Increased potency of $\alpha 1$ -adrenergic receptors to induce inositol phosphates production correlates with the up-regulation of $\alpha 1d$ /Gh α /phospholipase C $\delta 1$ signaling pathway in term rat myometrium

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Abstract

In the present study, we studied the potential regulation by rat myometrial $\alpha 1$ -adrenergic receptors ($\alpha 1$ -AR) of the newly identified Gh α protein/phospholipase C $\delta 1$ (PLC $\delta 1$) signaling pathway and compared myometrial inositol phosphates (InsP) production and activity of the uterine circular muscle in response to $\alpha 1$ -AR activation between mid-pregnancy and term. For this, we quantified the level of rat myometrial $\alpha 1$ -AR coupling to Gh α protein by photoaffinity-labeling, the cytosolic amount of PLC $\delta 1$ enzyme by immunoblotting, and the expression level of $\alpha 1$ -AR subtypes by RT-PCR. The results showed an increased level of $\alpha 1$ -AR/Gh α protein coupling and the amount of PLC $\delta 1$ at term (+147 and +65% respectively, versus mid-pregnancy). This was correlated with an up-regulation of $\alpha 1d$ -AR subtype (+70% versus mid-pregnancy). Incubation of myometrial strips with phenylephrine (Phe), a global $\alpha 1$ -agonist, increased InsP production in a dose-dependent manner at both mid-pregnancy and term, but with an enhanced potency (tenfold decrease in EC₅₀ value) at term. Phe also dose-dependently induced contraction of the circular muscle at both mid-pregnancy and term. However, unlike InsP response, no amelioration of potency was observed at term. Similar results were obtained with the endogenous agonist norepinephrine. Our results show, for the first time, that rat myometrial $\alpha 1d$ -AR/Gh α /PLC $\delta 1$ signaling pathway is up-regulated at term. This is associated with an increased potency of $\alpha 1$ -AR to elicit InsP production but not uterine contraction at this period. It is thus hypothesized that $\alpha 1$ -AR, through activation of Gh α /PLC $\delta 1$ system, are not primarily involved in the initiation of labor but may rather regulate responses such as myometrial cell proliferation or hypertrophy.

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Introduction

It is well established that catecholamines (norepinephrine, epinephrine) regulate uterine contractility. The first evidence came from Rüsse and Marshall's works, which showed that norepinephrine, through activation of α -adrenergic receptors (α -AR), enhances the effects of uterotonic agents by improving the excitability of the uterine muscle in the guinea pig (Rüsse & Marshall 1970). Moreover, Legrand and Maltier have reported evidences for a noradrenergic transmission in the control of both pregnancy and parturition in rat. For instance, administration of propranolol, a β -AR antagonist, advanced pregnancy (Maltier & Cavaillé 1975). In contrast, administration to near term rats of prazosin, a powerful α -AR antagonist, reduced myometrial excitability and delayed parturition (Legrand & Maltier 1986).

Norepinephrine signals through nine different G-protein-coupled ARs divided into three subfamilies, namely $\alpha 1$ ($\alpha 1a$, $\alpha 1b$, $\alpha 1d$), $\alpha 2$ ($\alpha 2a$, $\alpha 2b$, $\alpha 2c$), and β ($\beta 1$, $\beta 2$, $\beta 3$). Using specific radioligands, we have shown that β - and $\alpha 2$ -AR are co-localized in the longitudinal muscle, whereas $\alpha 1$ -AR are expressed in the circular layer of rat myometrium (Legrand *et al.* 1991, 1993). Co-activation of β - and $\alpha 2$ -AR increases myometrial cAMP production at mid-pregnancy (Mhaouty *et al.* 1995), thereby resulting in a relaxation of the longitudinal uterine smooth muscle. Near term, progesterone withdrawal associated with increase in estradiol concentrations trigger desensitization of β -adrenergic signaling pathway (Cohen-Tannoudji *et al.* 1991) and $\alpha 2$ -AR shift to inhibitors of adenyl cyclase (Mhaouty *et al.* 1995). These events probably participate in the initiation of labor.

Activation of global $\alpha 1$ -AR triggers phospholipase C (PLC)-mediated degradation of phosphatidyl-inositol in plasma membrane preparations from late pregnant rat and human myometrium (Breuiller-Fouche *et al.* 1991, Limon-Boulez *et al.* 1997). Different mammalian PLC isoforms have been described including four β (PLC β 1–4), two γ (γ 1–2), four δ (δ 1–4), and ϵ . Among the different subfamilies of PLCs, PLC β members are triggered by G-protein-coupled receptors. In many smooth muscle cell types, stimulation of the Gq α /PLC β system is intimately linked to contraction through inositol phosphates (InsP $_3$)-dependent liberation of Ca $^{2+}$ from intracellular stores. In line with these data, we have shown that the up-regulation of rat myometrial PLC β 1 and PLC β 3 and Gq α protein correlates well with the increased uterine responsiveness to oxytocin and carbachol at term (Houdeau *et al.* 2005). In pregnant and parturient rat myometrium, we have also shown that stimulation of $\alpha 1$ -AR results in the activation of Gh α protein pathway (Dupuis *et al.* 2004). Gh α protein is a bifunctional enzyme with both transglutaminase and GTPase activities (Nakaoka *et al.* 1994). By virtue of its GTP binding/GTPase activity, Gh α protein acts as a signaling molecule. Nevertheless, unlike the Gq α subunit, Gh α protein does not interact with the members of the PLC β family but selectively activates PLC δ 1 enzyme (Das *et al.* 1993, Feng *et al.* 1996, Baek *et al.* 2001). The role of the Gh α /PLC δ 1 signaling pathway in smooth muscle cells remains to be clearly identified. PLC δ 1 is, however, known to be induced during the cell cycle or by stress responses (Yagisawa *et al.* 2006).

Therefore, in light of all these data, the present work was undertaken to compare the level of rat myometrial $\alpha 1$ -AR/Gh α coupling, the expression pattern of $\alpha 1$ -AR subtypes and PLC δ 1 enzyme, and the potency of $\alpha 1$ -AR to induce both myometrial InsP production and uterine contraction of the circular muscle between mid-pregnancy and term.

Results

Quantification of Gh α /PLC δ 1 signaling components in mid-pregnant and parturient rat

We recently reported that Gh α protein, a new class of GTP-binding proteins, functionally interacts with the rat myometrial $\alpha 1$ -AR (Dupuis *et al.* 2004). In order to determine whether Gh α protein plays an important role in the transduction of rat myometrial $\alpha 1$ -AR, we compared by photoaffinity labeling studies $\alpha 1$ -AR/Gh α coupling between mid-pregnancy and term. Figure 1A illustrates the net incorporation of [α - 32 P]GTP into Gh α protein following activation of $\alpha 1$ -AR by 100 μ M Phe. Our results show that $\alpha 1$ -AR/Gh α coupling occurred on day 12 of pregnancy and significantly increased at term (2.47- and 2.1-fold increases at term versus days 12 and 15 of pregnancy respectively; $P < 0.05$). We also quantified, by immunoblotting, the cytosolic amount of PLC δ 1 enzyme, the known target of $\alpha 1$ -AR/Gh α coupling. Results illustrated in Fig. 1B show that the

Figure 1 (A) Quantification of [α - 32 P]GTP-photolabeled Gh α protein following stimulation of $\alpha 1$ -AR by 100 μ M phenylephrine at days 12 (D12), 15 (D15), and 20 (D20) of pregnancy and at term (T). Results are expressed as percentage of basal photolabeled Gh α (without phenylephrine) and are means $\pm$ S.E.M. of three independent experiments done in duplicate. ^a $P < 0.05$ versus D12 and D15. (B) Upper panel: representative western blots for PLC δ 1 at pregnancy on days 12 (D12), 15 (D15), and 20 (D20) of pregnancy and at term (T); lower panel: quantitative data are expressed as percentage of term and are means $\pm$ S.E.M. of four independent experiments. ^a $P < 0.05$ versus D12 and D15; ^b $P < 0.05$ versus D12, D15, and D20.

amount of PLC δ 1 increases progressively from day 12 to term when it finally reaches a maximal level (+65% at term versus day 12 of pregnancy; $P < 0.05$).

Altogether, these results indicate that the rat myometrial $\alpha 1$ -AR/Gh α /PLC δ 1 signaling pathway is up-regulated at the end of pregnancy.

Correlation between rat myometrial $\alpha 1$ d- expression and $\alpha 1$ -AR/Gh α coupling

It is well known that Gh α protein selectively interacts with $\alpha 1$ b- and $\alpha 1$ d- but not with $\alpha 1$ a-subtype (Chen *et al.* 1996). We thus characterized the expression of rat myometrial $\alpha 1$ -adrenergic subtypes using RT-PCR technique. Our results show that the three $\alpha 1$ -subtypes are present in rat

Figure 2 Myometrial mRNA expression of $\alpha 1$ -AR subtypes during pregnancy. (A) Total RNA isolated from rat myometrium at days 12 (D12), 15 (D15), and 20 (D20) of pregnancy and at term (T) was reverse-transcribed and amplified for $\alpha 1a$ -, $\alpha 1b$ -, and $\alpha 1d$ -AR subtypes. (B) The average results from three independent experiments are expressed as percentage of T. ^a $P < 0.05$ versus previous stages of pregnancy; ^b $P < 0.05$ versus D12 and D15.

myometrium but exhibit a differential expression during the course of pregnancy (Fig. 2A). Indeed, $\alpha 1a$ - and $\alpha 1d$ -transcripts were detected from day 12 of pregnancy until term with a significant lower level of expression (-69% , $P < 0.05$) for $\alpha 1a$ - and a progressive increase ($+70\%$, $P < 0.05$) for $\alpha 1d$ - between day 12 of pregnancy and term (Fig. 2B). In contrast, detectable levels of $\alpha 1b$ -mRNAs were observed only from day 15 of pregnancy, with a significant decreased expression at term (-50% versus day 15, $P < 0.05$).

The clear-cut increase in $\alpha 1d$ mRNA in late pregnant myometrium concomitant with the sharp decrease in its $\alpha 1b$ counterpart at term lead us to suggest that $\alpha 1d$ -AR might be a good candidate for the activation of Gh α /PLC $\delta 1$ system. Due to the lack of enough selective tools that could discriminate in functional studies between $\alpha 1b$ - and $\alpha 1d$ -subtypes, we examined the relationship between the amount of $\alpha 1$ -AR/Gh α protein coupling and $\alpha 1b$ or $\alpha 1d$ expression. The change in the expression of $\alpha 1d$ -mRNA correlated strongly with the change in the amount of $\alpha 1$ -AR/Gh α protein coupling ($r^2 = 0.905$ for $\alpha 1d$ versus 0.277 for $\alpha 1b$; Fig. 3A and B). As a positive control, we assessed the relationship between the myometrial amounts of $\alpha 1$ -AR/Gh α protein coupling and PLC $\delta 1$. Figure 3C shows a highly significant positive correlation ($r^2 = 0.840$), indicating a tight link between these two events.

Altogether, these data strongly suggest the involvement of rat myometrial $\alpha 1d$ -AR in the regulation of Gh α protein/PLC $\delta 1$ signaling pathway at the end of pregnancy.

Myometrial InsP response to the activation of $\alpha 1$ -AR in mid-pregnant and term rat

Myometrial strips from mid-pregnant or parturient rats were incubated with increasing concentrations of Phe, a global $\alpha 1$ -AR agonist. As basal InsP production significantly increases at term (Houdeau *et al.* 2005), Phe-induced InsP accumulation was expressed as the percentage of its corresponding basal in order to evaluate the net responses due to the specific activation of $\alpha 1$ -AR. Results illustrated in Fig. 4A show that Phe dose-dependently enhanced InsP production at both mid-pregnancy and term. The E_{max} was unchanged between day 15 of pregnancy and term, while the EC_{50} value was significantly decreased at term ($P < 0.05$, Table 1) as evidenced by the leftward shift in the dose-response curve. Pre-treatment of myometrial strips with $100 \mu M$ prazosin, an $\alpha 1$ -AR antagonist, completely blocked InsP production in response to maximally effective Phe ($1 \mu M$) in both pregnant and term rats (Fig. 5). This indicates that InsP response is due to the specific activation of $\alpha 1$ -AR at both stages of pregnancy.

These results show that the up-regulation of rat myometrial $\alpha 1$ -AR/Gh α /PLC $\delta 1$ signaling pathway is associated with an amelioration of PLC/InsP response at term.

Figure 3 A study of correlation between the amount of $\alpha 1$ -AR-coupled Gh α protein and the expression of $\alpha 1b$ (A), $\alpha 1d$ (B), and PLC $\delta 1$ (C) in pregnant rat myometrium.

Uterine contraction induced by rat myometrial $\alpha 1$ -AR at mid-pregnancy and term

Since PLC activation is generally linked to uterine contraction, we compared, for the first time, InsP production and contraction of the circular layer of smooth muscle uterus in response to $\alpha 1$ -AR activation. We have previously reported that $\alpha 1$ -AR are mainly located in the uterine circular muscle (Legrand *et al.* 1991). This was confirmed by our recent functional studies, which showed that Phe specifically enhances the activity of the uterine circular muscle and has no effect on the longitudinal layer (Mhaouty-Kodja *et al.* 2004). We thus studied the contractile activity of the circular muscle layer in response to increasing concentrations of Phe. All uterine strips exhibited spontaneous

Figure 4 (A) Myometrial inositol phosphates (InsP) production in response to increasing concentrations (0.1 nM–1 mM) of phenylephrine (Phe) at day 15 of pregnancy (D15) and at term (T). (B) Contractile activity of the circular layer of myometrium in response to increasing concentrations (0.1 nM–1 mM) of phenylephrine (Phe) at D15 of pregnancy and term. (C) A comparison of the effect of increasing concentrations of Phe and norepinephrine (NE) on contractile activity of the circular layer of myometrium at term. Results are expressed as the percentage of basal InsP production (A) or spontaneous contraction (B and C) and are means $\pm$ S.E.M. of three to six experiments done in triplicate. ^a $P < 0.05$ versus the same concentration at D15.

rhythmic contractions a few minutes after being mounted on the bath (data not shown). As illustrated in Fig. 1B, Phe elicited a dose-dependent increase in uterine activity at both mid-pregnancy and term. An

Table 1 EC₅₀ values for contractile activity of the rat uterine circular muscle and myometrial InsP production elicited by phenylephrine on day 15 of pregnancy and at term.

	Day 15	Term
Contraction	1 $\pm$ 0.2 μ M	0.7 $\pm$ 0.3 μ M
InsP production	41 $\pm$ 16 nM	4 $\pm$ 1 nM*

Numbers are means $\pm$ S.E.M. ($n=3-6$). * $P<0.05$ versus day 15 of pregnancy.

analysis of the sigmoid dose–response curves revealed no changes in the potency and maximal response for Phe between mid-pregnancy and term (Fig. 4B, Table 1). When challenged with the endogenous agonist norepinephrine, uterine strips exhibited a dose-dependent contraction (Fig. 4C) with an EC₅₀ value at the micromolar range ($2 \pm 0.4 \mu$ M) similar to that obtained for Phe. Again, no change was observed in the maximal response (not shown).

Altogether, these results show that the up-regulation of $\alpha 1$ -AR/ $Gh\alpha$ protein/PLC $\delta 1$ and the amelioration of InsP response at term are not primarily linked to contractile activity of the circular muscle.

Discussion

We report, in the present study, an up-regulation of $\alpha 1$ -AR/ $Gh\alpha$ protein/PLC $\delta 1$ signaling pathway in term rat myometrium. Indeed, both the level of $\alpha 1$ -AR/ $Gh\alpha$ protein coupling and the amount of PLC $\delta 1$ significantly increased at this period. The enhanced $\alpha 1$ -AR/ $Gh\alpha$ interaction could be explained by the induction of $Gh\alpha$ expression at both mRNAs and protein levels as reported previously (Dupuis *et al.* 2004). In addition, we also

showed that, among the $Gh\alpha$ -protein-coupled $\alpha 1$ -AR, the expression of $\alpha 1b$ -AR was down-regulated, whereas that of $\alpha 1d$ -AR was significantly enhanced at term. Interestingly, we found a good temporal correlation between $\alpha 1$ -AR/ $Gh\alpha$ coupling and $\alpha 1d$ -AR expression during the course of pregnancy. The regulatory factors underlying this up-regulation need to be determined. It is, however, interesting to note that we did not find any effect of progesterone or estradiol on $Gh\alpha$ expression (data not shown).

We also report that the up-regulation of $\alpha 1$ -AR/ $Gh\alpha$ protein/PLC $\delta 1$ signaling pathway correlates well with the increased potency of $\alpha 1$ -AR to induce InsP production (a tenfold lower EC₅₀ value) at term. This is in agreement with the increased Phe-stimulated PLC activity reported in plasma membrane preparations from term rat myometrium (Limon-Boulez *et al.* 1997). Surprisingly, this was not associated with an amelioration of uterine contraction in response to either Phe or the endogenous agonist norepinephrine. Parallel experiments where InsP production and contractile activity of the circular muscle were measured in response to oxytocin indicated a higher potency for this agonist to elicit both responses at term (Houdeau *et al.* 2005). This increased term sensitivity to oxytocin correlated well with the up-regulation of OTR, Gq α protein, and PLC $\beta 1$ /PLC $\beta 3$ enzymes as shown previously (Houdeau *et al.* 2005). Indeed, progesterone withdrawal and the increase in estradiol concentrations at the end of pregnancy induce expression of all these signaling molecules (Houdeau *et al.* 2005). As shown by our previous pharmacological studies (Limon-Boulez *et al.* 1997) and confirmed by the present study, rat myometrium expresses a heterogeneous population of $\alpha 1$ -AR. The three $\alpha 1$ -AR subtypes ($\alpha 1a$, $\alpha 1b$, and $\alpha 1d$) are present during the second half of pregnancy although they exhibit different expression patterns. Previous studies attempted to assign different roles to $\alpha 1$ -AR subtypes in the regulation of uterine contraction (Ducza *et al.* 2002, Mihalyi *et al.* 2003). It is, however, clear from the present study that, in physiological conditions where the three $\alpha 1$ -AR subtypes are co-activated by their endogenous ligand norepinephrine, contraction of the circular muscle is induced at term but not as potently as does oxytocin. Indeed, the EC₅₀ values calculated for norepinephrine and phenylephrine are at the micromolar range compared with the 20 nM obtained for oxytocin under similar experimental conditions (Houdeau *et al.* 2005). It thus appears that rat myometrial $\alpha 1$ -AR are not efficiently linked to the classical Gq α /PLC β system and consequently to uterine contraction. In line with this finding, we have previously shown that the Gq α /PLC β -coupled $\alpha 1a$ -AR subtype undergoes uncoupling at term (Limon-Boulez *et al.* 1997). This, together with the presently reported down-regulation of $\alpha 1b$ -AR expression and the emergence of the $\alpha 1d$ -AR/ $Gh\alpha$

Figure 5 Effect of an $\alpha 1$ -adrenergic antagonist prazosin (Praz, 100 μ M) on myometrial inositol phosphates (InsP) production elicited by 1 μ M phenylephrine (Phe) at day 15 of pregnancy (D15) and at term (T). Results are expressed as the percentage of basal InsP production without Phe or Praz and are means $\pm$ S.E.M. of three independent experiments. ^a $P<0.05$ versus basal InsP response; ^b $P<0.05$ versus InsP production in the presence of Phe.

PLC δ 1 signaling pathway, strongly suggests that α 1-AR preferentially signal through Gh α /PLC δ 1 at term.

In myometrium, the physiological meaning of the emergence of α 1d-AR/Gh α /PLC δ 1 signaling pathway in the late pregnant rat myometrium remains to be determined. However, it is interesting to note that the plasma membrane α 1-AR/Gh α interaction takes place at mid-pregnancy and progressively increases until term. One may then postulate that this signaling pathway participates to the myometrial cell hypertrophy or proliferation that occurs during this period. Indeed, Gh α protein was shown to mediate α 1-AR-induced hepatocyte proliferation (Wu *et al.* 2000) and activation of extracellular signal-regulated kinases in neonatal rat cardiomyocytes (Lee *et al.* 2003). In agreement with these results, we have recently shown that Gh α protein participates to the α 1-AR-mediated regulation of smooth muscle cell proliferation (Dupuis *et al.* 2004). Alternatively, the α 1/Gh α /PLC δ 1 system may also play a role in the preparation of uterus to labor by regulating the expression of some contraction-associated proteins. For instance, the directed overexpression of Gh α protein in cardiomyocytes resulted in the up-regulation of cyclooxygenase-2, prostaglandin F 2α receptors, and Gi proteins (Zhang *et al.* 2003). Mice lacking tissue transglutaminase II/Gh α protein do not seem to exhibit any alteration in the pregnancy or parturition processes. However, it is possible that compensatory mechanisms taking place during development mask the real importance of myometrial Gh α protein. One way to answer this question *in vivo* is to induce the conditional deletion of transglutaminase II/Gh α in pregnant myometrium by using the smooth muscle-inducible CreERT system as was recently described by Döring *et al.* (2006).

In conclusion, the present work describes the up-regulation of different transduction components involved in the newly identified α 1/Gh α /PLC δ 1 signaling pathway in rat myometrium between mid-pregnancy and term. The increased α 1-AR/Gh α coupling as well as α 1d-AR and PLC δ 1 expression correlates well with the increased potency of α 1-AR to induce myometrial InsP production but not uterine contraction at term. This lead us to hypothesize that myometrial α 1d-AR, through activation of Gh α /PLC δ 1 system, are not primarily involved in the initiation of labor but may either induce the expression of contraction-associated proteins during the phase of uterine preparation to labor and/or regulate other uterine responses like myometrial cell hypertrophy or proliferation.

Materials and Methods

Animals

Sprague–Dawley rats were obtained from Janvier (Le Genest, France). The females were caged with males overnight and successful mating was determined by the presence of spermatozoa in the vaginal smear (day 1 of pregnancy).

Animals were killed by cervical dislocation at days 12, 15, 20 of pregnancy or at term during the expulsion of fetoplacental units, following the guidelines laid down by the NIH Guide. The uterine horns were quickly isolated, cut open lengthwise and the fetoplacental units removed. The myometrium was then freed of the adherent endometrium except for uterine tension studies.

RT-PCR

Using SuperScript Reverse Transcriptase kit (Gibco BRL Life Technologies), 5 μ g total RNA was reverse-transcribed and the resulting cDNA was stored at -80°C . PCR amplification was performed with 1/20 volume of each RT reaction using specific upstream and downstream primers for α 1a-, α 1b-, and α 1d-AR subtypes and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (Mhaouty-Kodja *et al.* 2001, Dupuis *et al.* 2004). For semi-quantitative analysis, PCR cycle profiles were conducted and the cycle number (25 cycles) was chosen from the linear portion of the curve. The PCR products were separated by electrophoresis on ethidium bromide containing 2% agarose gel.

Preparation of myometrial fractions

Rat myometrium was homogenized in buffer A (50 mM Tris (pH 7.3), 100 mM NaCl, 2 mM MgCl $_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.32 M sucrose) for Western blot analysis, and in buffer B (10 mM HEPES (pH 7.5), 250 mM sucrose, 5 mM EGTA) for photoaffinity labeling experiments supplemented with a cocktail of protease inhibitors (Sigma–Aldrich). After 10 min centrifugation at 4°C , the supernatants were collected and submitted to 100 000 *g* centrifugation at 4°C for 1 h to separate plasma membranes from cytosol. The pellet containing plasma membranes was resuspended in a homogenization buffer and the protein concentration of plasma membrane and cytosolic fractions was determined according to Bradford (1976) with BSA as standard. Samples were stored at -80°C until use.

Western blot analysis

Cytosolic samples (20 μ g protein) from rat myometrium were separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Perkin–Elmer Life Sciences, Paris, France). The blots were blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% non-fat-dried milk and incubated for 1 h at room temperature with monoclonal anti-PLC δ 1 (Upstate Biotechnology, Euromedex, Souffelweyersheim, France) diluted 1:500. Incubation with secondary anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:10 000 dilution) was carried out for 45 min at room temperature. Immunoreactive bands were visualized by the chemiluminescence detection system (Amersham Pharmacia Biotech) and quantified by densitometric scanning followed by computer analysis using the NIH image 1.62 program (<http://rsb.info.nih.gov/ni-image/download.html>).

Photoaffinity labeling

Photoaffinity labeling of Gh α with [α - ^{32}P]GTP (3000 Ci/mmol, Perkin–Elmer Life Sciences) was carried out as described previously (Dupuis *et al.* 2004). Briefly, plasma membrane fractions (200 μg protein) were incubated with 10 μCi [α - ^{32}P]GTP for 10 min at 30 °C in a labeling buffer in the absence or presence of 100 μM Phe (Sigma–Aldrich). Reactions were stopped in ice and the samples were irradiated with u.v. light (254 nm) for 20 min and solubilized with Laemmli solution for 1 h at room temperature. The samples were then subjected to SDS-PAGE in 7.5% gels and transferred to the PVDF membranes. The membranes were first autoradiographed and then immunodetected with a monoclonal anti-Gh α diluted 1:500 from NeoMarkers (Interchim, Monluçon, France). The obtained levels of labeled Gh α were normalized with the corresponding amount of immunodetected protein. Densitometric scanning followed by computer analysis using NIH image 1.62 program was used to determine the levels of labeled and immunodetected Gh α protein.

Measurement of myometrial InsP accumulation

InsP production was measured as described previously (Mhaouty-Kodja *et al.* 2001). Myometrial strips were incubated at 37 °C for 4 h with 7 μCi myo-[^3H]inositol (10–25 Ci/mmol, Perkin–Elmer Life Sciences) in 1 ml Krebs bicarbonate buffer. Increasing concentrations (0.1 nM–1 mM) of Phe were added after 10 min incubation of the myometrial strips with 10 mM LiCl (Sigma–Aldrich) in Krebs bicarbonate buffer. Assays were stopped 15 min later by freezing the strips in liquid N₂. When used, 100 μM pazosin was added 15 min before the addition of 100 μM Phe.

Strips were homogenized in 7% trichloroacetic acid and the obtained supernatant was extracted with diethyl ether, neutralized with Tris-base, and then chromatographed over an anion-exchange resin (AG1-X8, Bio-Rad Laboratories). Total InsP eluted with 1 M ammonium formate/0.1 M formic acid (Sigma–Aldrich) were counted by liquid scintillation in a 1214 Rack-beta spectrometer (LKB, Turku, Finland) for tritium.

Uterine contraction studies

Uterine strips, 4 mm long, were prepared from pregnant rats and mounted in organ baths containing 8 ml Krebs solution as described previously (Mhaouty-Kodja *et al.* 2001). We measured isometric contractions of the circular muscle using a Bioscience UF1 tension transducer (Phymep, Paris, France) under 0.7 g resting force. A 30 min equilibration period was allowed before adding cumulative doses of Phe (0.1 nM–1 mM). The concentration–response curves were recorded by computerized calculation of the integral under the tension/time curve for 3 min using Prism 4 software (Graph Pad, San Diego, CA, USA) for sigmoidal dose–response (nonlinear regression fit). Isometric changes in tissue tension and maximal effects (E_{max}) were expressed as a percentage above the spontaneous activity in the absence of agonists, and potency as EC_{50} .

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical significance was assessed by Student's *t*-test for unpaired data. $P < 0.05$ was considered significant.

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References

- Baek KJ, Kang SK, Damron DS & Im M-J 2001 Phospholipase C $\delta 1$ is a guanine nucleotide exchanging factor for transglutaminase II (G α h) and promotes $\alpha 1\text{B}$ -adrenoceptor-mediated GTP binding and intracellular calcium release. *Journal of Biological Chemistry* **276** 5591–5597.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Breuiller-Fouche M, Doualla-Bell Kotto Maka F, Geny B & Ferre F 1991 Alpha-1 adrenergic receptor: binding and phosphoinositide breakdown in human myometrium. *Journal of Pharmacology and Experimental Therapeutics* **258** 82–87.
- Chen S, Lin F, Iismaa S, Lee KN, Birckbichler PJ & Graham RM 1996 $\alpha 1$ -adrenergic receptor signaling via Gh is subtype specific and independent of its transglutaminase activity. *Journal of Biological Chemistry* **271** 32385–32391.
- Cohen-Tannoudji J, Vivat V, Heilmann J, Legrand C & Maltier JP 1991 Regulation by progesterone of the high-affinity state of myometrial β -adrenergic receptor and of adenylate cyclase activity in the pregnant rat. *Journal of Molecular Endocrinology* **6** 137–145.
- Das T, Baek KJ, Gray C & Im M-J 1993 Evidence that the Gh protein is a signal mediator from $\alpha 1$ -adrenoceptor to a phospholipase C. II. Purification and characterization of a Gh-coupled 69-kDa phospholipase C and reconstitution of $\alpha 1$ -adrenoceptor, Gh family, and phospholipase C. *Journal of Biological Chemistry* **268** 27390–27397.
- Döring B, Shynlova O, Tsui P, Eckardt D, Janssen-Bienhold U, Hofmann F, Feil S, Feil R, Lye SJ & Willecke K 2006 Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition. *Journal of Cell Science* **119** 1715–1722.
- Ducza E, Gaspar R & Falkay G 2002 Altered levels of mRNA expression and pharmacological reactivity of $\alpha 1$ -adrenergic receptor subtypes in the late-pregnant rat myometrium. *Molecular Reproduction and Development* **62** 343–347.
- Dupuis M, Lévy A & Mhaouty-Kodja S 2004 Functional coupling of rat myometrial $\alpha 1$ -adrenergic receptors to Gh α /tissue transglutaminase 2 during pregnancy. *Journal of Biological Chemistry* **279** 19257–19263.
- Feng J-F, Rhee SG & Im M-J 1996 Evidence that phospholipase $\delta 1$ is the effector in the Gh (transglutaminase II)-mediated signaling. *Journal of Biological Chemistry* **271** 16451–16454.
- Houdeau E, Lévy A & Mhaouty-Kodja S 2005 Up-regulation of rat myometrial phospholipases C $\beta 1$ and C $\beta 3$ correlates with increased term sensitivity to carbachol and oxytocin. *Journal of Endocrinology* **187** 197–204.
- Lee K-H, Lee N, Lim S, Jung H, Ko Y-G, Park H-Y, Jang Y, Lee H & Hwang K-C 2003 Calreticulin inhibits the MEK1,2-ERK1,2 pathway in $\alpha 1$ -adrenergic receptor/Gh-stimulated hypertrophy of neonatal rat cardiomyocytes. *Journal of Steroid Biochemistry and Molecular Biology* **84** 101–107.
- Legrand C & Maltier JP 1986 Evidence for a noradrenergic transmission in the control of parturition in the rat. *Journal of Reproduction and Fertility* **76** 415–424.
- Legrand C, Vivat V, Rigolot C & Maltier JP 1991 Selective distribution of alpha-1 and beta adrenoceptors in pregnant rat uterus visualized by autoradiography. *Journal of Pharmacology and Experimental Therapeutics* **256** 767–772.
- Legrand C, Mhaouty S, Rigolot C & Maltier JP 1993 Heterogeneity of alpha-2 adrenoceptors in pregnant rat uterus: identification of subtypes and autoradiographic distribution. *Journal of Pharmacology and Experimental Therapeutics* **266** 439–449.

- Limon-Boulez I, Mhaouty-Kodja S, Coudouel N, Benoit de Coignac A, Legrand C & Maltier JP** 1997 The $\alpha 1B$ -adrenergic receptor subtype activates the phospholipase C signaling pathway in rat myometrium at parturition. *Biology of Reproduction* **57** 1175–1182.
- Maltier JP & Cavaille F** 1975 Effects of injections of monoamine oxidase inhibitor or saline into the uterus in late pregnancy on uterine catecholamine levels related to abnormal parturition in rats. *Journal of Endocrinology* **67** 371–383.
- Mhaouty S, Cohen-Tannoudji J, Bouet-Alard R, Limon-Boulez I, Maltier JP & Legrand C** 1995 Characteristics of the $\alpha 2$ -/ $\beta 2$ -adrenergic receptor-coupled adenylyl cyclase system in rat myometrium during pregnancy. *Journal of Biological Chemistry* **270** 11012–11016.
- Mhaouty-Kodja S, Houdeau E, Cohen-Tannoudji J & Legrand C** 2001 Catecholamines are not linked to myometrial phospholipase C and uterine contraction in late pregnant and parturient mouse. *Journal of Physiology* **536** 123–131.
- Mhaouty-Kodja S, Houdeau E & Legrand C** 2004 Regulation of myometrial phospholipase C system and uterine contraction by β -adrenergic receptors in mid-pregnant rat. *Biology of Reproduction* **70** 570–576.
- Mihalyi A, Gaspar R, Csonka D & Falkay G** 2003 Synergism between $\beta 2$ -adrenoceptor agonists and subtype-selective $\alpha 1A$ -adrenoceptor antagonists in the tocolytic effect on pregnant rat uterus *in vitro*. *Clinical and Experimental Pharmacology and Physiology* **30** 164–167.
- Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im M-J & Graham RM** 1994 Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* **264** 1593–1596.
- Russe MW & Marshall JM** 1970 Uterine response to adrenergic nerve stimulation in the guinea pig. *Biology of Reproduction* **3** 13–22.
- Wu J, Liu S-H, Zhu J-L, Norton PA, Nojiri S, Hoek JB & Zern MA** 2000 Roles of tissue transglutaminase in ethanol-induced inhibition of hepatocyte proliferation and $\alpha 1$ -adrenergic signal transduction. *Journal of Biological Chemistry* **275** 22213–22219.
- Yagisawa H, Okada M, Naito Y, Sasaki K, Yamaga M & Fujii M** 2006 Coordinated intracellular translocation of phosphoinositide-specific phospholipase C- δ with the cell cycle. *Biochimica et Biophysica Acta* **1761** 522–534.
- Zhang Z, Vezza R, Plappert T, McNamara P, Lawson JA, Austin S, Pratico D, St-John Sutton M & FitzGerald GA** 2003 COX-2-dependent cardiac failure in Gh/tTG transgenic mice. *Circulation Research* **92** 1153–1161.

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