^{2+} sensitization at mid-pregnancy. However, the absence of a change in RhoA and Rho kinase expression in mid-pregnant myometrium (Fig. 2*A*) does not support a direct role for RhoA or Rho kinase, suggesting the interference of an inhibitory mechanism. We therefore hypothesized that the observed upregulation of Rnd3 expression could be involved in the loss of GTPγS-induced Ca^{2+} sensitization at mid-pregnancy.

Farnesyl-transferase inhibitor restored Ca^{2+} sensitization at mid-pregnancy

Rho proteins required isoprenylation at the C-terminus of the proteins to be able to perform their functions. Inhibition of prenylation prevented the membrane localization of the Rho proteins and inhibited their activities (Cohen *et al.* 2000). Rnd proteins share this

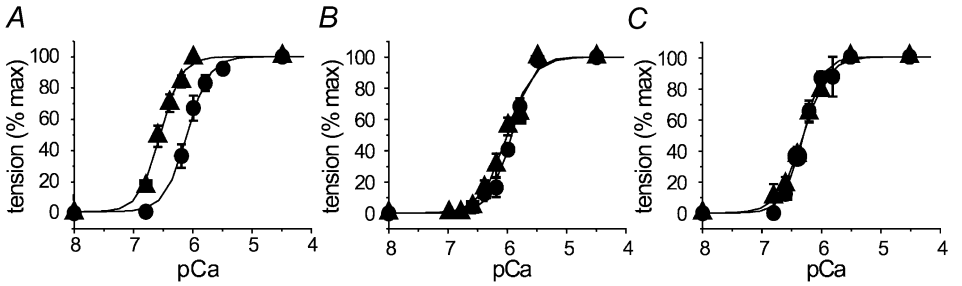

Figure 4. pCa–tension relationships in permeabilized uterine smooth muscle strips

PCa–tension relationships were determined in permeabilized muscle strips from non-pregnant (*A*), mid-pregnant (*B*) and late-pregnant (*C*) myometrium by cumulatively increasing [Ca^{2+}] (pCa 8–4.5), in the absence (●) and presence of 10 μM GTPγS (▲). Tension responses were expressed as the percentage of the maximal response induced at pCa 4.5.

property with other Rho proteins and we have previously demonstrated that non-prenylated Rnd proteins lose their inhibitory activity on RhoA-dependent processes (Loirand *et al.* 1999). Contrary to RhoA, which is geranylgeranylated, Rnd proteins are farnesylated (Nobes *et al.* 1998). This particular property allows the selective inhibition of Rnd protein prenylation using farnesyl-transferase inhibitor (Chardin, 1999). Manumycin A is a farnesyl-transferase inhibitor that binds to the farnesyl-pyrophosphate binding site of farnesyl-transferase (Hara *et al.* 1993). We analysed the effect of manumycin A on the Ca^{2+} sensitivity of mid-pregnant myometrium. While the treatment with manumycin A (10 μM, 12 h) did not affect the resting Ca^{2+} sensitivity (pCa_{50} = 5.95 ± 0.03, n = 5; $P > 0.5$), it restored the GTPγS-induced Ca^{2+} sensitization, as illustrated by the leftward shift of the pCa–tension relationship and the significant increase in the pCa_{50} value to 6.27 ± 0.03 (n = 5; $P < 0.001$ in comparison with the resting value) (Fig. 5). The Ca^{2+} sensitization induced by GTPγS in manumycin A-treated mid-pregnant myometrium muscle strips was completely inhibited by 10 μM Y-27632 (Fig. 5). Similar manumycin A treatment of non-pregnant myometrium had no effect on the pCa–tension relationship either in control conditions or in the presence of GTPγS, indicating that manumycin A did not alter RhoA–Rho kinase signalling (not shown). Similar results have been obtained with the cell-permeable farnesyl-transferase peptidomimetic inhibitor I (FTase inhibitor I) that competed with the acceptor proteins, suggesting that the observed inhibitory action with these pharmacological compounds was not due to non-selective effects (not shown). These results thus indicate that a farnesylated active protein mediated the inhibition of RhoA–Rho kinase-dependent Ca^{2+} sensitization in mid-pregnant myometrium. The absence of effect of manumycin A on non-pregnant myometrium and the upregulation of Rnd3 expression suggests that Rnd3 might be the farnesylated protein responsible for the loss of RhoA–Rho kinase-dependent Ca^{2+} sensitization at mid-pregnancy.

The inhibition of Ca^{2+} sensitization is balanced by RhoA and Rho kinase at the end of pregnancy

As the upregulation of Rnd3 detected at mid-pregnancy is still observed in late-pregnant myometrium, we also assessed the effect of manumycin A in late-pregnant myometrium. Figure 6*A* shows that at this stage of pregnancy, the farnesyl-transferase inhibitor did not modify the resting pCa_{50} (6.26 ± 0.02, n = 5, $P > 0.5$). In addition, contrary to observations in mid-pregnant myometrium, GTPγS remained inefficient in manumycin A-treated late-pregnant myometrium (Fig. 6*A*, pCa_{50} = 6.30 ± 0.03, n = 5, $P > 0.2$). This result suggests that, despite its upregulation, Rnd3 did not exert an inhibitory effect on the Ca^{2+} sensitivity of the contractile apparatus at late pregnancy. Therefore, it is possible that the upregulation of RhoA and Rho kinase observed in late-pregnant myometrium leads to a constitutive activation of the RhoA–Rho kinase pathway, which is responsible for the high resting Ca^{2+} sensitivity of the contractile proteins

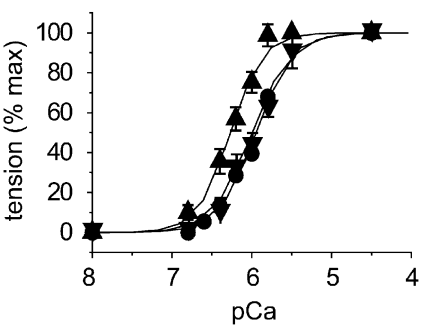

Figure 5. Farnesyl-transferase inhibitor restored Ca^{2+} sensitization at mid-pregnancy

Muscle strips from mid-pregnant myometrium were treated with 10 μM manumycin A for 12 h. pCa–tension relationships were then determined in permeabilized strips by cumulatively increasing [Ca^{2+}] (pCa 8–4.5), in the absence (●) and presence of 10 μM GTPγS without (▲) or with 10 μM Y-27632 (▼). Tension responses were expressed as the percentage of the maximal response induced at pCa 4.5.

and the absence of a GTPγS effect at late pregnancy. Measurements of the amount of active GTP-bound RhoA by pull-down assay were in agreement with this hypothesis (Fig. 6*B*). The amount of active RhoA was similar in non-pregnant and mid-pregnant myometrium, in agreement with the similar resting pCa–tension relationships in both types of samples. In late-pregnant myometrium, the high resting Ca^{2+} sensitivity correlated with a 6.8-fold ± 1.1-fold increase in the amount of active GTP-bound RhoA, relative to the β-actin content ($n = 3$; Fig. 6*B*). According to these data, inhibition of the RhoA–Rho kinase pathway in late-pregnant myometrium under resting conditions would produce a rightward shift of the pCa–tension relationship. Indeed, the Rho kinase inhibitor Y-27632 (10 μM) decreased the resting Ca^{2+} sensitivity, resulting in a significant shift of the pCa–tension relationship towards higher [Ca^{2+}] ($pCa_{50} = 5.90 \pm 0.01$ *versus* 6.22 ± 0.02 μM in control, $n = 5$, $P < 0.005$) (Fig. 6*C*) while the protein kinase C (PKC) inhibitor GF109203x (5 μM) had no effect ($pCa_{50} = 6.25 \pm 0.02$ *versus* 6.25 ± 0.03 in control, $n = 4$, $P > 0.5$). Y-27632 (10 μM) had no effect on the resting Ca^{2+} sensitivity of the contractile proteins in non-pregnant and mid-pregnant myometrium. These observations are therefore in agreement with a major functional consequence of the upregulation of RhoA and Rho kinase, the activity of which could balance the inhibitory effect of Rnd3 on the Ca^{2+} sensitivity of contractile proteins at the end of pregnancy.

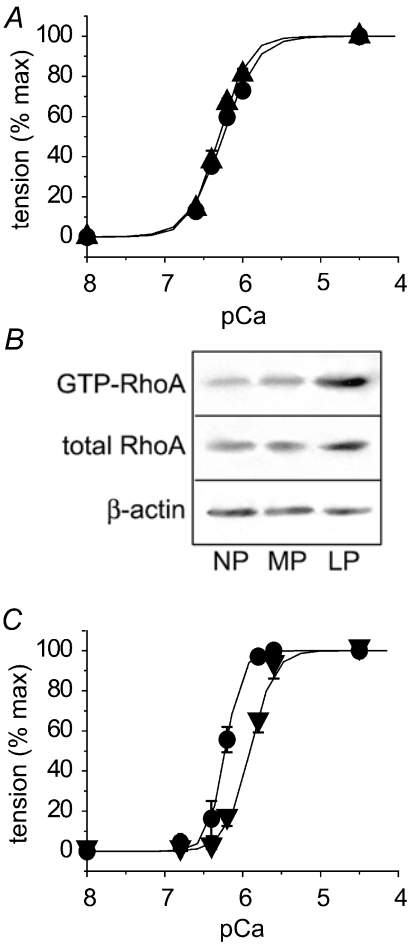

Figure 6. Effect of Rho kinase inhibition and farnesyl-transferase inhibitor on Ca^{2+} sensitivity at late pregnancy

A, muscle strips from late-pregnant myometrium were treated with 10 μM manumycin A for 12 h. pCa–tension relationships were then determined in permeabilized strips by cumulatively increasing [Ca^{2+}] (pCa 8–4.5), in the absence (●) and presence of 10 μM GTPγS (▲). *B,* measurement of the amount of active GTP-bound RhoA by pull-down assay in non-pregnant (NP), mid-pregnant (MP) and late-pregnant (LP) myometrium. Total RhoA and β-actin amounts were also assessed by Western blot in each sample. Results shown are representative of three independent experiments. *C,* pCa–tension relationships obtained in muscle strips from late-pregnant myometrium under control conditions (●) and in the presence of 10 μM Y-27632 (▼). Tension responses were expressed as the percentage of the maximal response induced at pCa 4.5.

DISCUSSION

By a combination of functional and genomic approaches, the present study demonstrates the time-dependent regulation of the Rho–Rho kinase-mediated Ca^{2+} sensitization of contractile proteins in pregnant myometrium, consisting of inhibition of this mechanism at mid-pregnancy followed by upregulation at term. Our results suggest that co-ordinated regulation of Rho protein expression (RhoA and Rnd3) during pregnancy might underlie this phenomenon and therefore participate in both the quiescence of the myometrium during pregnancy and the increase in contractile activity at term.

cDNA array technology holds considerable potential for improving our understanding of physiological or pathophysiological conditions inducing modification of several cellular functions probably related to a change in expression of numerous genes. The dramatic increase in uterine growth, as well as the modulation of contractile properties during pregnancy, requires dynamic remodelling of the myometrial smooth muscle–extracellular matrix interaction and intracellular signalling. We therefore focused on alterations of gene expression involved in cell signalling and interaction. This approach is valid since we were able to confirm changes in gene expression previously identified by individual analysis. This includes upregulation of the extracellular matrix regulator metalloproteinase (MMP)-9, tissue inhibitors of matrix metalloproteinases (TIMP)-1 and TIMP-2, already shown to be increased during pregnancy (Roh *et al.* 2000; Zhao *et al.* 2002). Similarly, by showing upregulation of insulin-like growth factor (IGF) binding proteins (IGFBP) 2 and 6, our results confirmed previous data (Cerro & Pintar, 1997) and further support a role of IGF signalling in the regulation of uterine muscular growth during pregnancy. In addition, our results describe a co-ordinated modulation

of various regulators of Rho protein signalling, involved in the regulation of cell growth and Ca^{2+} sensitization of the contractile apparatus (Somlyo & Somlyo, 1994; Van Aelst & D'Souza-Schorey, 1997). In particular we show for the first time, the expression of Rnd3 in myometrium and its upregulation during pregnancy.

Ca^{2+}-sensitizing mechanisms, which allow the development of maintained tension at low Ca^{2+} concentrations, are major mechanisms regulating smooth muscle contraction. The role of the Rho–Rho kinase-dependent pathway in agonist-induced contraction in myometrium was recently suggested by the use of the Rho kinase inhibitor Y-27632. Rho kinase inhibition induced relaxation of oxytocin-induced contraction in human myometrium at term (Moran *et al.* 2002). In rat myometrium, the sensitivity of oxytocin-induced contraction to Y-27632 was significantly increased at the end of pregnancy compared with non-pregnant samples (Tahara *et al.* 2002), suggesting that RhoA–Rho kinase pathway was upregulated at the end of pregnancy and may participate in increased myometrium contractility at the onset of labour. Our present data are in agreement with these previous reports. In addition, by direct measurements of Ca^{2+} sensitivity in permeabilized muscle strips, we demonstrate for the first time that the increased expression of RhoA and Rho kinase at the end of pregnancy is associated with a constitutive activation of the RhoA–Rho kinase-mediated Ca^{2+} sensitization (Fig. 6*B*). This confirms the involvement of the RhoA–Rho kinase signalling pathway in the generation of powerful uterine contractions at the time of parturition. Other mechanisms have been shown to control Ca^{2+} sensitivity of smooth muscle contraction including phosphorylated CPI-17, which is also a negative regulator of MLCP. Phosphorylation of CPI-17 could be inhibited by either Y-27632 or GF109203x, suggesting that multiple kinases, such as Rho kinase or PKC, could mediate CPI-17 phosphorylation (Kitazawa *et al.* 2000). However, the PKC delta isoform (PKC δ isoform), recognized as the major CPI-17 kinase, is also inhibited by Y-27632 (Eto *et al.* 2001). The role of CPI-17 and its expression have been shown to depend on smooth muscle type. In tonic arterial smooth muscle, which possesses a high CPI-17 expression, PKC–CPI-17 signalling is a dominant contributor towards Ca^{2+} sensitivity of the contraction. In contrast, PKC–CPI-17 signalling makes a minor contribution to Ca^{2+} sensitivity in phasic visceral smooth muscle, that has a low CPI-17 content (Woodsome *et al.* 2001). In myometrium, GTPγS-induced Ca^{2+} sensitization was not affected by GF109203x and the increased sensitivity observed in late-pregnant myometrium was not changed in the presence of GF109203x. This suggests a minor role of PKC–CPI-17 in the regulation of Ca^{2+} sensitivity during pregnancy. The inhibitory effect of Y-27632 on GTPγS-induced Ca^{2+} sensitization indicates that Rho kinase is the major regulator of Ca^{2+} sensitivity. However, we could not rule out the involvement of CPI-17 as a downstream target of Rho kinase involved in the regulation of MLCP activity.

The major finding of the present study is that inhibition of RhoA–Rho kinase-dependent Ca^{2+} sensitization occurred at mid-pregnancy, which could contribute to the low contractile activity required during the major part of pregnancy to avoid pre-term labour. The upregulation of Rnd3 and the effect of farnesyl-transferase inhibitor, which restored RhoA–Rho kinase-mediated Ca^{2+} sensitization in mid-pregnant myometrium, strongly support a role for Rnd3 in the inhibition of RhoA–Rho kinase-mediated Ca^{2+} sensitization.

Rnd3 constitutes with Rnd1 and Rnd2, a subset of Rho proteins with particular properties (Nobes *et al.* 1998). Contrary to other Rho proteins, Rnd proteins lack GTPase activity. Consequently they are constitutively in the active GTP-bound form and attached to the membrane. The involvement of Rnd proteins in signalling pathways and cell functions is therefore directly related to their expression levels, while the role of other Rho proteins essentially depends on their activation–inactivation cycle. Whereas the majority of Rho proteins required geranyl-geranylation to be active, Rnd proteins have to be farnesylated (Foster *et al.* 1996). Although the functional consequence of this difference is not known, this peculiarity of Rnd proteins offers the opportunity to use farnesyl-transferase inhibitors to block their functions. Rnd proteins can be inhibited by treatment with farnesyl-transferase inhibitors for less than 18 h whereas longer treatment (more than 24 h) is required to inhibit farnesylated Ras proteins (Chardin, 1999). Expression of Rnd proteins in fibroblasts promoted disassembly of actin filament structures and loss of cell adhesion, contrary to RhoA that induced actin stress fibre formation. In smooth muscle, Rnd proteins inhibit agonist- and GTPγS-induced Ca^{2+} sensitization by specifically interfering with a RhoA-dependent mechanism (Loirand *et al.* 1999), suggesting that equilibrium between Rnd proteins and RhoA expression and activity might determine the Ca^{2+} sensitivity of the contractile apparatus. Indeed, this study shows that this mechanism contributes to the physiological regulation of uterine smooth muscle contraction during pregnancy. Upregulation of Rnd3 expression at mid-pregnancy correlated with the suppression of RhoA–Rho kinase-mediated Ca^{2+} sensitization. Therefore, the amplitude of agonist-induced contraction should be reduced, irrespective of the nature of the contractile agonist and the expression level of its membrane receptor. Although the mechanism of the inhibitory action of Rnd3 on the RhoA–Rho kinase pathway has not been investigated in this study, the observation that upregulation of Rnd3 at mid-pregnancy did not modify basal RhoA activity suggests that Rnd3 acts downstream of RhoA. These data are therefore in agreement with Rnd3 binding to Rho

kinase and the consequent inhibition of its enzyme activity (Riento *et al.* 2003). The inhibitory action of Rnd3 over-expression on the contractile activity was compensated at the end of pregnancy by the over-expression of RhoA and Rho kinase, which led to a basal high sensitivity of the contractile proteins to Ca^{2+}. Accordingly, a small rise in intracellular Ca^{2+} would be able to trigger powerful contraction at term.

Little is known regarding the mechanisms that control Rho family protein expression. However, the modulation of Rnd3 transcript expression during pregnancy is consistent with the previous demonstration that expression of Rnd proteins was increased by oestrogen and more efficiently by progesterone (Loirand *et al.* 1999). In rabbits, the circulating level of progesterone reached its maximal level at mid-pregnancy (Challis *et al.* 1973) and hence could account for the over-expression of Rnd3 up to the end of pregnancy. The mechanism of action of steroid hormones on Rnd protein expression is not elucidated. However, it has been recently demonstrated that activation of the Raf-MEK-extracellular signal-regulated kinase-induced expression of Rnd3 (Hansen et *al.* 2000). The activity of extracellular signal-regulated kinases was increased by oestrogen and progesterone in uterine smooth muscle (Ruzycky, 1996) and could therefore be involved in the increase of Rnd3 expression occurring during pregnancy.

In conclusion, these data demonstrate the time-dependent regulation of the RhoA–Rho kinase-mediated Ca^{2+} sensitization during the course of pregnancy, with a depression of this mechanism at mid-pregnancy followed by a constitutive activation near term, which correlates with changes in Rnd3 and RhoA expression. Our results thus suggest that the RhoA–Rho kinase signalling pathway and its regulators might thus present potential targets for the development of new treatments for pre-term labour.

REFERENCES

Boyle MB, MacLusky NJ, Naftolin F & Kaczmarek LK (1987). Hormonal regulation of K^+-channel messenger RNA in rat myometrium during oestrus cycle and in pregnancy. *Nature* **330**, 373–375.

Cerro JA & Pintar JE (1997). Insulin-like growth factor binding protein gene expression in the pregnant rat uterus and placenta. *Dev Biol* **184**, 278–295.

Challis JR, Davies J & Ryan KJ (1973). The concentrations of progesterone, estrone and estradiol-17 beta in the plasma of pregnant rabbits. *Endocrinology* **93**, 971–976.

Chardin P (1999). Rnd proteins: a new family of Rho-related proteins that interfere with the assembly of filamentous actin structures and cell adhesion. *Prog Mol Subcell Biol* **22**, 39–50.

Cohen LH, Pieterman E, van Leeuwen RE, Overhand M, Burm BE, van der Marel GA & van Boom JH (2000). Inhibitors of prenylation of Ras and other G-proteins and their application as therapeutics. *Biochem Pharmacol* **60**, 1061–1068.

Coleman HA, Hart JD, Tonta MA & Parkington HC (2000). Changes in the mechanisms involved in uterine contractions during pregnancy in guinea-pigs. *J Physiol* **523**, 785–798.

Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y & Brautigan DL (2001). Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. *J Biol Chem* **276**, 29072–29078.

Europe-Finner GN, Phaneuf S, Watson SP & Lopez Bernal A (1993). Identification and expression of G-proteins in human myometrium: up-regulation of G alphas in pregnancy. *Endocrinology* **132**, 2484–2490.

Foster R, Hu KQ, Lu Y, Nolan KM, Thissen J & Settleman J (1996). Identification of a novel human Rho protein with unusual properties: GTPase deficiency and *in vivo* farnesylation. *Mol Cell Biol* **16**, 2689–2699.

Fujihara H, Walker LA, Gong MC, Lemichez E, Boquet P, Somlyo AV & Somlyo AP (1997). Inhibition of RhoA translocation and calcium sensitization by *in vivo* ADP-ribosylation with the chimeric toxin DC3B. *Mol Biol Cell* **8**, 2437–2447.

Gong MC, Iizuka K, Nixon G, Browne JP, Hall A, Eccleston JF, Sugai M, Kobayashi S, Somlyo AV & Somlyo AP (1996). Role of guanine nucleotide-binding proteins – ras-family or trimeric proteins or both – in Ca^{2+} sensitization of smooth muscle. *Proc Natl Acad Sci U S A* **93**, 1340–1345.

Hansen SH, Zegers MM, Woodrow M, Rodriguez-Viciana P, Chardin P, Mostov KE & McMahon M (2000). Induced expression of Rnd3 is associated with transformation of polarized epithelial cells by the Raf-MEK-extracellular signal-regulated kinase pathway. *Mol Cell Biol* **20**, 9364–9375.

Hara M, Akasaka K, Akinaga S, Okabe M, Nakano H, Gomez R, Wood D, Uh M & Tamanoi F (1993). Identification of Ras farnesyltransferase inhibitors by microbial screening. *Proc Natl Acad Sci U S A* **90**, 2281–2285.

Hirshman CA & Emala CW (1999). Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho. *Am J Physiol* **21**, L653–661.

Horiuti K (1988). Mechanism of contracture on cooling of caffeine-treated frog skeletal muscle fibres. *J Physiol* **398**, 131–148.

Inoue Y & Sperelakis N (1991). Gestational change in Na^+ and Ca^{2+} channel current densities in rat myometrial smooth muscle cells. *Am J Physiol* **260**, C658–663.

Kitazawa T, Eto M, Woodsome TP & Brautigan DL (2000). Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J Biol Chem* **275**, 9897–9900.

Kupittayanant S, Burdyga T & Wray S (2001). The effects of inhibiting Rho-associated kinase with Y-27632 on force and intracellular calcium in human myometrium. *Pflugers Arch* **443**, 112–114.

Lee YH, Hwang MK, Morgan KG & Taggart MJ (2001). Receptor-coupled contractility of uterine smooth muscle: from membrane to myofilaments. *Exp Physiol* **86**, 283–288.

Loirand G, Cario-Toumaniantz C, Chardin P & Pacaud P (1999). The Rho-related protein Rnd1 inhibits Ca^{2+} sensitization of rat smooth muscle. *J Physiol* **516**, 825–834.

Lopez Bernal A, Europe-finner GN, Phaneuf S & Watson SP (1995). Preterm labour: a pharmacological challenge. *Trends Pharmacol Sci* **16**, 129–133.

Moran CJ, Friel AM, Smith TJ, Cairns M & Morrison JJ (2002). Expression and modulation of Rho kinase in human pregnant myometrium. *Mol Hum Reprod* **8**, 196–200.

Moore F, Da Silva C, Wilde JI, Smarason A, Watson SP & Lopez Bernal A (2000). Up-regulation of p21- and RhoA-activated protein kinases in human pregnant myometrium. *Biochem Biophys Res Commun* **269**, 322–326.

Niiro N, Nishimura J, Sakihara C, Nakano H & Kanaide H (1997). Up-regulation of rho A and rho-kinase mRNAs in the rat myometrium during pregnancy. *Biochem Biophys Res Commun* **230**, 356–359.

Nobes CD, Lauritzen I, Mattei MG, Paris S, Hall A & Chardin P (1998). A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol* **141**, 187–197.

Otto B, Steusloff A, Just I, Aktories K & Pfitzer G (1996). Role of Rho proteins in carbachol-induced contractions in intact and permeabilized guinea-pig intestinal smooth muscle. *J Physiol* **496**, 317–329.

Ren XD, Kiosses WB & Schwartz M (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J* **18**, 578–585.

Riento K, Guasch RM, Garg R, Jin B & Ridley AJ (2003). RhoE binds to ROCK I and inhibits downstream signaling. *Mol Cell Biol* **23**, 4219–4229.

Roh CR, Oh WJ, Yoon BK & Lee JH (2000). Up-regulation of matrix metalloproteinase-9 in human myometrium during labour: a cytokine-mediated process in uterine smooth muscle cells. *Mol Hum Reprod* **6**, 96–102.

Ruzycky AL (1996). Effects of 17 beta-estradiol and progesterone on mitogen-activated protein kinase expression and activity in rat uterine smooth muscle. *Eur J Pharmacol* **300**, 247–254.

Schoenwaelder SM & Burridge K (1999). Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* **11**, 274–286.

Somlyo AP (1997). Signal transduction. Rhomantic interludes raise blood pressure. *Nature* **389**, 908–909.

Somlyo AP & Somlyo AV (1994). Signal transduction and regulation in smooth muscle. *Nature* **372**, 231–236.

Taggart MJ, Lee YH & Morgan KG (1999). Cellular redistribution of PKCalpha, rhoA, and ROKalpha following smooth muscle agonist stimulation. *Exp Cell Res* **251**, 92–101.

Tahara M, Morishige K, Sawada K, Ikebuchi Y, Kawagishi R, Tasaka K & Murata Y (2002). RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology* **143**, 920–929.

Togashi H, Emala CW, Hall IP & Hirshman CA. (1998). Carbachol-induced actin reorganization involves Gi activation of Rho in human airway smooth muscle cells. *Am J Physiol* **274**, L803–809.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M & Narumiya S (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990–994.

Van Aelst L & D'Souza-Schorey C (1997). Rho GTPases and signaling networks. *Genes Dev* **11**, 2295–22322.

Woodsome TP, Eto M, Everett A, Brautigan DL & Kitazawa T (2001). Expression of CPI-17 and myosin phosphatase correlates with Ca^{2+} sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. *J Physiol* **535**, 553–564.

Zhao YG, Xiao AZ, Cao XM & Zhu C (2002). Expression of matrix metalloproteinase -2, -9 and tissue inhibitors of metalloproteinase -1, -2, -3 mRNAs in rat uterus during early pregnancy. *Mol Reprod Dev* **62**, 149–158.

Zhong C, Chrzanowska-Wodnicka M, Brown J, Shaub A, Belkin AM & Burridge K (1998). Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J Cell Biol* **141**, 539–551.

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