British Journal of Pharmacology (2004) 141, 831–841
www.nature.com/bjp

The human near-term myometrial β_3-adrenoceptor but not the β_2-adrenoceptor is resistant to desensitisation after sustained agonist stimulation

*,[1,2]C. Rouget, [2]M. Breuiller-Fouché, [1,3]F.J. Mercier, [2]M.J. Leroy, [4]C. Loustalot, [1]E. Naline, [5]R. Frydman, [7]T. Croci, [8]E.J. Morcillo, [1]C. Advenier & *,[1,6]M. Bardou

[1]UPRES EA220-Pharmacology, UFR Biomédicale des Saints Pères, 45 rue des Saints Pères, Paris 75006, France; [2]INSERM U361, Pavillon Baudelocque, 123 Bd de Port-Royal, Paris 75014, France; [3]Department of Anaesthesiology at CHU Antoine Béclère, 157 rue de la Porte de Trivaux, Clamart 92141, France; [4]Department of Gynaecology CHU du Bocage, 21 Bd de Lattre de Tassigny, BP 1542, Dijon 21000, France; [5]Department of Gynaecology at CHU Antoine Béclère, 157 rue de la Porte de Trivaux, Clamart 92141, France; [6]Laboratory of Cardiovascular Physiopathology and Pharmacology, Faculty of Medicine, 7 Bd Jeanne d'Arc, BP 87900, Dijon 21079, France; [7]Research Centre Sanofi-Midy, Via Piranesi, 38, Milan 20137, Italy and [8]Department of Pharmacology, University of Valencia, Av. Blasco Ibañez 17, Valencia 46010, Spain

1 In order to compare the β_2- and β_3-adrenoceptor (β-AR) desensitisation process in human near-term myometrium, we examined the influence of a pretreatment of myometrial strips with either a β_2- or a β_3-AR agonist (salbutamol or SR 59119A, respectively, both at 10 μM, for 5 and 15 h) on the relaxation and the cyclic adenosine monophosphate (cAMP) production induced by these agonists.

2 To assess some of the mechanisms potentially implicated in the β-AR desensitisation process, we studied the influence of such treatment on the number of β_2- and β_3-AR binding sites, the β_2- and β_3-AR transcripts expression and the phosphodiesterase 4 (PDE4) activity.

3 Salbutamol, but not SR 59119A, concentration–response curve (CRC) was shifted by a 15 h salbutamol preincubation, with a significant difference in $-\log EC_{20}$ values (6.31 ± 0.13 vs 5.58 ± 0.24, for control and 15 h salbutamol pretreatment, respectively, $P<0.05$). Neither salbutamol nor SR 59119A CRCs were modified after a 15 h preincubation with SR 59119A.

4 A 15 h exposure of myometrial strips to salbutamol significantly reduced the salbutamol-induced (0.60 ± 0.26 vs 1.54 ± 0.24 pmol mg^{-1} protein, $P<0.05$), but not the SR 59119A-induced, cAMP production. No decrease in cAMP production was observed after a 15 h SR 59119A exposure.

5 A 15 h salbutamol exposure of myometrial strips significantly reduced the β_2- but not the β_3-AR binding site density, whereas no decrease in the number of β_2- and β_3-AR binding sites was observed after a 15 h SR 59119A treatment.

6 Neither PDE4 activity nor the β_2- and β_3-AR mRNA expression levels were affected by salbutamol or SR 59119A treatments.

7 Our results indicate that β_3-AR, but not β_2-AR, are resistant to the agonist-induced desensitisation. In our model, β_2-AR desensitisation is mediated by a decreased number of β_2-AR that was not explained by transcriptional regulation of the receptor.
British Journal of Pharmacology (2004) **141,** 831–841. doi:10.1038/sj.bjp.0705616

Keywords: Human myometrium; tocolysis; desensitisation; β_2-adrenoceptor; β_3-adrenoceptor; cyclic AMP; phosphodiesterase; β_2- and β_3-adrenoceptor mRNA; salbutamol; SR 59119A; preterm delivery

Abbreviations: AR, adrenoceptor; cAMP, cyclic adenosine monophosphate; CRC, concentration–response curve; ICYP, (−)-[^{125}I]-iodocyanopindolol; PDE, phosphodiesterase

Introduction

Despite preventative measures, the incidence of premature birth (a gestational age at birth of less than 37 completed gestational weeks) remains relatively high, at around 6%, in developed countries (Goldenberg & Rouse, 1998; Ananth *et al*., 2001). The reduction in the number of premature deliveries depends on the early and specialised management of pregnancy and parturition, including the development of new tocolytic agents (Tsatsaris *et al*., 2001). β_2-adrenoceptor (β_2-AR) agonists are the most common tocolytic agents used worldwide, but a loss of responsiveness to β-mimetics occurs physiologically in late-pregnant myometrium and after chronic exposure to these drugs (Berg *et al*., 1983; Litime *et al*., 1989). In addition, β-mimetics are known to be responsible for serious foetal and maternal side effects. For these different reasons, the use of β-mimetics as tocolytic agents has been repeatedly questioned (The Canadian Preterm Labor Investigators Group, 1992; Berkman *et al*., 2003).

Originally subclassified into β_1- and β_2-AR (Lands *et al*., 1967a, b), another subtype, the β_3-AR subtype has since been reported (Emorine *et al*., 1989). β_3-AR have been shown to

*Author for correspondence;
E-mail: celine.rouget@biomedicale.univ-paris5.fr
Advance online publication: 9 February 2004

mediate lipolysis in white adipose tissue and thermogenesis in brown adipose tissue (Arch *et al*., 1984; Zaagsma & Nahorski, 1990; Lonnqvist *et al*., 1995), and also to inhibit the contractile activity of ileum and colon (Bond & Clarke, 1988; Manara & Bianchetti, 1990; Bardou *et al*., 1998). The presence of a functional β_3-AR was recently demonstrated in the human near-term myometrium (Bardou *et al*., 2000). In this work, the stimulation of myometrial tissue by a specific β_3-AR agonist led to a higher degree of relaxation than specific stimulation by β_2-AR agonist, and was associated with a cAMP production. Several arguments suggest that β_3-AR might be less prone than β_2-AR to desensitisation (Carpene *et al*., 1993; Nantel *et al*., 1995). This particularity may be due to a lack of recognition sites in β_3-AR for the cAMP-dependent protein kinase and for the β-AR kinase (Strosberg, 1993) implicated in the desensitisation process of β_2-AR.

Several processes have been implicated in β_2-AR desensitisation; for example, the downregulation of the receptor and its uncoupling from G-stimulating (Gs) protein after phosphorylation by kinases (Berg *et al*., 1985; Litime *et al*., 1989). Another mechanism accounting for the reduced responsiveness of myometrium to β_2-AR agonists might be the enhancement of the phosphodiesterase (PDE) capacity to degrade cyclic AMP (cAMP). PDE families represent the sole mechanism for inactivating cAMP or cGMP (Conti *et al*., 2003; Houslay & Adams, 2003). In human myometrium, among the five PDE families represented, it is now well established that the PDE4 family, specific for cAMP hydrolysis and inducible by its own substrate cAMP, is predominantly expressed (Leroy *et al*., 1989; Bardou *et al*., 1999). There is now defined evidence that cAMP induces an upregulation of PDE4, accelerating the degradation of this cyclic nucleotide (Perry *et al*., 2002). Interestingly, previous studies on cellular models such as U937 monocytes (Torphy *et al*., 1995) and human neutrophils (Ortiz *et al*., 2000) indicated that cAMP-PDE4 activity was increased after a treatment by β_2-agonists. In human myometrial cells, we demonstrated that compounds elevating intracellular cAMP level, such as 8-Br-cAMP or forskolin, were able to increase PDE4 activity (Mehats *et al*., 1999). This mechanism could explain the limited relaxant effect of cAMP on myometrial tissue.

The purpose of the present study was to assess the desensitisation process of β_2- and β_3-AR in human near-term myometrium. We investigated the effects of long-term exposure of myometrial strips to a β_2-AR agonist, salbutamol, or a β_3-AR agonist, SR 59119A, on the inhibition of spontaneous contractile activity, on the cAMP production and on the number of β_2- and β_3-AR binding sites, induced by these agonists. Furthermore, we evaluated the possible implication of an upregulation of PDE4 activity or of a modulation in the mRNA expression levels for both β_2- and β_3-AR as some of the possible contributing process to desensitisation.

Methods

Biological samples

Myometrial biopsies were obtained from pregnant women who presented normal uncomplicated pregnancies, but who were delivered by elective caesarean section prior to the onset of labour (38–40th weeks of pregnancy) because of previously diagnosed cephalopelvic disproportion. Myometrial strips, excised from the longitudinal layer at the antiplacental site, were immediately placed in preoxygenated Krebs solution at 4°C (composition, mM: NaCl, 118; KCl, 5.4; $CaCl_2$, 2.5; KH_2PO_4, 0.6; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.7; ascorbic acid, 0.1) and transported immediately to the laboratory. Tissues were dissected free from serosa and used fresh (functional studies) or quickly frozen in liquid nitrogen and stored at −80°C after pretreatment (biochemical, binding and molecular studies). The use of human myometrial tissue for the experiments was approved by the 'Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale' (CCPPRB de Bourgogne, France), and by the Ethics Committee of the La Fe Hospital (Valencia, Spain). Written informed consent was obtained from all donors.

Tissue incubation process

Myometrial tissues were cut into strips (8–10 mm long by 2–3 mm in cross section) and incubated for 5 or 15 h in preoxygenated Krebs solution (composition as above) containing either a β_3-AR agonist, SR 59119A (10 μM), or a β_2-AR agonist, salbutamol (10 μM), or their respective solvents, at room temperature. We previously determined that maximal effects of salbutamol and SR 59119A were obtained at the concentration of 10 μM (Bardou *et al*., 2000).

Functional study

After the incubations described above, each muscle strip was suspended isometrically under a resting tension of 2 g in a 10 ml organ bath containing Krebs solution (composition as above) at 37°C, and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide (pH 7.4). The 2 g initial tension was chosen according to previous reports including ours (Bardou *et al*., 2000; Dennedy *et al*., 2001). One end of each strip was connected to a force–displacement transducer and tension changes were measured with Pioden strain gauges (UF1), amplified (EMKA, Paris, France), and recorded on a pen-writing oscillograph (Linseis, L65514, Munich, Germany). After 1 h, during which the myometrial strips were washed every 15 min and the resting tension readjusted to 2 g, the strips were allowed to equilibrate for a further 1 h until they showed regular spontaneous rhythmic contractile activity. Once contractions became regular in amplitude and frequency, cumulative concentration–response curves (CRCs) (from 0.1 to 10 μM) were established for each compound studied: SR 59119A (β_3-AR agonist) and salbutamol (β_2-AR agonist). The effect of each agonist was expressed as a percentage of the initial amplitude of spontaneous contractions. For all concentrations, the amplitude of contraction was recorded at steady state. Since 3–4 h were necessary for the construction of a cumulative CRC (the contact time for each concentration was around 40 min), only one complete curve was obtained for each strip. E_{max} indicates the maximal response obtained at the maximal concentration tested (10 μM), for each agent. EC_{20} values indicate the concentration producing a 20% inhibition of tone, and were calculated using the GraphPad Prism 4.01 computer program (GraphPad Software, San Diego, CA, U.S.A.). For analysis, EC_{20} values were log transformed and expressed as $-\log EC_{20}$.

Biochemical study

cAMP assay After the 5 or 15 h pretreatment, myometrial strips were equilibrated in Krebs solution (composition as above) continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide at 37°C (pH 7.4) for 50 min, and then exposed to the agonists (SR 59119A or salbutamol, each at 10 μM) for 5 min (Andersson *et al*., 1980). During this 5-min time period, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), a nonselective inhibitor of cAMP-PDE activity, was added in order to prevent cAMP degradation (Fonteh *et al*., 1993; Bardou *et al*., 2000; Boulven *et al*., 2001). cAMP levels (expressed as pmol mg^{-1} protein) were determined after agonist pretreatments, and were compared with those obtained in time-matched paired strips incubated either in Krebs solution alone (basal value) or in Krebs solution containing the respective solvents of each agonist (solvent value). Tissues were then processed as previously reported by Bardou *et al*. (1999). The samples were immediately transferred into liquid nitrogen and homogenised in ice-cold 10% trichloroacetic acid. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C. The pH of the supernatant was neutralised by the addition of excess of calcium carbonate, followed by low-speed centrifugation. Aliquots of the supernatant were assessed for cAMP, using an enzyme immunoassay kit (RPN 225, Amersham Pharmacia Biotech, Little Chalfont, U.K.), following the instructions of the manufacturer.

cAMP-PDE assay Myometrial tissues, treated as described above, were homogenised in ice-cold homogenisation buffer (100 mg tissue ml^{-1}) with an Ultra-Turrax. The composition of the homogenisation buffer was 100 mM Tris-HCl (pH 7.4), 2 mM $MgSO_4$, 2 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol, 1 μM leupeptin, 10 μg ml^{-1} aprotinin, 25 μg ml^{-1} pefabloc, 130 μg ml^{-1} benzamidine and 50 μg ml^{-1} soybean trypsin inhibitor. cAMP PDE activity was determined using the Kincaid & Manganiello (1988) method. Activity was measured in high-affinity conditions with 1 μM cAMP as substrate, and was expressed as specific activity (pmol min^{-1} mg^{-1} protein). All assays were carried out in linearity conditions with respect to time and protein concentration. PDE4 activity was gauged as the fraction of total cAMP PDE activity that was inhibited by 10 μM rolipram, a selective PDE4 inhibitor. Protein concentrations were determined using the Bradford (1976) method with bovine serum albumin (BSA) as a standard. IBMX was not used in this part of the study.

Binding studies

After a 15 h incubation of myometrial strips with salbutamol or SR 59119A (as described above), crude membranes were obtained as reported previously (Breuiller *et al*., 1987). Membranes were prepared at a protein concentration of 1–3 mg ml^{-1}, in a cold 50 mM Tris-HCl (pH 7.4) buffer. Protein concentration was determined by the Bradford method. Binding studies were performed at 30°C for 60 min in a 0.25 ml crude membrane preparation (12 μg of protein per sample) that contained 50 mM Tris-HCl (pH 7.4), 154 mM NaCl, 0.1% BSA and (−)-[^{125}I]-iodocyanopindolol (ICYP) from 2 to 1000 pM (saturation analysis). The ICYP (specific activity, 2200 Ci $mmol^{-1}$) was obtained from Perkin-Elmer-Life Sciences (Boston, MA, U.S.A.). Reactions were terminated by dilution with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 0.01% Triton X-100 and filtration over glass-fiber filters (Whatman GF/C) presoaked in 0.1% BSA in 50 mM Tris-HCl (pH 7.4). The filters were washed with an additional 15 ml of 50 mM Tris-HCl (pH 7.4), 0.01% X-100 Triton, dried and counted on a Packard Gamma counter with 85% efficiency. In previous studies examining β_2-AR, specific binding was defined as the difference between the amount of ICYP bound in the absence (total binding) and the presence (nonspecific binding) of 500 μM unlabeled (−)-isoproterenol (Sigma). For β_3-AR binding studies, nonspecific binding was defined by the amount of ICYP bound in the presence of 500 μM unlabeled (±)-alprenolol (Sigma). Competition experiments were carried out at a fixed concentration of ICYP (400 pM) with increasing concentrations of competing agents, SR 59119A or salbutamol, and in the presence of 0.1 μM (−)-propranolol in order to block β_1- and β_2-AR (Roberts *et al*., 1993). At ligand concentrations near the ICYP dissociation constant (K_D), nonspecific binding was <15 and 30% of total binding for β_2- and β_3-AR, respectively. Results are expressed as means ± standard error mean (s.e.m.). The density of receptors (B_{max}) and dissociation constants (K_D) were calculated by Scatchard analysis of saturation curves and linear regression analysis using the GraphPad Prism 4.01 computer program (GraphPad Software, San Diego, CA, U.S.A.). The inhibition constants were calculated by the Cheng & Prusoff (1973) equation: $K_i = IC_{50}/(1 + [L]/K_D)$, where IC_{50} is the concentration of competing agent producing 50% of a maximal response, [L] is the concentration of ICYP used in the assay and K_D is the dissociation constant of ICYP, as determined by Scatchard analysis.

RT–PCR analysis

Total RNA was extracted from the pretreated myometrial strips using the Trizol reagent method (Gibco-Life Technologies, Cergy-Pontoise, France). Samples were homogenised in Trizol buffer (100 mg tissue ml^{-1}) with an Ultra-Turrax. RNA preparations were recovered by phenol/chloroform extraction, isopropanol precipitation and ethanol washing, according to the manufacturer's instructions. The first strand of complementary DNA (cDNA) was generated from 8 μg total RNA using random hexamers to prime the reverse transcription (RT) in a total reaction volume of 25 μl. Total RNA was denatured by heating at 72°C for 10 min and cooling immediately on ice. This preparation was then incubated with 800 U Moloney Murine Leukemia Virus reverse transcriptase M-MLV RT (Gibco-Life Technologies) in the presence of 10 mM dithiothreitol, 20 μM random hexamers (pd(N)6), 600 μM dNTP and 20 U ribosomal RNAsin ribonuclease inhibitor (Gibco-Life Technologies), for 60 min at 39°C. The reaction was stopped by heating at 95°C for 5 min, followed by cooling. RT products were stored at −20°C. Preparations achieved without reverse transcriptase were routinely used as a control of each RNA sample. No PCR product was detected in the absence of reverse transcriptase during the RT step, indicating that the RNA preparations were free from intact genomic DNA. Amplification was performed in 1 × PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 9.3) in a 25 μl total reaction volume. This contained 200 μM of each deoxy-NTP and 0.5 mM $MgCl_2$, together with 1 μM of each specific primer, sense and antisense, 1.25 U Taq DNA polymerase (Gibco-Life Technologies), and 2–3 μl of the

RT product. The primers were designed according to Roberts *et al*. (1997). The PCR conditions for amplification were 28 cycles for β_2-AR and 36 cycles for β_3-AR. The amplification profile for β_2- and β_3-AR consisted in denaturation at 94°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min.

After the indicated subsaturing cycles of denaturation and extension, a 15 μl aliquot from each reaction mixture was resolved by electrophoresis on 2% agarose gel (Agarose 1000, Gibco-Life Technologies), and visualised by ethidium bromide staining under UV light. The DNA molecular mass standard ladder consisted of fragments between 123 and 861 bp in multiples of 123 bp (λ123 DNA ladder, Gibco-Life Technologies). Additional validity control was achieved by a Southern blot analysis of the PCR product using an internal oligonucleotide probe as previously described (Roberts *et al*., 1997) (data not shown). Amplification of an endogenous marker, the human β_2-microglobulin cDNA, was used as an internal control, because its related protein is found on the surface of many nucleated cells (Suggs *et al*., 1981).

Drugs and solutions

The drugs and chemicals used and their sources were: *N*-((7-methoxy-1,2,3,4-tetrahydronaphtalen-(2*R*)-2-yl)methyl)-(2*R*)-2-hydroxy-2-(3-chlorophenyl)ethanamine hydro-chloride (SR 59119A) that was a gift from Sanofi-Synthelabo, Sanofi-Midy Research Centre (Milan, Italy), and salbutamol sulfate (Sigma, France). SR 59119A was dissolved in a mixture of absolute ethanol 30%, DMSO 2%, distilled water for the 10 μM solution and thereafter diluted in distilled water, whereas salbutamol was dissolved in distilled water. Drug concentrations are given as final bath concentrations.

Statistical analysis

In the functional experiments, differences among groups were determined by analysis of variance (ANOVA), followed by the Bonferroni-corrected *t*-test. In biochemical and binding experiments, differences among groups were determined by Student's *t*-test for paired data. All differences were considered significant when $P<0.05$.

Results

Viability after 15 h pretreatment

In order to assess the viability of the myometrial smooth muscle tissue, we compared the amplitude of spontaneous contractions after the resting and equilibration period of either freshly harvested and set-up tissues or tissues incubated for a 15 h period in Krebs solution at room temperature. The amplitudes for spontaneous contractions were 4.81 ± 0.65 and 4.19 ± 0.45 g, respectively ($n=21$ for each time, difference not statistically significant).

Effect of a long-term exposure to a β_2-AR agonist, salbutamol, or to a β_3-AR agonist, SR 59119A, on the further inhibition of spontaneous contractions of near-term myometrial strips induced by the same agonists

Without pretreatment, salbutamol and SR 59119A both inhibited the spontaneous contractions of myometrial strips in a concentration-dependent manner (Figures 1a and b). A 15 h pretreatment of the strips with salbutamol (10 μM) induced a significant shift (Figure 1a) of the CRC of the same agonist, whereas the CRC of SR 59119A was not modified (Figure 1b). The effect of salbutamol obtained at the maximal concentration tested (10 μM) was not significantly affected by long-term treatment with this agonist (41 ± 9 vs 27 ± 8% for control and 15 h salbutamol pretreatment experiments, respectively), whereas a significant difference was reached for the $-\log EC_{20}$ values (6.31 ± 0.13 vs 5.58 ± 0.24, for control and 15 h salbutamol pretreatments, respectively, $P<0.05$). The pretreatment of myometrial strips with SR 59119A (10 μM) did

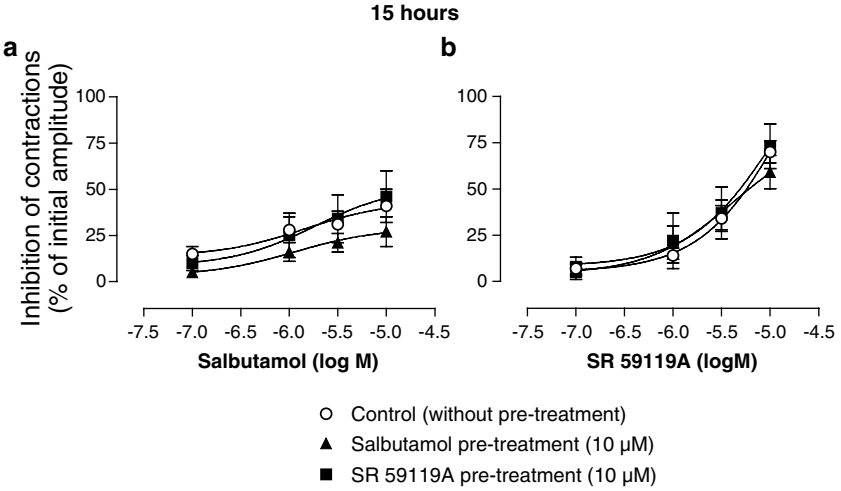

Figure 1 Effect of a 15 h pretreatment with salbutamol (10 μM) or with SR 59119A (10 μM) on the (a) salbutamol- or (b) SR 59119A-induced inhibition of spontaneous contractions of human near-term myometrial strips. Results are expressed as mean ± s.e.m. of seven experiments performed on myometrial strips obtained from seven women. A statistically significant difference for the concentration–response curves for salbutamol between salbutamol pretreatment and control experiments was determined (ANOVA, $P<0.05$).

not affect significantly either the CRC of the same agonist (Figure 1b) or the CRC of salbutamol (Figure 1a). A 5 h pretreatment of the strips with either salbutamol or SR 59119A did not significantly affect the two CRC (data not shown).

Effect of salbutamol or SR 59119A pretreatment on cAMP production induced by the same agonists

The cAMP production was measured in myometrial strips pretreated with salbutamol or with SR 59119A for 5 or 15 h. Results were expressed as mean ± s.e.m. of cAMP increase (pmol min^{-1} mg^{-1} protein) over basal value (3.19 ± 0.39 and 2.96 ± 0.41 pmol min^{-1} mg^{-1} protein at 5 and 15 h, respectively). In time-matched control experiments (5 and 15 h), salbutamol and SR 59119A (both at 10 μM) were both able to induce cAMP production. The increase in cAMP production was not statistically different between salbutamol and SR 59119A in 5 and 15 h time-matched controls (Figures 2a–d). The 15 h pretreatment with salbutamol significantly decreased the ability of this agonist to stimulate cAMP production (0.60 ± 0.26 pmol min^{-1} mg^{-1} protein after 15 h pretreatment with salbutamol vs 1.54 ± 0.24 pmol min^{-1} mg^{-1} protein without pretreatment, $P<0.05$) (Figure 2c). The decrease in salbutamol-induced cAMP production obtained after a 5 h pretreatment with salbutamol did not reach statistical significance (Figure 2a).

Neither a 5 nor a 15 h pretreatment with salbutamol affected the SR 59119A-induced cAMP production (Figures 2b and d). Indeed, the 5 or 15 h pretreatments with SR 59119A were without effect either on the salbutamol-induced or the SR 59119A-induced cAMP production (Figures 2a–d).

Effect of salbutamol or SR 59119A pretreatment on the number of β_2- and β_3-AR binding sites

Pharmacological characterisation of β-AR in human pregnant myometrium was accomplished by performing saturation and competition-binding studies using ICYP as a labelled ligand. Saturation radioligand-binding studies were performed to quantify the density of β-AR subtypes in membrane preparations of control myometrial tissue, and after salbutamol or SR 59119A pretreatment. In control myometrial tissue, Scatchard analysis of the saturation curve demonstrated the appearance of a two-site plot (data not shown), indicating both a low-affinity site ($pK_D = 8.98 \pm 0.11$; $B_{max} = 116.2 \pm 19.0$ fmol mg^{-1}

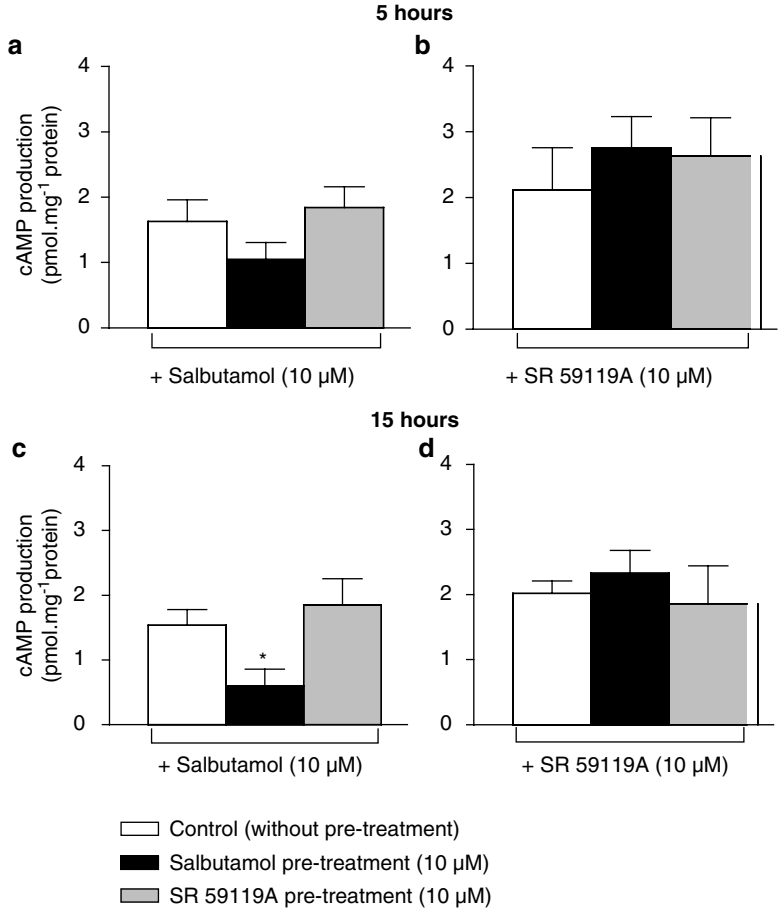

Figure 2 Effect of a 5 h (a, b) or a 15 h (c, d) pretreatment with salbutamol (10 μM) or SR 59119A (10 μM) on the further salbutamol- and SR 59119A-induced cAMP production of human near-term myometrial strips. Results are expressed as mean ± s.e.m. of cAMP increase (in pmol min^{-1} mg^{-1} protein) over basal value (3.19 ± 0.39 and 2.96 ± 0.41 pmol min^{-1} mg^{-1} protein at 5 and 15 h, respectively). Six experiments were performed on myometrial strips obtained from six women. The asterisk indicates a cAMP level that was statistically different from control value (*$P<0.05$).

Table 1 Effect of a 15 h exposure of myometrial strips to salbutamol (10 μM) or to SR 59119A (10 μM) on the number of β_2- and β_3-AR binding sites and on the pK_D values

		Control	*Salbutamol pretreatment*	*SR 59119A pretreatment*
β_2-AR	B_{max}	7.6±1.3	4.7±1.0*	10.7±2.7
	pK_D	10.55±0.17	10.70±0.10	10.23±0.14
β_3-AR	B_{max}	116.2±19.0	107.5±14.4	127.5±26.0
	pK_D	8.98±0.11	8.88±0.07	8.95±0.11

Results are expressed as mean±s.e.m. of pK_D and of maximal binding (B_{max} in fmol mg^{-1} protein). Experiments were performed on five myometrial membrane preparations obtained from five women. [a]The asterisk indicates the number of binding sites that were statistically different from control ($P<0.05$).

of protein) and a higher affinity site ($pK_D = 10.55 \pm 0.17$; $B_{max} = 7.6 \pm 1.3$ fmol mg^{-1} of protein) (Table 1). Consistent with our previous report where β-AR binding properties were described in human pregnant myometrium (Breuiller *et al.*, 1987), the high-affinity binding site found here resembled that of a β_2-AR profile. The identity of the low-affinity binding site was confirmed by competition-binding experiments. As shown in Figure 3a, ICYP binds to a class of receptors that demonstrate pharmacologic specificity consistent with the β_3-AR subtype: the selective β_3-AR agonist SR 59119A was more potent than the selective β_2-AR agonist salbutamol in inhibiting ICYP binding.

Scatchard analysis revealed that the number of β_2-AR binding sites was significantly reduced in the membrane preparations of the 15 h salbutamol-treated strips compared to the control strips ($B_{max} = 4.7 \pm 1.0$ vs 7.6 ± 1.3 fmol mg^{-1} of protein, $P<0.05$) (Table 1). However, a 15 h exposure to SR 59119A did not significantly modify the number of β_2-AR binding sites. pK_D values for 15 h salbutamol and 15 h SR 59119A exposed membrane preparations were similar to that of control (Table 1). By contrast, Scatchard analysis of saturation curves demonstrated that the number of β_3-AR binding sites was not modified either by a 15 h salbutamol or by a 15 h SR 59119A treatment ($B_{max} = 107.5 \pm 14.4$ and 127.5 ± 26.0 fmol mg^{-1} of protein in membrane preparations of 15 h salbutamol- and 15 h SR 59119A-treated strips, respectively) (Table 1). pK_D values for β_3-AR in the membrane preparations of 15 h salbutamol- and of 15 h SR 59119A-treated strips were unchanged in comparison to strips without pretreatment.

The effect of salbutamol or SR 59119A pretreatment on the agonist-binding properties of β_2- and β_3-AR was further analysed (Figure 3). In control membrane preparations, SR 59119A competed at the propranolol-resistant ICYP-binding site with a pK_i value of 6.58±0.68 (Figure 3a). Competition-binding data were similar in membrane preparations of 15 h salbutamol- ($pK_i = 6.19 \pm 0.33$) and 15 h SR 59119A- ($pK_i = 6.76 \pm 0.49$) treated myometrial strips (Figures 3b and c). It should be noted that the shallow slope for these curves indicates that two sites may be present but cannot be significantly differentiated by computer analysis. The β_2-AR agonist salbutamol did not compete at the β_3-AR-binding sites (Figures 3a–c).

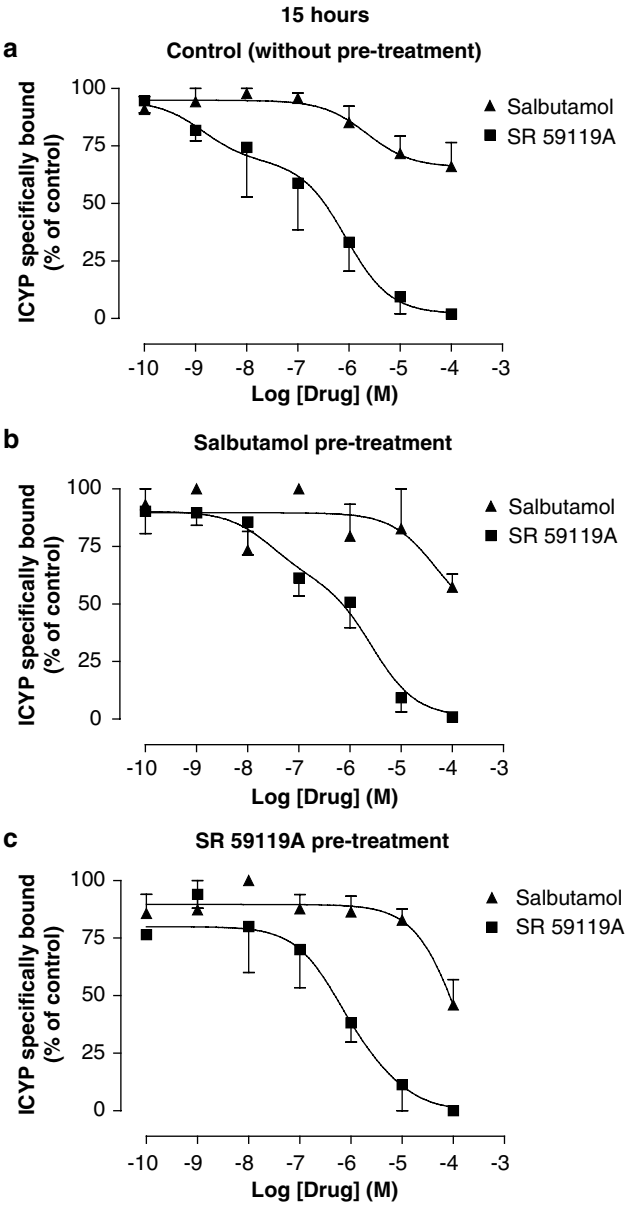

Figure 3 Competition between ICYP and SR 59119A or salbutamol for β_3-AR binding sites in membrane preparations of (a) control, (b) 15 h salbutamol- and (c) 15 h SR 59119A-treated pregnant myometrial strips. Results are given for three to four different pregnant women.

Analysis of β_2- and β_3-AR mRNA expression in near-term myometrial strips treated with salbutamol or SR 59119A

Using an RT–PCR method, we analysed the expression of β_2- and β_3-AR transcripts in myometrial strips treated either with 10 μM salbutamol or SR 59119A. Amplification of β_2-microglobulin cDNA, the internal control, allowed a semi-quantitative analysis of the β_2- and β_3-AR transcripts expression, as we verified the successful normalisation of RNA amounts by obtaining equivalent intensity for the β_2-microglobulin band in control vs treated strips. None of the treatments (5 and 15 h) with salbutamol or with SR 59119A induced a change in β_2- and β_3-AR mRNA expression (Figures 4 and 5).

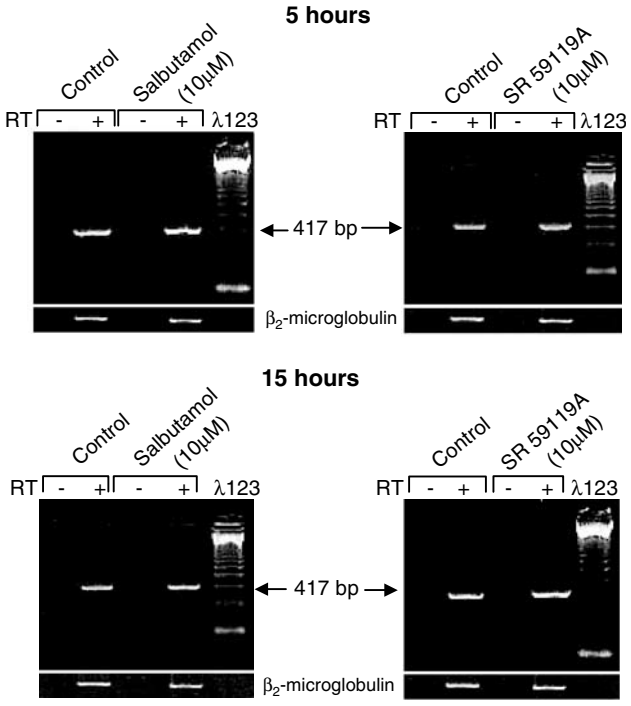

Figure 4 Effect of a 5 or 15 h treatment with salbutamol (10 μM) or SR 59119A (10 μM) on the β_2-AR transcript expression of human near-term myometrium. The ethidium bromide-stained gel pictures are representative of five separate experiments with the myometrium of different women. DNA molecular mass standards appear in the lane at the far right.

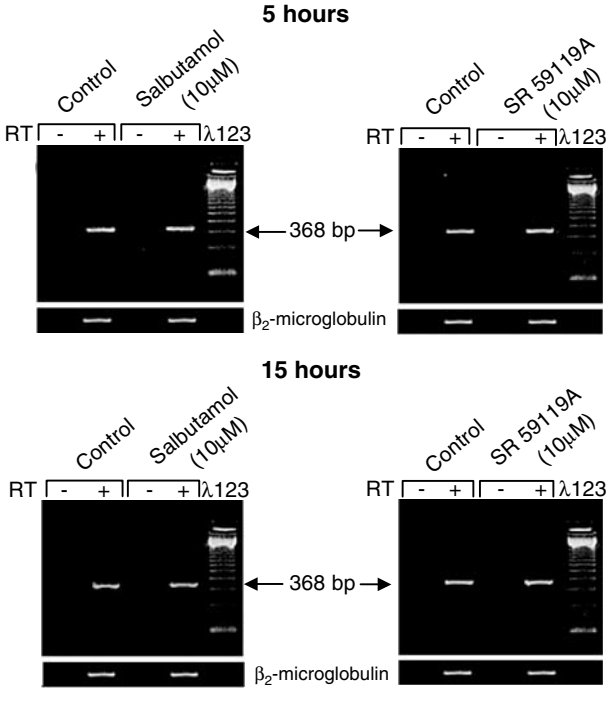

Figure 5 Effect of a 5 or 15 h treatment with salbutamol (10 μM) or SR 59119A (10 μM) on the β_3-AR transcripts expression of human near-term myometrium. The ethidium bromide-stained gel pictures are representative of five separate experiments with myometrium of different women. DNA molecular mass standards appear in the lane at the far right.

Influence of salbutamol or SR 59119A pretreatment on cAMP-PDE4 activity

In order to assess whether cAMP-PDE4 activity could be affected by β_2- or β_3-AR agonist treatment, we measured the PDE4 activity in homogenates of myometrial strips treated either with salbutamol or with SR 59119A. No increase in PDE4 activity was observed after 5 or 15 h of treatment with salbutamol or SR 59119A (Figure 6). We also controlled so that the total cAMP-PDE activity was not modified by these treatments (data not shown).

Discussion

The purpose of the present study was to examine whether desensitisation occurred in human near-term myometrium after a sustained stimulation of β_2- and β_3-AR with their respective agonists. In our experimental conditions, we confirmed that, without pretreatment of the myometrial strips, both salbutamol and SR 59119A were able to inhibit spontaneous contractions and to induce cAMP production. These findings provide confirmation of β_2- and β_3-AR functional integrity, and positive coupling with the adenylyl cyclase pathway (Bardou *et al*., 2000). Our functional and biochemical results demonstrate that β_2-AR undergoes functional desensitisation after long-term exposure to salbutamol, which is associated with a significant decrease in cAMP production. As it was shown in binding experiments, this desensitisation occurs at the receptor level. Indeed, the density of β_2-AR binding sites was decreased after a 15 h exposure of myometrial strips to salbutamol. In contrast, a sustained stimulation by the β_3-AR agonist SR 59119A did not modify its subsequent functional effect and the number of β_3-AR binding sites remained unchanged after such treatment. Our results provide strong evidences for resistance of β_3-AR to the agonist-induced desensitisation in human near-term myometrium. Nevertheless, our binding experiments show that β_3-AR are more numerous than β_2-AR in near-term myometrium, while it was admitted that the predominant subtype was the β_2-AR subtype in this tissue (Hayashida *et al*., 1982; Breuiller *et al*., 1987). β_3-AR have not been previously detected, probably because of insensitive experimental conditions such as unadapted (i.e. too low) ICYP concentration needed to recognise the β_3-AR subtype. Indeed, the pharmacological properties of the human β_3-AR differ from those of conventional β_1- and β_2-AR. The main differences include atypically low affinity for conventional β-AR antagonist radioligands such as ICYP, as well as low potencies for β-AR reference agonists (Emorine *et al*., 1994).

Our results are in general agreement with the numerous literature on myometrial β_2-AR desensitisation. For example, it was shown that treatment of pregnant women (Berg *et al*., 1985; Engelhardt *et al*., 1997), or *in vitro* treatment of human myometrial tissue (Andersson *et al*., 1980) with a β_2-AR agonist was associated with a loss in efficacy of the β_2-AR agonist, due to a reduction of the number of β_2-AR binding sites.

We hypothesised that part of the β_2-AR desensitisation process consists in a transcriptional regulation of their receptors and that long-term treatment with salbutamol would be associated with a decrease in β_2-AR mRNA levels, as it was

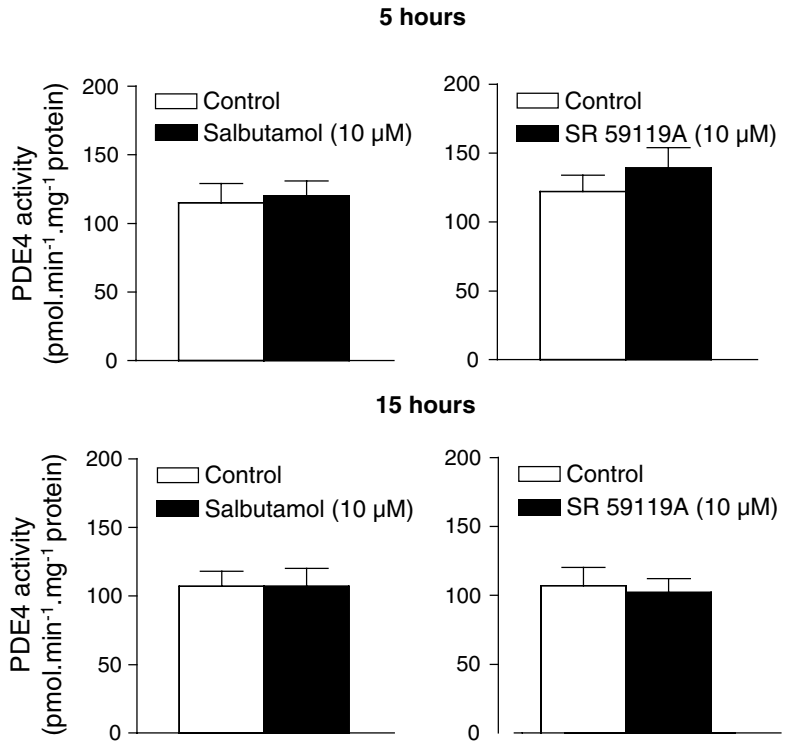

Figure 6 Effect of a 5 or 15 h treatment with salbutamol (10 μM) or SR 59119A (10 μM) on the PDE4 activity of human near-term myometrial strips. Results are expressed as mean ± s.e.m. of six experiments performed on myometrial strips obtained from six women.

previously described in pregnant rats with isoprenaline, a nonselective β-AR agonist (Lecrivain *et al*., 1998). Contrary to the report of Lecrivain *et al*. (1998), in our study, the decrease in the number of β_2-AR binding sites was not associated with a significant change in the β_2-AR mRNA levels. But our results are in accordance with the study by Engelhardt *et al*. (1997) performed in human myometrial biopsies treated with fenoterol, a selective β_2-AR agonist.

We might also hypothesise that an increased degradation of cAMP by PDE4 could play a role in β_2-AR desensitisation. This mechanism has been well documented in several systems including S49 lymphoma cells, platelets, hepatocytes, adipocytes and Sertoli cells (for review, Giembycz, 1996; Perry *et al*., 2002). It has been demonstrated, in different human blood cells, that treatment with either β_2-AR agonists (Manning *et al*., 1996; Seybold *et al*., 1998; Ortiz *et al*., 2000) or with a cAMP structural analogue, 8-Bromo-cAMP, leads to an upregulation of PDE4 enzymes. More recently, new evidences for a role of cAMP on PDE4 promoters have been defined (Vicini & Conti, 1997; D'Sa *et al*., 2002; Le Jeune *et al*., 2002). Furthermore, it has been shown, in an *in vivo* model of rat pulmonary β_2-AR desensitisation induced by salbutamol, that the β_2-AR binding site density was significantly reduced, and associated with an increase in the PDE3 and PDE4 activities in lung membranes of these animals (Finney *et al*., 2000). Interestingly, the implication of an upregulation of PDE4 in the adaptation of myometrium to long-term treatment with β_2-AR has been emphasised by Mehats *et al*. (1999). Furthermore, Bardou *et al*. (1999) and Mehats *et al*. (2001) have both shown that pretreatment of myometrial strips of women at term with a PDE4-selective inhibitor potentiates the relaxant effect of salbutamol. In our model, the long-term exposure of myometrial strips to salbutamol did not modify the PDE4 activity. Such an absence of PDE4 increase can partly be explained by the degree of intensity of the intracellular cAMP rise (1.5–2-fold) observed following β-AR agonist treatment. This increase might be too weak, with regard to the 10-fold cAMP increase observed in forskolin-treated myometrial cells, to induce PDE4 *de novo* synthesis (Mehats *et al*., 1999). Another reason for the lack of PDE4 upregulation observed in our study might be linked to specific cAMP intracellular compartmentalisation, as already described (Jurevicius & Fischmeister, 1996; Zaccolo & Pozzan, 2002).

The myometrial β_2-AR desensitisation process may also occur through other different pathways that have not been assessed in the present study. Simon *et al*. (2001) demonstrated that G-protein-coupled receptor kinase (GRK) activation, particularly GRK2, could be an important mediator of myometrial β_2-AR desensitisation. Another possible mechanism for β_2-AR desensitisation is uncoupling to adenylyl cyclase, as it was described either in pregnant rat myometrium (Lecrivain *et al*., 1998) or in the human failing heart (Bohm *et al*., 1997). This finding could be attributed either to an increased activity and gene expression of β-AR kinase leading to phosphorylation and uncoupling of receptors (Bohm *et al*., 1997), or to an increase in the functional activity of Gi-proteins, negatively coupled with the cAMP-signalling pathway (Lecrivain *et al*., 1998). We can also hypothesise that modifications in the coupling mechanisms (Gs proteins) between receptors and the catalytic component of adenylyl cyclase are implicated in the loss of β_2-AR adenylyl cyclase stimulation in the myometrium after long-term exposure to a

selective β_2-AR agonist, as it has been shown to occur at the end of pregnancy (Litime *et al*., 1989).

The originality of our work is to demonstrate for the first time that, at the end of pregnancy, the myometrial β_3-AR seems to be less prone to sustained agonist-induced desensitisation. Such refractoriness of β_3-AR to desensitisation has already been suggested in other models. For example, it was reported that both the rat β_3-AR expressed in white adipocytes (Granneman, 1992) and the human β_3-AR transfected in Chinese Hamster Ovary (β_3-CHO) cells (Nantel *et al*., 1993) were completely resistant to short-term agonist-promoted desensitisation, whereas β_2-CHO cells were not (Chambers *et al*., 1994). Our functional studies showed that human myometrial β_3-AR is resistant to desensitisation. The biochemical approach demonstrated the lack of increased PDE4 activity, consequent to a long-term treatment of myometrial strips with SR 59119A, and the binding experiments showed the lack of downregulation of β_3-AR binding sites. Furthermore, we did not notice any modification in the β_3-AR mRNA levels. These results are conflicting with those of Thomas *et al*. (1992), who demonstrated that a long-term treatment of 3T3-F442A adipocytes with a β_3-AR agonist led to an upregulation of β_3-AR mRNA and receptor expression. This might suggest that β_3-AR regulation is tissue specific. Indeed Chaudhry & Granneman (1994) demonstrated that β_3-AR desensitisation is dependent upon the cellular background in which this receptor is expressed. In this study, the mechanism responsible for β_3-AR desensitisation does not appear to involve cAMP-dependent phosphorylation or downregulation of the receptor. Oriowo (1998) also described that in the rat lower oesophageal activation of β_3-AR was not linked to either ATPase-, Ca^{2+}-dependent K^+ channels or to the NO/cGMP signalling pathway. We found that, in the human near-term myometrium, β_3-AR stimulation was not associated with an increase of cGMP production (Bardou *et al*., 2000).

In conclusion, we have demonstrated that, in contrary to β_2-AR, β_3-AR are resistant to agonist-induced desensitisation in near-term human myometrium. Interestingly, long-term treatment with salbutamol did not affect the inhibitory properties and the ability to induce cAMP production of SR 59119A, and reciprocally. This study confirms the potential therapeutic interest of β_3-AR agonists, particularly for women requiring rescue therapy after failure of tocolysis with a β_2-AR agonist. Although their clinical properties remain to be demonstrated, the use of these new molecules could be of interest in pharmacological treatment of preterm labour.

This study was supported in part by Grant FIS01/1231 from 'Fondo Investigaciones Sanitarias' of the Ministry of Health of Spain. We are indebted to the surgical teams of the Services of Obstetrics and Gynaecology at the University Hospitals, Antoine Béclère Hospital (Clamart, France), CHU du Bocage Hospital (Dijon, France) and La Fe Hospital (Valencia, Spain) for making the myometrial tissue available to us. Special thanks are due to Dr Alan Barkun and Mrs Ruth Bardou for their careful reading of this manuscript, and to Laurent Morisot for technical support.

References

ANANTH, C.V., MISRA, D.P., DEMISSIE, K. & SMULIAN, J.C. (2001). Rates of preterm delivery among Black women and White women in the United States over two decades: an age–period–cohort analysis. *Am. J. Epidemiol.,* **154,** 657–665.

ANDERSSON, R.G., BERG, G., JOHANSSON, S.R. & RYDEN, G. (1980). Effects of non-selective and selective beta-adrenergic agonists on spontaneous contractions and cyclic AMP levels in myometrial strips from pregnant women. *Gynecol. Obstet. Invest.,* **11,** 268–293.

ARCH, J.R., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNITT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). Atypical beta-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature,* **309,** 163–165.

BARDOU, M., CORTIJO, J., LOUSTALOT, C., TAYLOR, S., PERALES-MARIN, A., MERCIER, F.J., DUMAS, M., DENEUX-THARAUX, C., FRYDMAN, R., MORCILLO, E. & ADVENIER, C. (1999). Pharmacological and biochemical study on the effect of selective phosphodiesterase inhibitors on human term myometrium. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **360,** 457–463.

BARDOU, M., DOUSSET, B., DENEUX-THARAUX, C., SMADJA, C., NALINE, E., CHAPUT, J.C., NAVEAU, S., MANARA, L., CROCI, T. & ADVENIER, C. (1998). *In vitro* inhibition of human colonic motility with SR 59119A and SR 59104A: evidence of a beta3-adrenoceptor-mediated effect. *Eur. J. Pharmacol.,* **353,** 281–287.

BARDOU, M., LOUSTALOT, C., CORTIJO, J., SIMON, B., NALINE, E., DUMAS, M., ESTEVE, S., CROCI, T., CHALON, P., FRYDMAN, R., SAGOT, P., MANARA, L., MORCILLO, E.J. & ADVENIER, C. (2000). Functional, biochemical and molecular biological evidence for a possible beta(3)-adrenoceptor in human near-term myometrium. *Br. J. Pharmacol.,* **130,** 1960–1966.

BERG, G., ANDERSSON, R.G. & RYDEN, G. (1983). *In vitro* study of phosphodiesterase-inhibiting drugs: a complement to beta-sympathomimetic drug therapy in premature labor? *Am. J. Obstet. Gynecol.,* **145,** 802–806.

BERG, G., ANDERSSON, R.G. & RYDEN, G. (1985). Beta-adrenergic receptors in human myometrium during pregnancy: changes in the number of receptors after beta-mimetic treatment. *Am. J. Obstet. Gynecol.,* **151,** 392–396.

BERKMAN, N.D., THORP JR, J.M., LOHR, K.N., CAREY, T.S., HARTMANN, K.E., GAVIN, N.I., HASSELBLAD, V. & IDICULA, A.E. (2003). Tocolytic treatment for the management of preterm labor: a review of the evidence. *Am. J. Obstet. Gynecol.,* **188,** 1648–1659.

BOHM, M., FLESCH, M. & SCHNABEL, P. (1997). Beta-adrenergic signal transduction in the failing and hypertrophied myocardium. *J. Mol. Med.,* **75,** 842–848.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the alpha- and beta-subtypes. *Br. J. Pharmacol.,* **95,** 723–734.

BOULVEN, I., PALMIER, B., ROBIN, P., VACHER, M., HARBON, S. & LEIBER, D. (2001). Platelet-derived growth factor stimulates phospholipase C-gamma 1, extracellular signal-regulated kinase, and arachidonic acid release in rat myometrial cells: contribution to cyclic 3′,5′-adenosine monophosphate production and effect on cell proliferation. *Biol. Reprod.,* **65,** 496–506.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.,* **72,** 248–254.

BREUILLER, M., ROUOT, B., LEROY, M.J., BLOT, P., KAPLAN, L. & FERRE, F. (1987). Adrenergic receptors in inner and outer layers of human myometrium near term: characterization of beta-adrenergic receptor sites by [125I]-iodocyanopindolol binding. *Gynecol. Obstet. Invest.,* **24,** 28–37.

CARPENE, C., GALITZKY, J., COLLON, P., ESCLAPEZ, F., DAUZATS, M. & LAFONTAN, M. (1993). Desensitization of beta-1 and beta-2, but not beta-3, adrenoceptor-mediated lipolytic responses of adipocytes after long-term norepinephrine infusion. *J. Pharmacol. Exp. Ther.,* **265,** 237–247.

CHAMBERS, J., PARK, J., CRONK, D., CHAPMAN, C., KENNEDY, F.R., WILSON, S. & MILLIGAN, G. (1994). Beta 3-adrenoceptor agonist-induced down-regulation of Gs alpha and functional desensitization in a Chinese hamster ovary cell line expressing a beta 3-adrenoceptor refractory to down-regulation. *Biochem. J.,* **303,** 973–978.

CHAUDHRY, A. & GRANNEMAN, J.G. (1994). Influence of cell type upon the desensitization of the beta 3-adrenergic receptor. *J. Pharmacol. Exp. Ther.,* **271,** 1253–1258.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CONTI, M., RICHTER, W., MEHATS, C., LIVERA, G., PARK, J.Y. & JIN, C. (2003). Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J. Biol. Chem.,* **278,** 5493–5496.

DENNEDY, M.C., FRIEL, A.M., GARDEIL, F. & MORRISON, J.J. (2001). Beta-3 *versus* beta-2 adrenergic agonists and preterm labour: *in vitro* uterine relaxation effects. *BJOG,* **108,** 605–609.

D'SA, C., TOLBERT, L.M., CONTI, M. & DUMAN, R.S. (2002). Regulation of cAMP-specific phosphodiesterases type 4B and 4D (PDE4) splice variants by cAMP signaling in primary cortical neurons. *J. Neurochem.,* **81,** 745–757.

EMORINE, L., BLIN, N. & STROSBERG, A.D. (1994). The human beta 3-adrenoceptor: the search for a physiological function (see comments). *Trends Pharmacol. Sci.,* **15,** 3–7.

EMORINE, L.J., MARULLO, S., BRIEND-SUTREN, M.M., PATEY, G., TATE, K., DELAVIER-KLUTCHKO, C. & STROSBERG, A.D. (1989). Molecular characterization of the human beta 3-adrenergic receptor. *Science,* **245,** 1118–1121.

ENGELHARDT, S., ZIEGER, W., KASSUBEK, J., MICHEL, M.C., LOHSE, M.J. & BRODDE, O.E. (1997). Tocolytic therapy with fenoterol induces selective down-regulation of beta-adrenergic receptors in human myometrium. *J. Clin. Endocrinol. Metab.,* **82,** 1235–1242.

FINNEY, P.A., BELVISI, M.G., DONNELLY, L.E., CHUANG, T.T., MAK, J.C., SCORER, C., BARNES, P.J., ADCOCK, I.M. & GIEMBYCZ, M.A. (2000). Albuterol-induced downregulation of Gsalpha accounts for pulmonary beta(2)-adrenoceptor desensitization *in vivo*. *J. Clin. Invest.,* **106,** 125–135.

FONTEH, A.N., WINKLER, J.D., TORPHY, T.J., HERAVI, J., UNDEM, B.J. & CHILTON, F.H. (1993). Influence of isoproterenol and phosphodiesterase inhibitors on platelet-activating factor biosynthesis in the human neutrophil. *J. Immunol.,* **151,** 339–350.

GIEMBYCZ, M.A. (1996). Phosphodiesterase 4 and tolerance to beta 2-adrenoceptor agonists in asthma. *Trends Pharmacol. Sci.,* **17,** 331–336.

GOLDENBERG, R.L. & ROUSE, D.J. (1998). Prevention of premature birth. *N. Engl. J. Med.,* **339,** 313–320.

GRANNEMAN, J.G. (1992). Effects of agonist exposure on the coupling of beta 1 and beta 3 adrenergic receptors to adenylyl cyclase in isolated adipocytes. *J. Pharmacol. Exp. Ther.,* **261,** 638–642.

HAYASHIDA, D.N., LEUNG, R., GOLDFIEN, A. & ROBERTS, J.M. (1982). Human myometrial adrenergic receptors: identification of the beta-adrenergic receptor by [3H]dihydroalprenolol binding. *Am. J. Obstet. Gynecol.,* **142,** 389–393.

HOUSLAY, M.D. & ADAMS, D.R. (2003). PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem. J.,* **370,** 1–18.

JUREVICIUS, J. & FISCHMEISTER, R. (1996). cAMP compartmentation is responsible for a local activation of cardiac Ca2+ channels by beta-adrenergic agonists. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 295–299.

KINCAID, R.L. & MANGANIELLO, V.C. (1988). Assay of cyclic nucleotide phosphodiesterase using radiolabeled and fluorescent substrates. *Methods Enzymol.,* **159,** 457–470.

LANDS, A.M., ARNOLD, A., MC AULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. (1967a). Differentiation of receptor systems activated by sympathomimetic amines. *Nature,* **214,** 597–598.

LANDS, A.M., LUDUENA, F.P. & BUZZO, H.J. (1967b). Differentiation of receptors responsive to isoproterenol. *Life Sci.,* **6,** 2241–2249.

LE JEUNE, I.R., SHEPHERD, M., VAN HEEKE, G., HOUSLAY, M.D. & HALL, I.P. (2002). Cyclic AMP-dependent transcriptional up-regulation of phosphodiesterase 4D5 in human airway smooth muscle cells. Identification and characterization of a novel PDE4D5 promoter. *J. Biol. Chem.,* **277,** 35980–35989.

LECRIVAIN, J.L., COHEN-TANNOUDJI, J., ROBIN, M.T., COUDOUEL, N., LEGRAND, C. & MALTIER, J.P. (1998). Molecular mechanisms of adenylyl cyclase desensitization in pregnant rat myometrium following *in vivo* administration of the beta-adrenergic agonist, isoproterenol. *Biol. Reprod.,* **59,** 45–52.

LEROY, M.J., CEDRIN, I., BREUILLER, M., GIOVAGRANDI, Y. & FERRE, F. (1989). Correlation between selective inhibition of the cyclic nucleotide phosphodiesterases and the contractile activity in human pregnant myometrium near term. *Biochem. Pharmacol.,* **38,** 9–15.

LITIME, M.H., POINTIS, G., BREUILLER, M., CABROL, D. & FERRE, F. (1989). Disappearance of beta-adrenergic response of human myometrial adenylate cyclase at the end of pregnancy. *J. Clin. Endocrinol. Metab.,* **69,** 1–6.

LONNQVIST, F., THOME, A., NILSELL, K., HOFFSTEDT, J. & ARNER, P. (1995). A pathogenic role of visceral fat beta 3-adrenoceptors in obesity (see comments). *J. Clin. Invest.,* **95,** 1109–1116.

MANARA, L. & BIANCHETTI, A. (1990). Further heterogeneity of the beta-adrenoceptor. The phenylethanolaminotetralines: new selective agonists for atypical beta-adrenoceptors. *Trends Pharmacol. Sci.,* **11,** 229–230.

MANNING, C.D., MCLAUGHLIN, M.M., LIVI, G.P., CIESLINSKI, L.B., TORPHY, T.J. & BARNETTE, M.S. (1996). Prolonged beta adrenoceptor stimulation up-regulates cAMP phosphodiesterase activity in human monocytes by increasing mRNA and protein for phosphodiesterases 4A and 4B. *J. Pharmacol. Exp. Ther.,* **276,** 810–818.

MEHATS, C., TANGUY, G., DALLOT, E., CABROL, D., FERRE, F. & LEROY, M.J. (2001). Is up-regulation of phosphodiesterase 4 activity by PGE2 involved in the desensitization of beta-mimetics in late pregnancy human myometrium? *J. Clin. Endocrinol. Metab.,* **86,** 5358–5365.

MEHATS, C., TANGUY, G., DALLOT, E., ROBERT, B., REBOURCET, R., FERRE, F. & LEROY, M.J. (1999). Selective up-regulation of phosphodiesterase-4 cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase variants by elevated cAMP content in human myometrial cells in culture. *Endocrinology,* **140,** 3228–3237.

NANTEL, F., BONIN, H., EMORINE, L.J., ZILBERFARB, V., STROSBERG, A.D., BOUVIER, M. & MARULLO, S. (1993). The human beta 3-adrenergic receptor is resistant to short term agonist-promoted desensitization. *Mol. Pharmacol.,* **43,** 548–555.

NANTEL, F., BOUVIER, M., STROSBERG, A.D. & MARULLO, S. (1995). Functional effects of long-term activation on human beta 2- and beta 3-adrenoceptor signalling. *Br. J. Pharmacol.,* **114,** 1045–1051.

ORIOWO, M.A. (1998). Neural inhibition in the rat lower esophageal sphincter: role of beta 3-adrenoceptor activation. *Gen. Pharmacol.,* **30,** 37–41.

ORTIZ, J.L., DASI, F.J., CORTIJO, J. & MORCILLO, E.J. (2000). Beta-adrenoceptor stimulation up-regulates phosphodiesterase 4 activity and reduces prostaglandin E2-inhibitory effects in human neutrophils. *Naunyn-Schmiedebergs Arch. Pharmacol.,* **361,** 410–417.

PERRY, S.J., BAILLIE, G.S., KOHOUT, T.A., MCPHEE, I., MAGIERA, M.M., ANG, K.L., MILLER, W.E., MCLEAN, A.J., CONTI, M., HOUSLAY, M.D. & LEFKOWITZ, R.J. (2002). Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science,* **298,** 834–836.

ROBERTS, S.J., MOLENAAR, P. & SUMMERS, R.J. (1993). Characterization of propranolol-resistant (−)-[125I]-cyanopindolol binding sites in rat soleus muscle. *Br. J. Pharmacol.,* **109,** 344–352.

ROBERTS, S.J., PAPAIOANNOU, M., EVANS, B.A. & SUMMERS, R.J. (1997). Functional and molecular evidence for beta 1-, beta 2- and beta 3-adrenoceptors in human colon. *Br. J. Pharmacol.,* **120,** 1527–1535.

SEYBOLD, J., NEWTON, R., WRIGHT, L., FINNEY, P.A., SUTTORP, N., BARNES, P.J., ADCOCK, I.M. & GIEMBYCZ, M.A. (1998). Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. Potential role in beta2-adrenoreceptor desensitization. *J. Biol. Chem.,* **273,** 20575–20588.

SIMON, V., MHAOUTY-KODJA, S., LEGRAND, C. & COHEN-TANNOUDJI, J. (2001). Concomitant increase of G protein-coupled receptor kinase activity and uncoupling of beta-adrenergic receptors in rat myometrium at parturition. *Endocrinology,* **142,** 1899–1905.

STROSBERG, A.D. (1993). Structure, function, and regulation of adrenergic receptors. *Protein Sci.,* **2,** 1198–1209.

SUGGS, S.V., WALLACE, R.B., HIROSE, T., KAWASHIMA, E.H. & ITAKURA, K. (1981). Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human beta 2-microglobulin. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 6613–6617.

THE CANADIAN PRETERM LABOR INVESTIGATORS GROUP (1992). Treatment of preterm labor with the beta-adrenergic agonist ritodrine. The Canadian Preterm Labor Investigators Group. *N. Engl. J. Med.,* **327,** 308–312.

THOMAS, R.F., HOLT, B.D., SCHWINN, D.A. & LIGGETT, S.B. (1992). Long-term agonist exposure induces upregulation of beta 3-adrenergic receptor expression *via* multiple cAMP response elements. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 4490–4494.

TORPHY, T.J., ZHOU, H.L., FOLEY, J.J., SARAU, H.M., MANNING, C.D. & BARNETTE, M.S. (1995). Salbutamol up-regulates PDE4 activity and induces a heterologous desensitization of U937 cells to prostaglandin E2. Implications for the therapeutic use of beta-adrenoceptor agonists. *J. Biol. Chem.,* **270,** 23598–23604.

TSATSARIS, V., PAPATSONIS, D., GOFFINET, F., DEKKER, G. & CARBONNE, B. (2001). Tocolysis with nifedipine or beta-adrenergic agonists: a meta-analysis. *Obstet. Gynecol.,* **97,** 840–847.

VICINI, E. & CONTI, M. (1997). Characterization of an intronic promoter of a cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase gene that confers hormone and cAMP inducibility. *Mol. Endocrinol.,* **11,** 839–850.

ZAAGSMA, J. & NAHORSKI, S.R. (1990). Is the adipocyte beta-adrenoceptor a prototype for the recently cloned atypical 'beta 3-adrenoceptor'? *Trends Pharmacol. Sci.,* **11,** 3–7.

ZACCOLO, M. & POZZAN, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science,* **295,** 1711–1715.

(Received September 3, 2003
Revised October 9, 2003
Accepted November 7, 2003)