British Journal of Pharmacology (1997) 122, 1732–1738

Heterogeneity of α_2-adrenoceptors in human and rat myometrium and differential expression during pregnancy

Rachel Bouet-Alard, [1]Sakina Mhaouty-Kodja, Isabelle Limon-Boulez, Noelline Coudouel, Jean Paul Maltier & [2]Chantal Legrand

Laboratoire de Physiologie de la Reproduction, URA CNRS 1449, Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cedex 05, France

1 The aim of this study was first, to characterize α_2-adrenoceptor subtypes in human and rat pregnant myometrium and second, to investigate the possibility of a differential expression of the putative subtypes according to the stage of pregnancy.

2 In both species, specific [^{3}H]-rauwolscine binding was inhibited by five different compounds with an order of affinity characteristic of the one described for α_2-adrenoceptors (yohimbine $\geqslant$ clonidine > noradrenaline > phenylephrine > propranolol). Binding affinities (pK_i) for the compounds tested were, in human and rat, respectively: 7.63 and 8.93 for yohimbine, 6.91 and 8.71 for clonidine, 6.23 and 6.09 for noradrenaline, 5.37 and 5.73 for phenylephrine, 4.64 and 4.72 for propranolol.

3 By use of non-linear iterative curve fitting procedures and by fitting the data to a two-site model, analysis of [^{3}H]-rauwolscine inhibition binding curves performed in the presence of oxymetazoline (α_{2A}-selective), ARC239, prazosin or chlorpromazine (α_{2B}- and α_{2C}-selective) indicated that pregnant human and rat myometrium contain at least two pharmacologically distinct α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and/or α_{2C}). RNA blot analysis with probes specific for each cloned human and rat α_2-adrenoceptor subtype demonstrated that α_{2A}- and α_{2B}-subtypes were present in both species but α_{2C} seems to be expressed only in human tissues.

4 In the pregnant rat myometrium, subtype selective compounds competition curves revealed a predominant expression of α_{2A}-adrenoceptors at mid-pregnancy whereas, at term, α_{2A}- and α_{2B}-subtypes density reached approximately the same level (α_{2A} : α_{2B} ratio = 73 : 27 at mid-pregnancy and = 43 : 57 at term). In addition, quantification of α_{2A}- and α_{2B}-transcripts by densitometry, following data normalization with an oligo(dT)$_{12-18}$ probe, showed a pattern of expression comparable to the one characterized by pharmacological studies.

5 In conclusion, these data demonstrate heterogeneity of α_2-adrenoceptors in pregnant human and rat myometria and an alteration of the α_{2A}-/α_{2B}-subtypes expression pattern during rat pregnancy. Such observations lead us to suggest a multiple role for α_2-adrenoceptors in regulating specific functions of myometrium throughout the time course of pregnancy.

Keywords: α_2-Adrenoceptor subtypes; myometrium; pregnancy; pharmacology; Northern blotting

Introduction

To date, three human α_2-adrenoceptor subtype genes have been cloned, designated as α_2C10, α_2C2 and α_2C4 according to their chromosomal location (Kobilka *et al*., 1987; Regan *et al*., 1988; Lomasney *et al*., 1990; 1991). The α_2C10 gene product has been identified as the pharmacologically defined platelet-type α_{2A}-adrenoceptor, which exhibits a high affinity for oxymetazoline and low affinity for prazosin and ARC 239 (Bylund *et al*., 1992). The rat clone RG20 (rat glycosylated 20) shares 89% amino acid identity with human α_2C10. However, the rat RG20 clone is often referred to a distinct α_2-adrenoceptor subtype, $\alpha_{2A/D}$-adrenoceptor, mostly with regard to its lower affinity for yohimbine and rauwolscine when compared to the human α_{2A}-adrenoceptor (Chalberg *et al*., 1990; Lanier *et al*., 1991). The α_2C4 gene product seems to correspond to the opossum kidney cell line α_{2C}-adrenoceptor and the rat RG10 (Flordellis *et al*., 1991; Lanier *et al*., 1991; Voigt *et al*., 1991; Blaxall *et al*., 1994a,b) The α_2C2 gene product represents the human counterpart of the prototypical RNG (rat non glycosylated) – α_{2B}-adrenoceptor in neonatal rat lung and NG108 cell line (Lomasney *et al*., 1990; Zeng *et al*., 1990). The α_2C4 and α_2C2 gene products display similar ligand recognition binding properties, i.e. a high affinity for prazosin and ARC 239 but a low affinity for oxymetazoline.

In man, pharmacological studies have previously identified α_2-adrenoceptors on myometrium (Bottari *et al*., 1983; Breuiller *et al*., 1990; Dahle *et al*., 1993). However, in none of these studies were the subtype(s) expressed characterized or quantified. The purpose of this study was, first, to examine, in the human pregnant myometrium compared to the rat, the possible heterogeneity of α_2-adrenoceptors. For this, we performed competition experiments with [^{3}H]-rauwolscine (the most commonly used radioligand for the studies of α_2-adrenoceptors, see Bylund *et al*., 1992; Deupree *et al*., 1996) and subselective compounds. These experiments allowed us to demonstrate the existence of, at least, two pharmacologically distinct populations of α_2-adrenoceptors. α_2-Adrenoceptor subtypes were also identified by Northern blotting with specific probes. In addition, we also examined whether α_2-adrenoceptor transcript levels and corresponding protein expression vary in the rat myometrium during the course of pregnancy.

Methods

Animals

Animals were maintained in accordance with the guidelines for care and use of laboratory animals (National Research Council. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press, 1996).

[1] Present address: Institut de Pharmacologie, 27 rue du Bugnon, 1005 Lausanne, Suisse.
[2] Author for correspondence.

Sprague Dawley rats (250–300 g) (Iffa Credo, L'Arbresle, France) were maintained on a 10 h light (09 h 00 min to 17 h 00 min) and 14 h dark schedule at 22–24°C. 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). In our breeding colony, parturition occurs between 12 and 19 h on day 22 for 80% of rats (Legrand & Maltier, 1986). Pregnant rats were killed by cervical dislocation on days 10, 12, 15, 21 and 22. The uterus was quickly isolated, cut open lengthwise and the foetoplacental units removed. The myometrium was then freed of adherent endometrium by scraping with a glass slide and stored in liquid nitrogen until analysis.

Human tissue collection

Samples of myometrium from pre-term (42 weeks gestation) women not in labour were taken from the upper border of the uterine incision during caesarian sections indicated for cephalopelvic disproportion. Tissue samples were frozen and stored in liquid nitrogen until required. This investigation had the approval of the Research Ethics Committee of INSERM (Institut National de la Santé et de la Recherche Médicale). Human kidneys not suitable for transplantation were recovered and frozen until used.

Cell culture and transfection

Chinese hamster ovarian (CHO) cells were grown in Ham's F12 medium with 10% foetal bovine serum. CHO cells deficient in dihydrofolate reductase ($DHFR^-$) were grown in MEM-α without ribonucleotides and deoxyribonucleotides supplemented with 10% (v/v) foetal calf serum and methotrexate.

HT29 human colonic adenocarcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 5% (v/v) foetal bovine serum and 5% (v/v) newborn calf serum. All cell types were cultured in the presence of penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), fungizone (0.25 $\mu g\ ml^{-1}$) at 37°C in a 5% CO_2 atmosphere. Cells were plated in 10 cm dishes and harvested when 90% confluency was reached.

Stable expression of human α_2C10, α_2C2 or α_2C4 was obtained in CHO cells $DHFR^-$ by use of expression vector pMDR 901 or pBC. α_2C10 cDNA fragment *Nco*I/*Hinf*I (1377 pb) and α_2C4 cDNA fragment *Nco*I/*Hind*III (~2000 pb) ends were blunted, *Eco*RI/*Not*I linkers (Pharmacia) were added and cDNA fragments *Eco*RI were digested by *Not*I. The resulting cDNA fragments *Not*I were inserted in pMDR 901 (containing the DHFR cDNA) into the *Not*I site to obtain α_2C10 pMDR 901 and α_2C4 pMDR 901. α_2C2 cDNA was inserted in expression vector pBC as described previously (Lomasney *et al*., 1991) to obtain α_2C2 pBC. CHO cells $DHFR^-$ were transfected with α_2C10 pMDR 901 or α_2C4 pMDR 901 or cotransfected by α_2C2 pBC and pMDR 901 by use of the electroporation method (Barsoum, 1990). The $DHFR^+$ transfectants were then selected in 3 μM methotrexate, a competitive inhibitor of the DHFR enzyme. Under these experimental conditions, the selected clones expressed high levels of the protein encoded by the gene of interest (Barsoum, 1990).

CHO transfectants expressing the rat α_{2A}-(RG20), α_{2B}-(RNG) or α_{2C}-(RG10) adrenoceptor subtype were kindly provided by Dr C.S. Flordellis.

Myometrial plasma membrane preparation

Crude membranes from pregnant myometria were obtained as previously described (Maltier & Legrand, 1985) and then resuspended in incubation buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.4). Protein concentration was determined by the method of Schacterle & Pollack (1973) with bovine serum albumin as the standard. 5′-Nucleotidase activity was measured to determine the plasma membrane samples enrichment versus homogenate. Briefly, 5′ nucleotidase activity was assayed according to the method described by Vallières *et al*. (1978) by measuring inorganic phosphate (Pi) liberated from AMP at 45°C during 20 min in a solution containing 2 mM $MgCl_2$, 100 mM KCl and 8 mM 5′AMP, pH 9.0.

Radioligand binding studies

In binding studies myometrial plasma membranes (200–300 μg) were incubated as described previously at 25°C for 20 min with the required concentrations of [^{3}H]-rauwolscine (Bottari *et al*., 1983; Legrand *et al*., 1993). Non specific binding was defined in the presence of 10 μM phentolamine. Competitions studies were performed in the presence of increasing concentrations (10 pM–50 mM) of various competitors and 5 nM [^{3}H]-rauwolscine (a concentration near its K_D for that tissue). Reactions were stopped by rapid cold filtration over GF/C glass fibre filters (Whatman, Clifton, NJ) under vacuum. Radioactivity was counted by liquid scintillation in a 1214 Rack-beta spectrometer (LKB, Turku, Finland) with a counting efficiency of approximately 50%. All assays were performed in duplicate.

Data from saturation and competition studies were analysed by use of a non linear least squares curve-fitting Graph Pad programme (Graph Pad Software, San Diego, CA). Iterative curve fitting of both one-site and two-site models to experimental data provide IC_{50}. IC_{50} values were converted to K_i values by use of the equation of Cheng & Prusoff (1973). The two-site model was considered to be a better fit than the one-site when the *F*-test was greater than the tabulated value at the prescribed degrees of freedom ($P<0.05$).

RNA isolation and Northern blot analysis

Total RNA was extracted from myometrium and other tissue specimens (brain, spleen, lung, liver, kidney) by the caesium-trifluorate gradient method (Okayama *et al*., 1987) as described previously (Vivat *et al*., 1992). For CHO cells, total RNA was extracted by use of the RNA-B kit (Bioprobe). $Poly(A)^+$ RNA were purified from total RNA by use of prepacked oligo (dT) – cellulose columns (Pharmacia Biotech Inc.). $Poly(A)^+$ RNA (10 μg) were then electrophoresed in a 1% agarose gel containing formaldehyde and transferred to GeneScreen Plus membranes (DuPont-New England Nuclear) by capillary blotting.

Hybridization was performed as described previously (Cohen-Tannoudji *et al*., 1995). Labelled $oligo(dT)_{12-18}$ probe (Pharmacia) was used as an internal control to estimate the amount of $poly(A)^+$ RNA loaded in each well and transfer efficiency.

Probes

The cDNA fragments used for identification of α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor mRNAs were chosen from regions coding for the putative third intracellular loops of the α_2-adrenoceptors, since these sequences are divergent between the different subtypes. In the rat, the 333-base pair *Kpn*I–*Xmn*I and 466-base pair *Nae*I–*Stu*I fragments were derived, respectively, from the rat brain α_{2A} and α_{2C} cDNA (kindly provided by Dr D.E. Handy) and were used as subtype-specific probes (Handy *et al*., 1993). A fragment specific for the α_{2B}-subtype was amplified from rat genomic DNA by use of polymerase chain reaction. The primers 5′-CGCAGCCACTGCAGAGGTCT-3′ (sense) and 5′-AGTCGCCCCACTAGTCCCCT-3′ (antisense) were chosen and provided amplification of a 279-base pair sequence from nucleic acids 981 to 1259 (Zeng *et al*., 1990). The polymerase chain reaction product was subcloned into pCRII vector (Invitrogen) and its sequence was verified by the Sanger dideoxynucleotide termination method by means of T7 DNA polymerase (Pharmacia). In the human tissues, the plasmids pUC18α_2C10, pSP65α_2C4 and pBCα_2C2 containing the coding sequences of human α_2C10-, α_2C4- and α_2C2-adre-

noceptors were kindly provided by Dr S. Cotecchia. cDNA fragments from regions coding for the third intracellular loop of the receptor were selected as in the rat. The 950-base pair *Pst*I, 871-base bair *Xmn*I and 503-base pair *Hin*P1I fragments derived respectively from the human α_2C10, α_2C4 and α_2C2 were used as subtype specific probes.

All of the cDNA probes were labelled by random priming with [α-^{32}P]-dATP to a specific activity averaging 10^9 d.p.m. μg^{-1}. Unbound radioactivity was separated by gel filtration by use of Sephadex G-50 DNA grade (Pharmacia).

Compounds used

Compounds used in this study were as follows: [^{3}H]-rauwolscine, specific activity 80 Ci mmol^{-1} (Radiochemical Centre, Amersham, U.K.); phentolamine hydrochloride (Ciba-Geigy, Basle, Switzerland); oxymetazoline hydrochloride, chlorpromazine hydrochloride, (Sigma Chemical Company, St Louis, MO); ARC-239 bichloride (2-[2-[4(O-methoxy-phen)piperazine-1-yl]ethyl]4,4 dimethyl-1,3,2H-4H) isoquinolinedione) (Karl Thomae, Biberach); clonidine (Boehringer-Ingelheim, Germany); phenylephrine hydrochloride and yohimbine hydrochloride (Serva, Heidelberg, Germany). All cell culture media were from Gibco BRL/Life technologies (Eragny, France). All other chemicals of the highest grade commercially available were from Sigma.

Data analysis

Data are presented as mean $\pm$ s.e.mean. Data were compared by Student's *t* test for paired or unpaired data. Differences are presented as significant at *P* values less than 0.05.

Results

5′-Nucleotidase assays

5′-Nucleotidase activities were measured in homogenates and plasma membrane fractions prepared from pregnant rat myometrium on days 12, 15, 22 of pregnancy and at term. 5′-Nucleotidase was similarly enriched (2.7 fold) in plasma membrane fractions at all the stages of pregnancy in comparison with the values in homogenates (mean values are 364 ± 65 μmol Pi h^{-1} mg^{-1} protein and 133 ± 23 μmol Pi h^{-1} mg^{-1}, respectively), indicating that all samples contained a similar quantity of myometrial plasma membranes.

Characterization and quantification of myometrial α_2-adrenoceptors by radioligand binding studies

Scatchard plot analysis with [^{3}H]-rauwolscine showed in rat myometrium (mid pregnancy or term) and in human myometrium (late pregnancy) a B_{max} of 32 ± 4 fmol mg^{-1} and 25 ± 1 fmol mg^{-1} with K_D values of 8.3 ± 0.4 nM and 2.6 ± 1.1 nM, respectively. To confirm that the [^{3}H]-rauwolscine binding sites in human and rat pregnant myometria were α_2-adrenoceptors, competition studies were performed with five different compounds, either α_2-, α_1- or β-selective antagonists or agonists (Figure 1). The data were analysed by a non-linear iterative curve fitting procedure. Computer analysis of the binding data indicated that a one-site model fit the data significantly better than a two-site model. Results obtained (pK_i values for each competitor) are summarized in Table 1. A comparison of these values showed that [^{3}H]-rauwolscine binding was inhibited by these compounds with an order of affinity known to be the one described for α_2-adrenoceptors (yohimbine $\geq$ clonidine $>$ (−)-noradrenaline $>$ phenylephrine $>$ propranolol). The characterization of the specific pharmacological subtype(s) of α_2-adrenoceptor was carried out by performing another set of competition studies in the presence of various subtype-selective compounds such as oxymetazoline (α_{2A}-specific), chlorpromazine, prazosin, ARC 239 (α_{2B}-specific). All drugs yielded competition curves which were shallow and biphasic (Figure 2). The computer modelling for all these drugs best fitted for a two-site model, $P < 0.05$. After calculation of pK_i values, it appeared that, whatever their subtype-selectivity, all the compounds displayed a high affinity for the human and rat myometrium [^{3}H]-rauwolscine binding

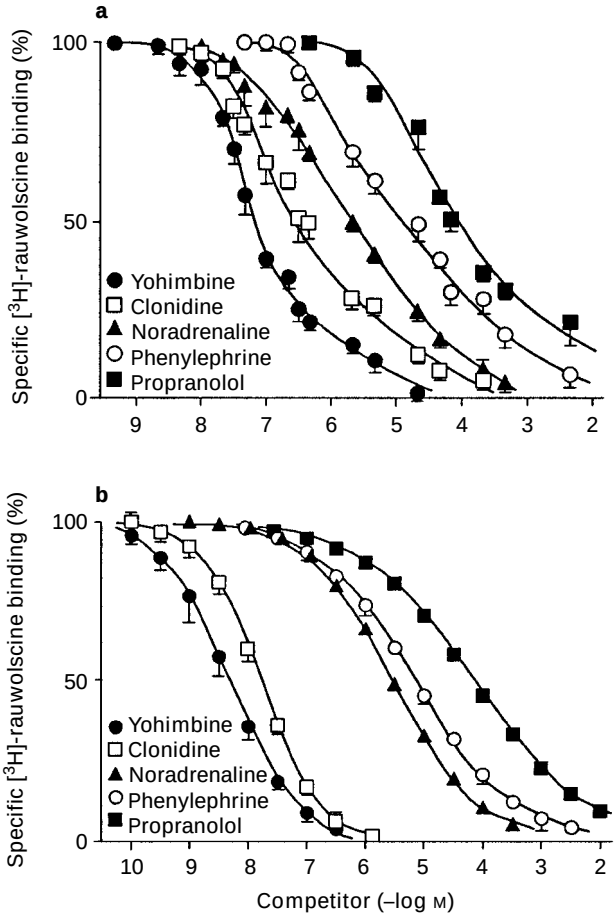

Figure 1 Inhibition of [^{3}H]-rauwolscine specific binding to membrane preparations obtained from preterm human myometrium (a) or from rat myometrium on day 21 of pregnancy (b) by increasing concentrations of yohimbine, clonidine, noradrenaline, phenylephrine and propranolol. [^{3}H]-rauwolscine binding is expressed as % of specific binding in the absence of competitor. Data points are mean vertical lines show s.e.mean for 2 to 4 separate experiments. pK_i values are indicated in Table 1.

Table 1 Binding affinities (pK_i) for different selective compounds at human and rat myometrial α_2-adrenoceptors

Competitors	*Human p*K$_i$	*Rat p*K$_i$
Yohimbine (antagonist α_2-selective)	7.63 ± 0.05	8.93 ± 0.17
Clonidine (agonist α_2-selective)	6.91 ± 0.09	8.71 ± 0.37
Noradrenaline (agonist α_2-selective)	6.23 ± 0.05	6.09
Phenylephrine (agonist α_1-selective)	5.37 ± 0.11	5.73 ± 0.11
Propranolol (antagonist β-selective)	4.64 ± 0.08	4.72

pK_i is the negative log of K_i. Affinities were determined by displacement of 5 nM [^{3}H]-rauwolscine by 15 concentrations of competing compounds. Values represent mean $\pm$ s.e.mean for 2–4 separate determinations. All assays were performed in duplicate.

sites (Table 2). These data are fully consistent with the presence of, at least, two α_2-adrenoceptor subtypes, α_{2A}- and α_{2B}- in pregnant myometria.

Characterization of α_2-adrenoceptor subtype expression by Northern blots

All the probes used in this study have been tested for their specificity on total RNA CHO cell line stably transfected with human α_2C10, α_2C2, α_2C4 or rat RG10, RG20 and RNG. Apart from a strong hybridization with the corresponding subtype probe, no cross hybridization has been detected (data not shown).

Human pregnant myometrium With ^{32}P labelled α_2C10 probe, two signals were observed at 3.8 kb and 4.7 kb in HT29 cells, human kidney, both known to express this receptor subtype, and in pregnant myometrium (Figure 3a). Both bands were specific with regard to α_2C10 transfected and control CHO cells. The weak band seen around 4.0 kb in CHO control was most likely due to non-specific hybridization to 28 S rRNA. In addition, the presence was noted of two strong specific 2.2 and

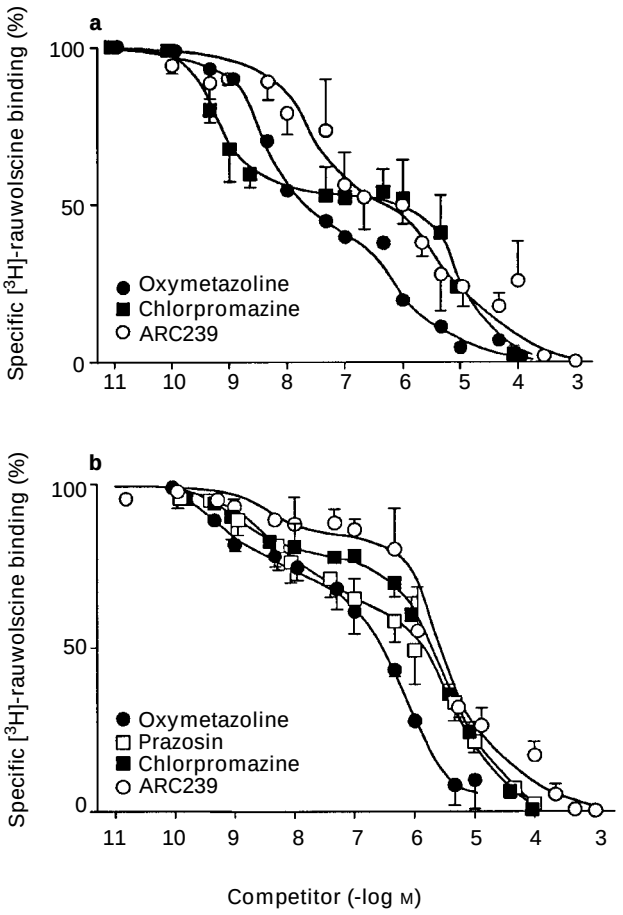

Figure 2 Inhibition of [^{3}H]-rauwolscine specific binding to membrane preparations obtained from preterm human myometrium (a) or from rat myometrium on day 21 of pregnancy (b) by increasing concentrations of oxymetazoline, prazosin, chlorpromazine and ARC 239. [^{3}H]-rauwolscine binding is expressed as % of specific binding in the absence of competitor. Data points are mean vertical lines show s.e.mean of 3–4 separate experiments. pK_i values for the high and low affinity sites are presented in Table 2.

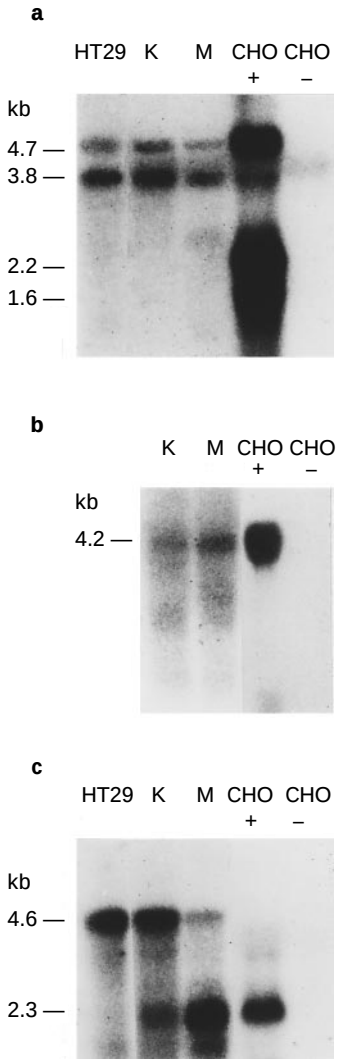

Figure 3 RNA blot analysis of α_2-adrenoceptor subtype transcripts expression in human myometrium. Poly(A)$^+$ RNA isolated from HT29 cells, kidney (K), preterm myometrium (M), transfected (CHO+) and control (CHO−) CHO cells were hybridized with ^{32}P-labelled α_2C10- (a), α_2C4- (b), α_2C2- (c) adrenoceptor cDNA probes. The position of co-electrophoresed RNA size markers (kb) is indicated.

Table 2 Binding affinities (pK_{iH} and pK_{iL}) for different subselective compounds at human and rat myometrical α_2-adrenoceptor subtypes

	Human		*Rat*	
Competitors	pK_{iH}	pK_{iL}	pK_{iH}	pK_{iL}
Oxymetazoline (α_{2A}-selective)	8.90	6.30	9.70±0.14	6.73±0.07
Prazosin (α_{2B}-selective)	Not done		9.00±0.10	5.91±0.06
Chlorpromazine (α_{2B}-selective)	9.83±0.37	5.48±0.16	9.43±0.20	5.94±0.06
ARC 239 (α_{2B}-selective)	7.94±0.23	3.99±0.85	9.71±1.05	6.09±0.12

pK_i is the negative log of K_i. The high affinity binding is expressed as pK_{iH}, the low affinity as pK_{iL}. Affinities were determined by displacement of 5 nM [^{3}H]-rauwolscine by 15 concentrations of competing compounds. Values represent mean±s.e.mean for 3–4 separate determinations, except for oxymetazoline in the human (1 experiment). All assays were performed in duplicate.

1.6 kb signals in α_2C10 transfected CHO cells, sizes which could correspond to the cleavage of the 3.8 Kb transcript. A specific probe hybridizing α_2C2 poly(A)$^+$ RNA, revealed a 4.2 kb band in human kidney and myometrium suggesting the presence of the α_{2B}-adrenoceptor subtype in both tissues (Figure 3b). With the α_2C4 probe, our data showed in myometrium a major and a minor band, respectively, at 2.3 kb and 4.6 kb. These two bands were also present in kidney. Nevertheless, only the 2.3 kb signal represented the α_{2C}-transcript, as confirmed by the positive signal obtained on CHO cells transfected with cDNA encoding for α_2C4 and the absence of a signal in control CHO and HT29 cells (Figure 3c). Altogether, these data strongly suggest that the human pregnant myometrium contains α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors.

Rat myometrium Hybridization of poly(A)$^+$ RNA with cDNA probe specific for the rat RG20-adrenoceptor showed a high level of the 3.8 kb transcript corresponding to the α_{2A}-subtype in pregnant rat myometrium. The identity of this band was confirmed by the fact that it was detected in brain and spleen and the absence of a signal in liver, which expresses only the α_{2B}-subtype. This probe also recognized a 4.7 kb band in all the tissues tested (Figure 4a). A 4.2 kb message encoding the

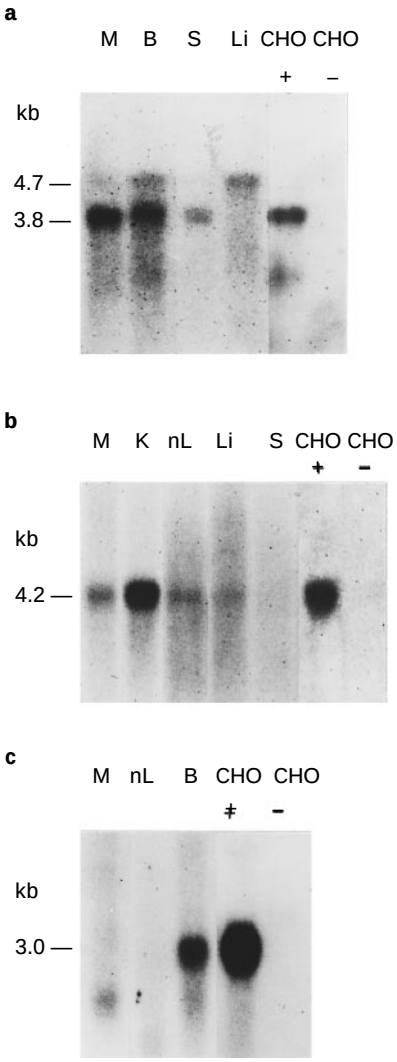

Figure 4 RNA blot analysis of α_2-adrenoceptor subtype transcripts expression in rat myometrium. Poly(A)$^+$ RNA isolated from late pregnant myometrium (M), brain (B), spleen (S), liver (Li), kidney (K), neonatal lung (nL), transfected (CHO+) and control (CHO−) CHO cells were hybridized to ^{32}P-labelled RG20- (a), RNG- (b) and RG10- (c) adrenoceptor cDNA probes. Position of co-electrophoresed RNA size markers (kb) are indicated.

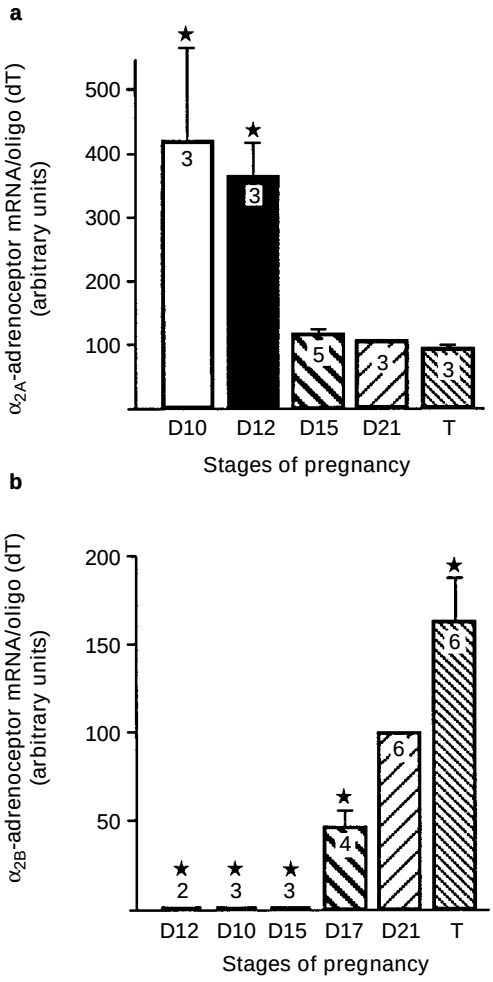

Figure 5 Changes of the steady-state level of RG20/α_{2A}- (a) and RNG/α_{2B}- (b) adrenoceptor mRNAs in rat myometrium from day 10 of pregnancy (D10) to term (T). For each stage, autoradiographic signals were quantified by densitometric scanning. Data are expressed as the changes in the density of each α_2-adrenoceptor subtype mRNA compared with values of day 21 (D21), as arbitrary units. Values are mean ± s.e.mean for the number of independent determinations indicated in parentheses. *Significantly different from day 21 ($P<0.05$).

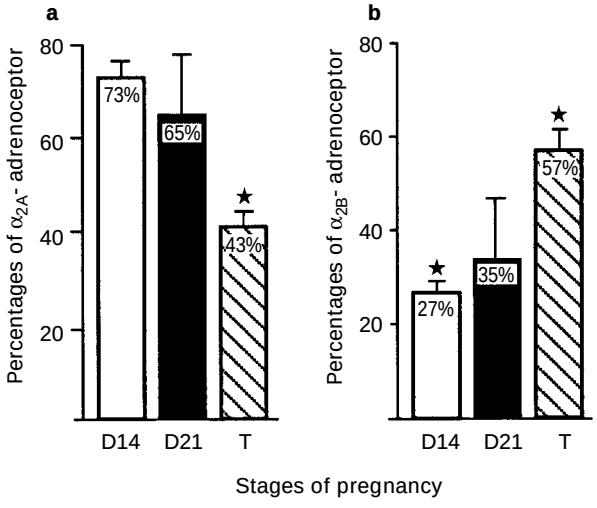

Figure 6 Relative proportions of the α_{2A}- (a) and α_{2B}- (b) adrenoceptor subtypes in pregnant rat myometria on day 14 (D14), day 21 (D21) of pregnancy and at term (T). Data are expressed as the percentage of high and low affinity sites calculated by use of standard computer techniques from inhibition curves of [^{3}H]-rauwolscine in competition with the α_{2A}- (oxymetazoline) and α_{2B}- (prazosin, ARC 239, chlorpromazine) subtype-selective compounds. Values are mean ± s.e.mean for 4 independent experiments. *Significantly different from previous stage ($P<0.05$).

RNG-adrenoceptor subtype was clearly detected in rat myometrium, as well as in the kidney, neonatal lung and liver, whereas the spleen, which expressed only the α_{2A}-subtype remained negative (Figure 4b). The α_{2C}-adrenoceptor probe specifically hybridized with a single 3 kb band in rat brain and RG10 CHO transfected cells. In contrast, no detectable signal was observed in pregnant rat myometrium whatever the stage of pregnancy (Figure 4c). In the pregnant rat myometrium, these results indicate the presence of only two adrenoceptor transcripts, α_{2A} and α_{2B}.

Differential expression of the α_{2A}- and α_{2B}-adrenoceptor subtypes in the rat myometrium during pregnancy

In the rat myometrium, quantification of the α_{2A}- and α_{2B}-transcripts with specific probes, as well as analysis of competition curves, clearly demonstrated the differential expression of α_{2A}- and α_{2B}-subtypes during the course of pregnancy. Indeed, the α_{2A}-adrenoceptor mRNA was 4 fold higher at mid pregnancy than in the later stages where it remained constant (Figure 5a). Conversely, with the RNG probe, an α_{2B} message could not be clearly detected until day 17. Then, its level increased by 110% from day 17 to term (Figure 5b). By performing competition studies with selective compounds at mid pregnancy and term, we defined the relative proportion of the α_{2A}-subtype versus the α_{2B}-subtype. Figure 6 shows that the density ratio of the two sites $\alpha_{2A}:\alpha_{2B}$ estimated at 73:27 on day 15 of pregnancy, was estimated at 43:57 at the time of parturition. So, in the pregnant rat myometrium, pharmacological data revealed a predominant expression of α_{2A}-adrenoceptors at mid-pregnancy whereas, at term, α_{2B}- and α_{2A}-subtype density reached approximately the same level. Thus, in rat, our results from competition studies are in general concordance with those obtained with Northern blot.

Discussion

The purpose of this study was to detect the heterogeneous expression of α_2-adrenoceptor subtypes in pregnant human and rat myometria. In both human and rat myometria, we showed that [^{3}H]-rauwolscine, a selective α_2-adrenoceptor antagonist (Perry & U'Prichard, 1981) binds to receptors with pharmacological characteristics of the α_2-adrenoceptor. Indeed, competition binding by various adrenoceptor antagonists or agonists exhibited the rank order of affinity expected for the α_2-adrenoceptor, i.e. yohimbine $\geqslant$ clonidine > noradrenaline > phenylephrine > propranolol. These results, as those obtained by Scatchard plot on human and rat pregnant myometrium, support previous radioligand binding data (Bottari *et al*., 1983; Maltier & Legrand, 1985; Dahle *et al*., 1993).

Recently, several studies have focused on tissue distribution of α_2-adrenoceptor subtypes in various species. Nevertheless, no information is currently available on different types of α_2-adrenoceptors expressed in rat and human myometrium. We first evaluated the different α_2-adrenoceptor subtypes expressed in the pregnant myometrium by performing competitive binding assays with the subtype-discriminating α_2-adrenoceptor ligands oxymetazoline (α_{2A}-selective), prazosin, chlorpromazine and ARC 239 (α_{2B}-selective). These compounds have been shown previously to be capable of discriminating between the three α_2-adrenoceptor subtypes in various peripheral tissues, brain or cell lines and in stable transfected cells (Bylund *et al*., 1988; 1992; Marjamäki *et al*., 1992; 1994). Binding of [^{3}H]-rauwolscine was inhibited by all these ligands. The inhibition curves of [^{3}H]-rauwolscine binding by these compounds were shallow and the results significantly warranted modelling with a two-site fit. Hence, the curves can be resolved into a high affinity site and a low affinity site. The high affinity site for oxymetazoline identified in both the human and rat myometria is consistent with the presence of an α_{2A}-adrenoceptor subtype. The low affinity site for oxymetazoline which had a higher affinity for chlorpromazine, a good discriminator for the α_{2B}-adrenoceptor, probably represents the α_{2B}-subtype. However, we could not exclude the presence of the α_{2C}-subtype since ARC 239 and prazosin show little or no discrimination between α_{2B}- and α_{2C}-subtypes (Bylund *et al*., 1992).

In tissues expressing more than one subtype it is difficult, by the assessment of the subtype selectivity of α_2-adrenoceptor ligands, to distinguish unequivocally between the subtypes. Thus, mRNA detection with human or rat specific cDNA probes was used as a complementary tool to discriminate between α_2-adrenoceptor subtypes. In addition, no data have been published on the myometrium whereas, in several human and rat tissues the distribution of α_2-adrenoceptor transcripts determined by PCR or Northern blotting has been determined (Perälä *et al*., 1992; Eason & Liggett, 1993; Blaxall *et al*., 1994b). In the rat, three α_2-adrenoceptor subtype genes designated RG20, RNG and RG10 are expressed in kidney and brain whereas RNG is strongly expressed only in the kidney and RG20 in the spleen (Handy *et al*., 1993). All the three human α_2C10, α_2C2 and α_2C4 genes have been also identified in kidney (Eason & Liggett, 1993). Using these tissues and transfected CHO cells as controls, we demonstrated the presence of α_{2A}- and α_{2B}-transcripts in both human and rat myometria, whereas α_{2C}-messenger was only detected in the human tissues. In human tissues, the hybridization studies performed with the α_2C10 probe clearly indicated the existence of two mRNA variants migrating respectively at 3.8 and 4.7 kb which probably result from post-transcriptional modification. Such observations have also been obtained in rat spleen, brain and kidney but the specificity of the 4.7 kb band was uncertain from these data (Flordellis *et al*., 1990; Handy *et al*., 1993). With the α_2C4 probe, the only specific signal is the 2.3 kb band as shown by the result of the hybridization of α_2C4 transfected CHO cells RNA. In the rat, immunoprecipitation of solubilized receptors from myometrial membranes, with specific α_2-adrenoceptor antibodies (Kurose *et al*., 1993) corroborate our Northern blot and pharmacological studies. Indeed, unpublished data obtained in our laboratory clearly demonstrate the presence, in the late pregnant myometrium, of a solubilized protein with an apparent molecular mass of 43 kDa, similar to the α_{2B}-adrenoceptor immunoprecipitated from the neonatal rat lung. This myometrial protein was not detected in early stages of pregnancy. In addition, with the RG10 antibody, the corresponding α_{2C}-adrenoceptor has not been immunoprecipitated whatever the stage of pregnancy.

In conclusion, the present data extend previous findings on the expanded distribution of α_{2A}-, α_{2B}- and/or α_{2C}-adrenoceptors in various tissues and species by demonstrating the heterogeneity of α_2-adrenoceptors in the pregnant human and rat myometrium. The most striking difference between human and rat is that the α_{2C}-transcript is strongly expressed in the human pregnant myometrium and is not found in the rat. This led us to postulate that this difference probably reflects a species-specific expression of this receptor subtype. Furthermore, in the rat myometrium, α_{2A}- and α_{2B}-adrenoceptors showed a differential expression during the course of pregnancy. The functional relevance of these results remains to be defined.

This study was partly supported by a grant from INSERM, Contract 92 07 10. We thank Dr S. Cotecchia for providing the cDNA for human α_2C10, α_2C4 and α_2C2-adrenoceptors, Drs D.E. Handy and C.S. Flordellis for providing the cDNA for rat RG20 and RG10 adrenoceptors. We also thank Dr H. Kurose for providing the rat RNGα_2 and RG10α_2-antibodies, and Drs R. Frydman and F. Olivennes (Service de Gynécologie-Obstétrique, Hôpital A. Béclère, Clamart) for providing us human myometria.

References

BARSOUM, J. (1990). Introduction of stable high copy number DNA into chinese hamster ovary cells by electroporation. *DNA Cell Biol.*, **9,** 293 – 300.

BLAXALL, H.S., CERUTIS, D.R., HASS, N.A., IVERSEN, L.J. & BYLUND, D.B. (1994a). Cloning and expression of the alpha-2C-adrenergic receptor from the OK cell line. *Mol. Pharmacol.*, **45,** 176 – 181.

BLAXALL, H.S., HASS, N.A. & BYLUND, D.B. (1994b). Expression of α_2-adrenergic receptor genes in rat tissues. *Receptor*, **4,** 191 – 197.

BOTTARI, S.P., VOKAER, A., KAIVEZ, E., LESCRAINIER, J.P. & VAUQUELIN, G. (1983). Identification and characterization of α_2-adrenergic receptors in human myometrium by [^{3}H] rauwolscine binding. *Am. J. Obstet. Gynecol.*, **146,** 639 – 643.

BREUILLER, M., ROUOT, B., LEROY, M.J., BLOT, P. & FERRE, F. (1990). Different α_2-adrenoceptor densities in the circular and longitudinal layers of human near-term myometrium as revealed by [^{3}H]idazoxan binding studies. *Gynecol. Obstet. Invest.*, **26,** 14 – 20.

BYLUND, D.B., BLAXALL, H.S., IVERSEN, L.J., CARON, M.G., LEFKOWITZ, R.J. & LOMASNEY, J.W. (1992). Pharmacological characteristics of α_2-adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol. Pharmacol.*, **42,** 1 – 5.

BYLUND, D.B., RAY-PRENGER, C. & MURPHY, T.J. (1988). Alpha-$_{2A}$ and alpha-$_{2B}$ adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.*, **245,** 600 – 607.

CHALBERG, S.C., DUDA, T., RHINE, J. & SHARMA, R.K. (1990). Molecular cloning, sequencing and expression of an α_2-adrenergic receptor complementary DNA from rat brain. *Mol. Cell. Biochem.*, **97,** 161 – 172.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22,** 3099 – 3108.

COHEN-TANNOUDJI, J., MHAOUTY, S., ELWARDY-MEREZAK, J., LECRIVAIN, J.L., ROBIN, M.T., LEGRAND, C. & MALTIER, J.P. (1995). Regulation of myometrial Gi2, Gi3, and Gq expression during pregnancy. Effects of progesterone and estradiol. *Biol. Reprod.*, **53,** 55 – 64.

DAHLE, L.O., ANDERSON, R.G.G., BERG, G., HURTIG, M. & RYDEN, G. (1993). Alpha-adrenergic receptors in human myometrium: changes during pregnancy. *Gynecol. Obstet. Invest.*, **36,** 75 – 80.

DEUPREE, J.D., HINTON, K.A., CERUTIS, D.R. & BYLUND, D.B. (1996). Buffers differentially alter the binding of [^{3}H]Rauwolscine and [^{3}H]RX821002 to the alpha-2 adrenergic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **278,** 1215 – 1227.

EASON, M.G. & LIGGETT, S.B. (1993). Human α_2-adrenergic receptor subtype distribution: widespread and subtype-selective expression of α2C10, α2C4 and α2C2 mRNA in multiple tissues. *Mol. Pharmacol.*, **44,** 70 – 75.

FLORDELLIS, C.S., CASTELLANO, M., FRANCO, R., ZANNIS, V.I. & GAVRAS, H. (1990). Expression of multiple α_2-adrenergic receptor messenger RNA species in rat tissues. *Hypertension*, **15,** 881 – 886.

FLORDELLIS, C.S., HANDY, D.E., BRESNAHAN, M.R., ZANNIS, V.I. & GAVRAS, H. (1991). Cloning and expression of a rat brain α_{2B}-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 1019 – 1023.

HANDY, D.E., FLORDELLIS, C.S., BOGDANOVA, N.N., BRESNAHAN, M.R. & GAVRAS, H. (1993). Diverse tissue expression of rat α_2-adrenergic receptor genes. *Hypertension*, **21,** 861 – 865.

KUROSE, H., ARRIZA, J.L. & LEFKOWITZ, R. (1993). Characterization of α_2-adrenergic receptor subtype-specific antibodies. *Mol. Pharmacol.*, **43,** 440 – 450.

KOBILKA, B.K., MATSUI, H., KOBILKA, T.S., YANG-FENG, T.L., FRANCKE, U., CARON, M.G., LEFKOWITZ, R.J. & REGAN, J.W. (1987). Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science*, **238,** 650 – 656.

LANIER, S.M., DOWNING, S., DUZIC, E. & HOMEY, C.J. (1991). Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor. *J. Biol. Chem.*, **266,** 10470 – 10478.

LEGRAND, C. & MALTIER, J.P. (1986). Evidence for a noradrenergic transmission in the control of parturition in the rat. *J. Reprod. Fert.*, **76,** 415 – 424.

LEGRAND, C., MHAOUTY, S., RIGOLOT, C. & MALTIER, J.P. (1993). Heterogeneity of Alpha-2 adrenoceptors in pregnant rat uterus: identification of subtypes and autoradiographic distribution. *J. Pharmacol. Exp. Ther.*, **266,** 439 – 449.

LOMASNEY, J.W., COTECCHIA, S., LEFKOWITZ, R.J. & CARON, M.G. (1991). Molecular biology of α-adrenergic receptors: implications for receptor classification and for structure/function relationships. *Biochem. Biophys. Acta*, **1095,** 127 – 139.

LOMASNEY, J.W., LORENZ, W., ALLEN, L.F., KING, K., REGAN, J.W., YANG-FENG, T.L., CARON, M.G. & LEFKOWITZ, R.J. (1990). Expansion of the α_2-adrenergic receptor family: cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 5094 – 5098.

MALTIER, J.P. & LEGRAND, C. (1985). Characterization of α-adrenoceptors in myometrium of preparturient rats. *Eur. J. Pharmacol.*, **117,** 1 – 13.

MARJAMÄKI, A., ALA-UOTILA, S., LUOMALA, K., PERÄLÄ, M., JANSSON, C., JALKANEN, M., REGAN, J.W. & SCHEININ, M. (1992). Stable expression of recombinant human α_2-adrenoceptor subtypes in two mammalian cell lines: characterization with [^{3}H]rauwolscine binding, inhibition of adenylate cyclase and RNase protection assay. *Biochim. Biophys. Acta*, **1134,** 169 – 177.

MARJAMÄKI, A., LUOMALA, K., ALA-UOTILA, S. & SCHEININ, M. (1994). Use of recombinant human α_2-adrenoceptors to characterize subtype selectivity of antagonist binding. *Eur. J. Pharmacol.*, **246,** 219 – 226.

OKAYAMA, H., KAWAICHI, M., BROWNSTEIN, M., LEE, F., YOKOTA, T. & ATAI, K. (1987). High efficiency cloning of full-length cDNA. Construction and screening of cDNA expression libraries for mammalian cells. *Methods Enzymol.*, **154,** 3 – 28.

PERÄLÄ, M., HIRVONEN, H., KALIMO, H., ALA-UOTILA, S., REGAN, J.W., AKERMAN, K.E.O. & SCHEININ, M. (1992). Differential expression of two α_2-adrenergic receptor subtype mRNAs in human tissues. *Molec. Brain. Res.*, **16,** 57 – 63.

PERRY, B.D. & U'PRICHARD, D.C. (1981). [^{3}H]Rauwolscine (alpha-yohimbine): a specific antagonist radioligand for brain alpha-2 adrenergic receptors. *Eur. J. Pharmacol.*, **76,** 461 – 464.

REGAN, J.W., KOBILKA, T.S., YANG-FENG, T.L., CARON, M.G., LEFKOWITZ, R.J. & KOBILKA, B.K. (1988). Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 6301 – 6305.

SCHACTERLE, G.R. & POLLACK, R.L.(1973). Simplified method for the quantitative assay of small amount of proteins in biological materials. *Analyt. Biochem.*, **51,** 654 – 655.

VALLIERES, J., FORTIER, M. & BUKOWIECKI, L. (1978). Alpha- and beta-adrenergic receptors in isolated rabbit uterine membranes: study of receptors alterations by pregnancy. *Biol. Reprod.*, **19,** 318 – 325.

VIVAT, V., COHEN-TANNOUDJI, J., REVELLI, J.P., MUZZIN, P., GIACOBINO, J.P., MALTIER, J.P. & LEGRAND, C. (1992). Progesterone transcriptionally regulates the β_2-adrenergic receptor gene in pregnant rat myometrium. *J. Biol. Chem.*, **267,** 7975 – 7978.

VOIGT, M.M., MCCUNE, S.K., KANTERMAN, R.Y. & FELDER, C.C. (1991). The rat α_2C4 adrenergic receptor gene encodes a novel pharmacological subtype. *FEBS Lett.*, **278,** 45 – 50.

ZENG, D., HARRISON, J.K., D'ANGELO, D.D., BARBER, C.M., TUCKER, A.L., LU, Z. & LYNCH, K.R. (1990). Molecular characterization of a rat α_{2B}-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 3102 – 3106.

(Received June 16, 1997
Revised September 8, 1997
Accepted September 17, 1997)