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Different roles of α_2-adrenoceptor subtypes in non-pregnant and late-pregnant uterine contractility *in vitro* in the rat

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Abstract

The roles of the α_2-adrenoceptor (α_2-AR) subtypes (α_{2A}-, α_{2B}- and α_{2C}-AR) in uterine contractility have not been investigated. The aims of this study were to identify these receptors in the non-pregnant and the late-pregnant rat myometrium and to determine their roles in contractions. We found that the myometrial α_2-AR subtypes are involved differently in the control of late-pregnant contractions, while they have no influence on the contractions of the non-pregnant myometrium.

The myometrial expressions of the α_2-AR subtypes were determined by RT-PCR and Western blotting techniques. *In vitro* contractions were stimulated with noradrenaline, and its effect was modified with the selective antagonists BRL 44408 (α_{2A}), ARC 239 ($\alpha_{2B/C}$) and spiroxatrine (α_{2C}). cAMP production was followed by noradrenaline stimulation in the presence of isobutylmethylxanthine and forskolin, and alterations induced in it by the antagonists were determined with an Enzyme Immunoassay Kit. The most effective antagonist was tested on labour-induced uteri *in vitro*.

All the α_2-AR subtypes were identified in both non-pregnant and pregnant uteri. Noradrenaline was not able to contract the non-pregnant tissue in the presence of propranolol and doxazosin, while its contracting effect in the pregnant uteri was enhanced by BRL 44408, spiroxatrine and the combination BRL 44408 + spiroxatrine. ARC 239 exerted a strong inhibitory effect on noradrenaline-stimulated contractions. The increasing and the decreasing effects of the compounds were confirmed by the changes in the intracellular cAMP levels. The effect of ARC 239 on the labour-induced myometrium was similar to that on the 22-day-pregnant myometrium.

The stimulation of α_2-ARs does not evoke contractions in the non-pregnant uterus. The α_{2A}- and α_{2C}-ARs mediate decreases, while the α_{2B}-AR mediates an increase in the contractions in the 22-day-pregnant myometrium. These differences may offer new targets for drugs against premature contractions in pregnancy.

Keywords: α_2-Adrenoceptors; Rat; Subtype-selective antagonists; Pregnancy; Uterus; cAMP; Premature labour

The adrenergic system plays an important role in the control of uterine contractility. It is well known that stimulation of the myometrial β_2-adrenoceptors (β_2-ARs) increases the intracellular cAMP level and causes uterine relaxation (Stiles et al., 1984), while the α_1-AR agonists elicit contractions via increases in the intracellular inositol phosphate and Ca^{2+} levels (Michelotti et al., 2000). The roles of the α_2-ARs, however, are not fully clear from the aspect of myometrial contractility. Earlier studies proved the presence of α_2-ARs in the uterus in several species, e.g. rat, swine and human (Bottari et al., 1985; Kyozuka et al., 1988; Taneike et al., 1995). Kyozuka et al. (1988) suggested that there was no connection between the non-pregnant myometrial α_2-ARs and contractility. Non-subtype-selective α_2-AR agonists were found to increase the contractions of the isolated pregnant uterus both in swine and in human (Kitazawa et al., 2000; Sia et al., 2005). Other authors claimed that pregnancy may alter the signal transduction processes of some ARs, including α_2-ARs, which may modify the response mediated by the ARs (Zhou et al., 2000).

The identification of the α_2-AR subtypes (Bylund et al., 1994) led to a more complicated background as concerns the understanding and interpretation of the roles of the α_2-ARs in

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myometrial contractions. All three α_2-AR subtypes (α_{2A}-AR, α_{2B}-AR and α_{2C}-AR) have been identified in the human myometrium, while only α_{2A}-ARs and α_{2B}-ARs have been found in the rat uterus (Bouet-Alard et al., 1997). To date there has been no investigation of the roles of the different α_2-AR subtypes in the control of uterine smooth muscle contractions. The existence of α_2-AR subtype-selective compounds offers a good possibility for determination of the functions of the given subtypes.

The aims of this study were to identify the α_2-AR subtypes in the non-pregnant and the late-pregnant rat myometrium and to determine their roles in contractions.

1. Experimental procedures

1.1. Housing and handling of the animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1813-1/2002). Sprague–Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3 °C; the relative humidity was 30–70% and the light/dark cycle was 12/12 h. They were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) with tap water available *ad libitum*. The animals were sacrificed by CO_2 inhalation.

1.2. Mating of the animals

Mature female (180–200 g) and male (240–260 g) rats were mated in a special mating cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4–5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day-pregnant animals.

1.3. RT-PCR studies

1.3.1. Tissue isolation

Uterine tissues from non-pregnant and 22-day-pregnant animals were rapidly removed, and the embryonic tissues were separated. The uteri were frozen in liquid nitrogen and then stored at −70 °C until total RNA extraction.

1.3.2. Total RNA preparation

Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate–phenol–chloroform by the procedure of Chomczynski and Sacchi (1987). After precipitation with isopropanol, the RNA was washed three times with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 μl DNase- and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm.

1.3.3. RT-PCR

The RNA (0.5 μg) was denatured at 70 °C for 5 min in a reaction mixture containing 20 μM oligo(dT) (Hybaid Corp., UK), 20 U RNase inhibitor (Hybaid Corp., UK), 200 μM dNTP (Sigma–Aldrich, USA) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl and 5 mM $MgCl_2$ in a final reaction volume of 20 μl. After the mixture had been cooled to 4 °C, 20 U MMLV reverse transcriptase (GIBCO, UK) and RNase H Minus (Promega, UK) were added, and the mixture was incubated at 37 °C for 60 min.

The PCR was carried out with 5 μl cDNA, 25 μl ReadyMix REDTaq PCR reaction mix (Sigma–Aldrich, USA), 2 μl 50 pM sense and antisense primers of the α_2-AR subtypes (GeneBank codes: NM_012739 for α_{2A}-AR; AF366899 for α_{2B}-AR; NM_138506 for α_{2C}-AR) and 16 μl DNase- and RNase-free distilled water. The coupling temperatures and numbers of cycles for the different α_2-AR subtypes were as follows: 57 °C, 32 cycles for α_{2A}-AR; 56 °C, 32 cycles for α_{2B}-AR; 59 °C, 36 cycles for α_{2C}-AR.

Glyceraldehyde-3-phosphate dehydrogenase primers were used as internal controls in all samples (Tso et al., 1985). The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp., UK). After the initial denaturation at 95 °C for 5 min, the reactions were taken through the previously determined number of cycles for each α_2-AR subtype: 60 s at 95 °C, 60 s at the appropriate coupling temperature and 60 s at 72 °C, followed by lowering of the temperature to 4 °C. This PCR protocol furnished optimized conditions and linear phase amplification for each of the primer sets employed. The optimum number of cycles for each set of primers was determined by performing kinetic analyses.

The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel with Kodak EDAS290 (Csertex Ltd., Hungary). For statistical evaluations, data were analysed by the use of ANOVA, followed by the Neuman–Keuls test.

1.4. Western blotting studies

Twenty micrograms of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in Series Standard Dual Cooled Units (BioRad, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Germany), using a semidry blotting technique (BioRad, Hungary). The membranes were blocked with 5% non-fat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween, overnight at 4 °C. After washing, the blots were incubated for 1 h at room temperature on a shaker with α_{2A}-, α_{2B}-, and α_{2C}-AR and β-actin polyclonal antibody (Santa Cruz Biotechnology, California, USA, 1:200) in the blocking buffer. Immunoreactive bands were visualized with the WesternBreeze Chromogenic Western blot immunedetection kit (Invitrogen, Hungary) and quantified. For statistical evaluations, data were analysed via the ANOVA, followed by the Neuman–Keuls test.

1.5. Isolated organ bath studies

Uteri were removed from non-pregnant (180–200 g), 22-day-pregnant (270–350 g), and labour-induced 20-day-pregnant rats (240–300 g). Muscle rings 5 mm long were sliced from the uterine horns and mounted in an organ bath (8 parallels) containing 10 ml de Jongh solution (in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH 7.4). The organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before experiments were undertaken, with a solution change every 15 min. The initial tension was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured and recorded with a gauge transducer and an S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Hungary), respectively. Contractions were elicited with noradrenaline (1×10^{-8} to 3×10^{-5} M) and cumulative concentration–response curves were constructed in each experiment in the presence of propranolol (10^{-5} M) and doxazosin (10^{-7} M) in order to avoid β- and α_1-adrenergic actions. α_2-AR antagonists (each 10^{-7} M) were left to incubate for 20 min before the administration of contracting agents. Following the addition of each concentration of noradrenaline, recording was performed for 300 s. Concentration–response curves were fitted and areas under curves (AUCs) were evaluated and analysed statistically with the Prism 4.0 (GraphPad Software, USA) computer program. From the AUC values, E_{max} and EC_{50} values were calculated (E_{max}: the maximum contracting effect of noradrenaline alone or in the presence of an α_2-AR antagonist; EC_{50}: the concentration of noradrenaline alone or in the presence of an α_2-AR antagonist which elicits half of the maximum contracting effect of noradrenaline). For statistical evaluations, data were analysed by two-tailed unpaired *t*-test.

1.6. Detection of myometrial cAMP

Uterine cAMP accumulation was measured with a commercial cAMP Enzyme Immunoassay Kit (Sigma–Aldrich, Hungary). Briefly, the kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the sample or an alkaline phosphatase molecule that has cAMP covalently attached to it. On a secondary antibody-coated microwell plate, the cAMP–antibody and the alkaline phosphatase–antibody complexes are conjugated. Following the addition of *p*-nitrophenyl phosphate, a substrate of alkaline phosphatase, the *p*-nitrophenol generated can be determined via its yellow colour at 405 nm. The more intense the colour, the lower the amount of intracellular cAMP.

Uterine tissue samples from non-pregnant and 22-day-pregnant rats were incubated in an organ bath (10 ml) containing de Jongh buffer (37 °C, perfused with carbogen). Isobutylmethylxanthine (10^{-3} M), doxazosin (10^{-7} M), propranolol (10^{-5} M) and the investigated subtype-selective α_2-AR antagonists (each 10^{-7} M) were incubated with the tissues for 20 min, and noradrenaline (3×10^{-6} M) was then added for 10 min. At the end of the noradrenaline incubation period, forskolin (10^{-5} M) was added for another 10 min, as described by Roberts et al. (1998). After this, the samples were immediately frozen and stored in liquid nitrogen until cAMP extraction. The tissue samples were next ground under liquid nitrogen, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at $600 \times g$ for 10 min. The supernatant was extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70 °C until the cAMP assay. The cAMP content was expressed in pmol (mg tissue)$^{-1}$. For statistical evaluations, data were analysed with ANOVA, followed by the Neuman–Keuls test.

1.7. Induction of premature labour in pregnant rat

The experimental premature labour procedure was carried out according to the model of Rechberger et al. (1996). Briefly, 19-day-pregnant rats were treated with s.c. antiprogesterone (mifepristone) at 3 mg/animal at 9.00 a.m. At 4.00 p.m., prostaglandin E_2 (0.5 mg/animal) was administered intravaginally. Our preliminary results had revealed that, after this treatment, the pregnant animals delivered between 9.00 and 10.00 a.m. on day 20 of pregnancy. Accordingly, the animals were sacrificed and uterine tissues were removed at 9.00 a.m., ensuring that the pregnant myometrium was very close to, but not after delivery.

1.8. Materials

Noradrenaline, isobutylmethylxanthine, forskolin, mifepristone, spiroxatrine and prostaglandin E_2 were purchased from Sigma–Aldrich, Hungary; BRL 44408 (2-[2*H*-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole) and ARC 239 (2-[2,4-(*O*-methoxyphenyl)-piperazin]-1-yl dihydrochloride were purchased from Tocris, UK. Doxazosin was donated by Pfizer Hungary Ltd., Hungary.

2. Results

2.1. RT-PCR and Western blotting studies

RT-PCR studies revealed the mRNAs of all three α_2-AR subtypes in both the non-pregnant and the 22-day-pregnant rat uteri. In the non-pregnant animals, there was a slight predominance of α_{2A}-AR mRNA (Fig. 1a and b), and in the pregnant myometrium a strong α_{2B}-AR mRNA predominance (Fig. 1c and d). The Western blotting analysis gave a result for the non-pregnant uteri similar to that from the RT-PCR studies (Fig. 2a and b). In the pregnant myometrium, the predominant

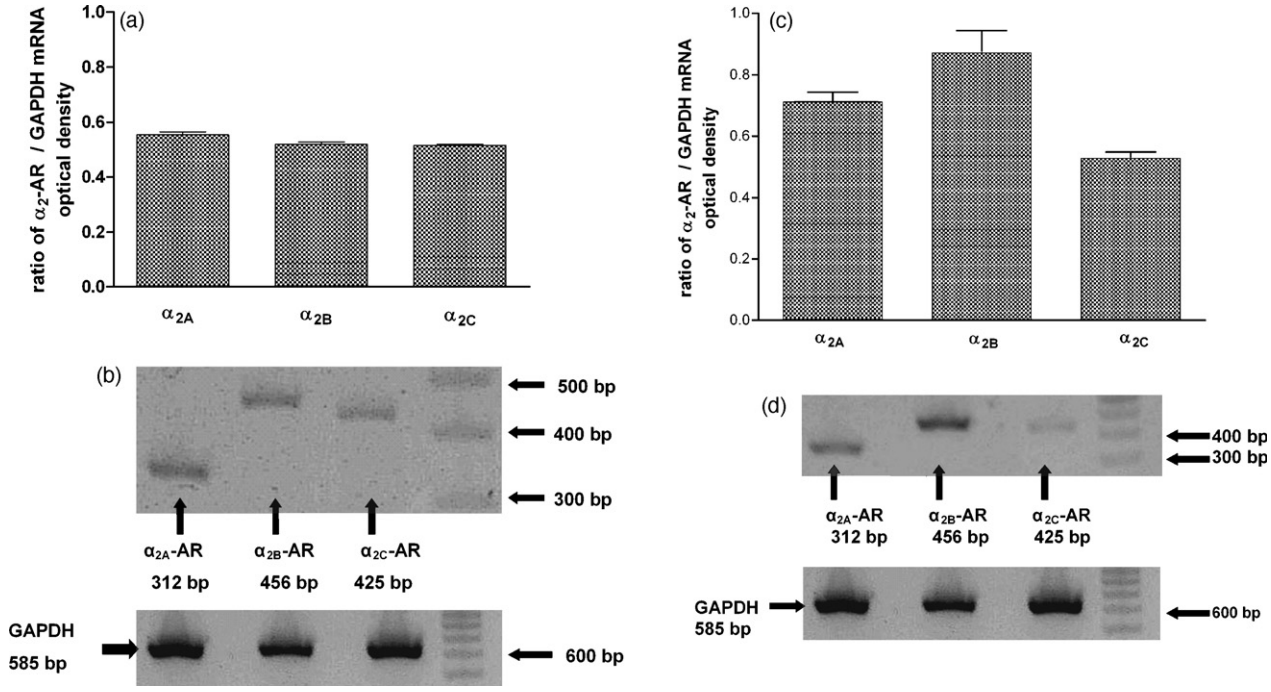

Fig. 1. The expressions of the mRNAs of the α_2-adrenoceptor (α_2-AR) subtypes in non-pregnant (a and b) and 22-day-pregnant (c and d) rat uteri ($n = 5$). (a and c) The results were expressed as the ratio of the optical densities of the AR subtype and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs (±S.E.M.). All subtype mRNAs were found in both the non-pregnant and the pregnant uteri. In the non-pregnant uteri, (a) a slight α_{2A}-AR mRNA predominance was found (levels of significance by ANOVA followed by the Neuman–Keuls test: α_{2A}-AR *vs.* α_{2B}-AR, $p < 0.01$; α_{2A}-AR *vs.* α_{2C}-AR, $p < 0.01$; α_{2B}-AR *vs.* α_{2C}-AR, $p > 0.05$). In the 22-day-pregnant uteri, (c) an α_{2B}-AR mRNA predominance was detected (levels of significance by ANOVA followed by the Neuman–Keuls test: α_{2B}-AR *vs.* α_{2A}-AR, $p < 0.05$; α_{2B}-AR *vs.* α_{2C}-AR, $p < 0.001$; α_{2A}-AR *vs.* α_{2C}-AR, $p < 0.01$). (b and d) GAPDH and α_2-AR subtype reverse transcription-polymerase chain reaction (RT-PCR) products from the total RNA from non-pregnant (b) and 22-day-pregnant (d) uteri. Each band contains RNA from the uterus of one animal. The 600, 312, 456 and 425 base pair (bp) RT-PCR products correspond to the mRNA of GAPDH, α_{2A}-, α_{2B}- and α_{2C}-AR, respectively.

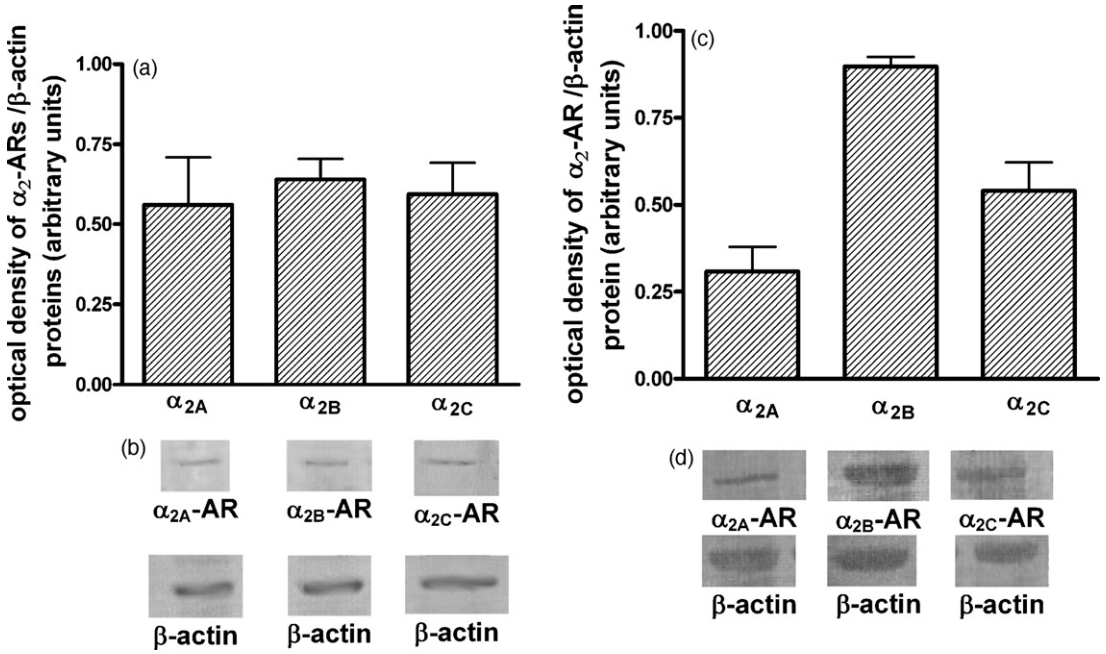

Fig. 2. The expressions of the α_2-adrenoceptor (α_2-AR) subtype protein in non-pregnant (a and b) and 22-day-pregnant (c and d) rat uteri ($n = 5$). (a and c) The results were expressed as the ratio of the optical densities of the receptor subtypes and β-actin proteins (±S.E.M.). All subtype proteins were found in both the non-pregnant and the pregnant uteri. In the non-pregnant uteri, (a) no differences were observed between the expressions of the proteins of the α_2-AR subtypes. In the 22-day-pregnant uteri, (c) an α_{2B}-AR protein predominance was detected (levels of significance by ANOVA followed by the Neuman–Keuls test: α_{2B}-AR *vs.* α_{2A}-AR, $p < 0.001$; α_{2B}-AR *vs.* α_{2C}-AR, $p < 0.01$; α_{2A}-AR *vs.* α_{2C}-AR, $p < 0.001$). (b and d) The α_2-AR subtype and β-actin Western blotting products from non-pregnant (b) and 22-day-pregnant (d) rat uteri. Each pair of bands (α_2-AR subtypes and β-actin) contains proteins from the uterus of one animal. Samples were subjected to gel electrophoresis on 10% polyacrylamide gel. The antibody binding was detected with an enhanced chemiluminescence detection system.

α_2-AR subtype protein was the α_{2B}-AR, while the optical density of the α_{2A}-AR protein was significantly lower than that of the α_{2C}-AR (Fig. 2c and d).

2.2. Isolated organ studies with non-pregnant and 22-day-pregnant myometria

Noradrenaline in the concentration range 10^{-8} to $10^{-4.5}$ M did not exert a contractile effect on the non-pregnant uterine rings, whereas vivid contractions were elicited by 25 mM KCl (data not shown). In the 22-day-pregnant myometrium, noradrenaline concentration-dependently increased the contractions, and these were slightly increased by the α_{2A}-AR antagonist BRL 44408 (Fig. 3). The $\alpha_{2B/C}$-AR antagonist ARC 239 significantly decreased the maximum effect of noradrenaline (Fig. 4), while the α_{2C}-AR antagonist spiroxatrine enhanced the noradrenaline-induced contractions (Fig. 5). The combination BRL 44408 + spiroxatrine also caused an increase in the maximum myometrium-contracting effect of noradrenaline (Fig. 6). The EC_{50} and E_{max} values of the curves are listed in Table 1.

2.3. cAMP studies

In the non-pregnant uterine tissue, BRL 44408, ARC 239, spiroxatrine, and the combination BRL 44408 + spiroxatrine did not influence the amount of cAMP produced in the presence of 3×10^{-6} M noradrenaline (data not shown). In the pregnant uteri, ARC 239 was able to increase the cAMP level produced by noradrenaline, while BRL 44408, spiroxatrine and the combination BRL 44408 + spiroxatrine caused significant decreases in the amount of myometrial cAMP (Fig. 7).

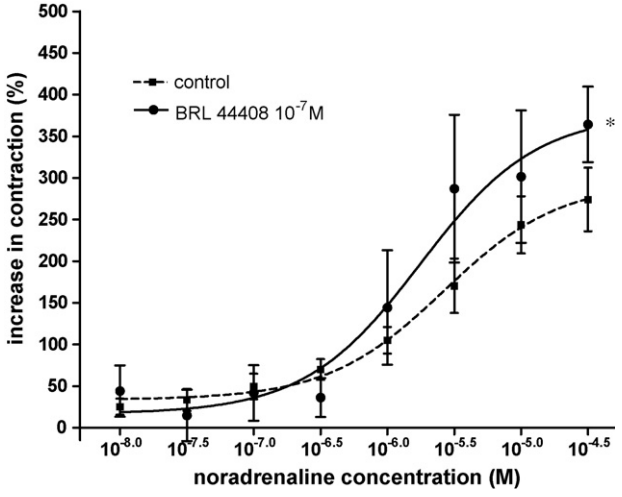

Fig. 3. Effect of the subtype-selective α_{2A}-adrenoceptor antagonist BRL 44408 on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath ($n = 8$). The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. $^*p < 0.05$. BRL 44408 at 10^{-7} M increased the maximum contracting effect of noradrenaline. For EC_{50} and E_{max} values, see Table 1.

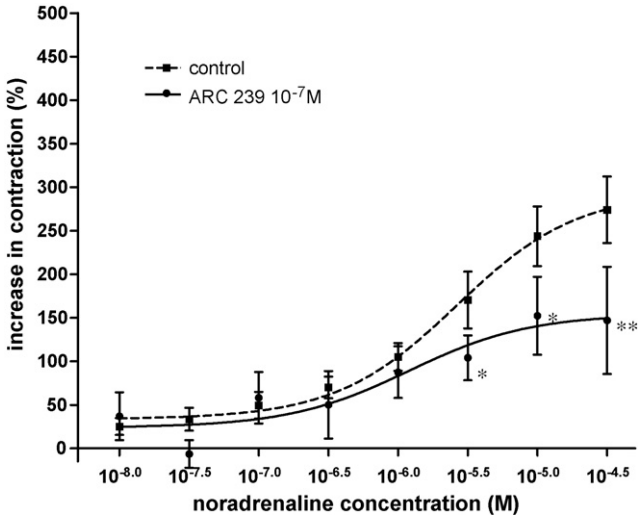

Fig. 4. Effect of the subtype-selective $\alpha_{2B/C}$-adrenoceptor antagonist ARC 239 on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath ($n = 8$). The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. $*p < 0.05$; $**p < 0.01$. ARC 239 at 10^{-7} M decreased the maximum contracting effect of noradrenaline. For EC_{50} and E_{max} values, see Table 1.

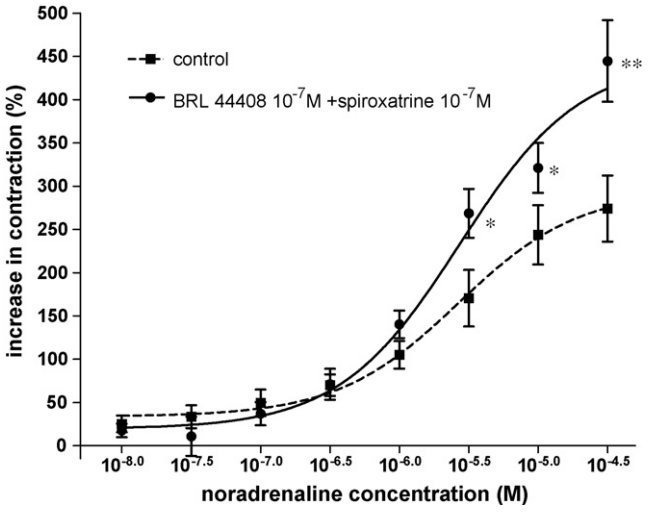

Fig. 6. Effects of the subtype-selective α_{2A}-adrenoceptor antagonist BRL 44408 and α_{2C}-adrenoceptor antagonist spiroxatrine on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath ($n = 8$). The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. $*p < 0.05$; $**p < 0.01$. BRL 44408 and spiroxatrine at 10^{-7} M increased the maximum contracting effect of noradrenaline. For EC_{50} and E_{max} values, see Table 1.

2.4. Isolated organ studies with myometria from hormonally induced preterm birth

Noradrenaline ($10^{-8.5}$ to 10^{-5} M) enhanced the contractions of labour-induced uterine rings, although its effect was less than that in the 22-day-pregnant animals. ARC 239 blocked the noradrenaline-evoked contractions (Fig. 8). The EC_{50} and E_{max} values of the curves are presented in Table 2.

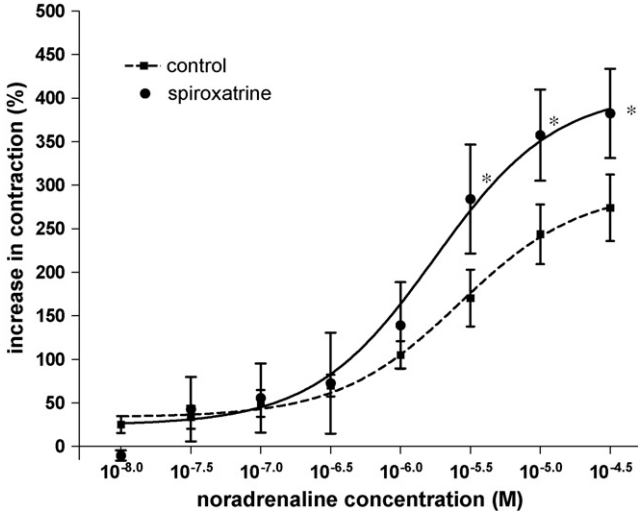

Fig. 5. Effect of the subtype-selective α_{2C}-adrenoceptor antagonist spiroxatrine on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath ($n = 8$). The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. $*p < 0.05$. Spiroxatrine at 10^{-7} M increased the maximum contracting effect of noradrenaline. For EC_{50} and E_{max} values, see Table 1.

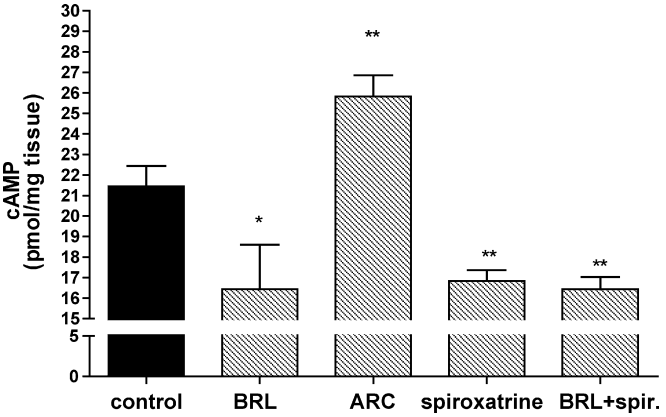

Fig. 7. Effects of the subtype-selective α_2-adrenoceptor antagonists on the myometrial cAMP level (pmol/mg tissue ± S.E.M.) stimulated by noradrenaline in the presence of isobutylmethylxanthine and forskolin (control) in the 22-day-pregnant rat ($n = 6$). The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). cAMP production was increased by IBMX (10^{-3} M) and forskolin (10^{-5} M). The statistical analyses were carried out with ANOVA followed by the Neuman–Keuls test. $*p < 0.05$; $**p < 0.01$. The antagonist concentrations were 10^{-7} M in each case. The myometrial cAMP produced by 3×10^{-6} M noradrenaline was increased by ARC 239 (ARC), while BRL 44408 (BRL), spiroxatrine and the combination BRL 44408 + spiroxatrine (BRL + spir.) decreased its level.

Table 1
Changes in the 22-day pregnant uterus-contracting effect of noradrenaline (EC_{50} and E_{max} values) in the presence of subtype-selective α_2-AR antagonists (10^{-7} M)

	Stimulated α_2-ARs	$EC_{50} \pm$ S.E.M. (M)	$E_{max} \pm$ S.E.M. (%)
Noradrenaline (control)	α_{2A}-, α_{2B}- and α_{2C}-ARs	$2.6 \times 10^{-6} \pm 0.8 \times 10^{-6}$	295.1 ± 30.3
Noradrenaline + BRL 44408	α_{2B}- and α_{2C}-ARs	$1.8 \times 10^{-6} \pm 0.9 \times 10^{-6}$	377.0 ± 51.8
Noradrenaline + ARC 239	α_{2A}-ARs	$1.2 \times 10^{-6} \pm 1.3 \times 10^{-6}$	154.4 ± 34.5
Noradrenaline + spiroxatrine	α_{2A}- and α_{2B}-ARs	$1.8 \times 10^{-6} \pm 1.1 \times 10^{-6}$	408.0 ± 41.5
Noradrenaline + BRL 44408 + spiroxatrine	α_{2B}-ARs	$2.7 \times 10^{-6} \pm 1.0 \times 10^{-6}$	446.8 ± 34.8

EC_{50}: the concentration of noradrenaline alone or in the presence of an α_2-AR antagonist which elicits half the maximum contracting effect of noradrenaline. E_{max}: maximum contracting effect of noradrenaline alone or in the presence of the α_2-AR antagonists.

Table 2
Changes in the labour-induced uterus-contracting effect of noradrenaline (EC_{50} and E_{max} values) in the presence of α_{2BC}-AR antagonist ARC 239 (10^{-7} M)

	Stimulated α_2-ARs	$EC_{50} \pm$ S.E.M. (M)	$E_{max} \pm$ S.E.M. (%)
Noradrenaline (control)	α_{2A}-, α_{2B}- and α_{2C}-ARs	$3.4 \times 10^{-7} \pm 1.6 \times 10^{-7}$	100.3 ± 100.4
Noradrenaline + ARC 239	α_{2A}-ARs	$3.1 \times 10^{-7} \pm 1.2 \times 10^{-7}$	58.6 ± 8.7

EC_{50}: the concentration of noradrenaline alone or in the presence of ARC 239 which elicits half the maximum contracting effect of noradrenaline. E_{max}: maximum contracting effect of noradrenaline alone or in the presence of ARC 239.

3. Discussion

The α_2-ARs, classified into three subtypes (α_{2A}-, α_{2B}- and α_{2C}-ARs), can be found at both presynaptic and postsynaptic sites of the adrenergic synapses (Philipp et al., 2002). The differences in the receptor subtypes and their various localizations are thought to be responsible for their different roles in, for example, the control of blood pressure (Link et al., 1996; Altman et al., 1999) and behaviour (Sallinen et al., 1999; Schramm et al., 2001). Little is known, however, of the roles of these receptor subtypes in the contractility of the uterine smooth muscle. Earlier, the localizations of the subtypes were studied in the late-pregnant rat myometrium, where they were found on the surface of the myometrial cells, while they were localized on both sides of the adrenergic synapses in the non-pregnant myometrium (Kyozuka et al., 1988; Legrand et al., 1993).

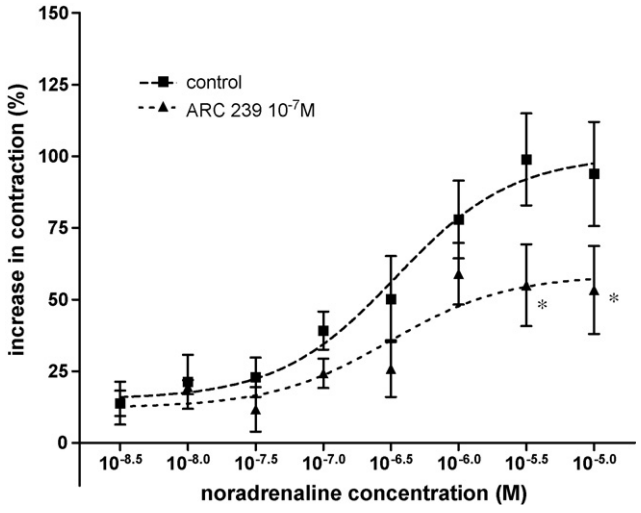

Fig. 8. Effect of the subtype-selective $\alpha_{2B/C}$-adrenoceptor antagonist ARC 239 on the noradrenaline-evoked contractions (control) in the labour-induced rat myometrium in an isolated organ bath ($n = 8$). Premature labour was induced by treatment with mifepristone s.c. 3 mg/animal) and prostaglandin E_2 (0.5 mg/animal) intravaginally on pregnancy day 19. The foetuses were usually delivered on the morning of day 20. Animals were sacrificed within 1 h before the anticipated birth. The studies were carried out in the presence of the β-adrenoceptor antagonist propranolol (10^{-5} M) and the α_1-adrenoceptor antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. $^*p < 0.05$. ARC 239 at 10^{-7} M significantly decreased the maximum contracting effect of noradrenaline. For EC_{50} and E_{max} values, see Table 2.

We identified all three α_2-AR subtypes in both the non-pregnant and the 22-day-pregnant rat uteri. An earlier study did not detect α_{2C}-AR in the rat myometrium, though in that work (Bouet-Alard et al., 1997) a radioligand-binding technique was used, which has a lower specificity as compared with our RT-PCR and Western blotting techniques, using α_2-AR subtype-specific primers and polyclonal antibodies, respectively.

The roles of α_2-AR subtypes in myometrial contractility were investigated via the effects of subtype-selective antagonists on the noradrenaline-stimulated contractions. Noradrenaline was ineffective on the non-pregnant uteri, whereas KCl enhanced the contractions. These results suggest no coupling between the α_2-ARs and adenylyl cyclase, which was supported by cAMP studies, where the presence of the antagonist did not alter the tissue cAMP production. This finding reaffirmed the earlier report on the lack of connection between α_2-ARs and contractions in the non-pregnant rat myometrium (Kyozuka et al., 1988).

Noradrenaline elicited contractions in the late-pregnant uteri, which were mediated via the α_2-ARs because of the presence of α_1- and β-AR blockers (doxazosin and propranolol). Although the antagonists for the α_2-AR subtypes are not very selective, we used three compounds with acceptable selectivity for one or two subtypes. BRL 44408, ARC 239 and spiroxatrine were earlier found to be selective for α_{2A}-AR, $\alpha_{2B/C}$-AR and α_{2C}-AR, respectively (Uhlen et al., 1992, 1997; Renouard et al., 1994). Each of these compounds displays various affinities for the 5-HT_{1A} receptors (Barrett et al., 1989;

Meana et al., 1996) and spiroxatrine also exerts effects in the dopaminergic system (Costall and Naylor, 1978), but these effects are likely to have only a low impact on the uterine contractions stimulated by noradrenaline.

The three subtype-selective compounds offered a possibility to investigate the results of the stimulation of only one or two α_2-AR subtypes in the contractions and in the changes in tissue cAMP level, which are crucial in the control of the smooth muscle contraction and relaxation (Pierce et al., 2002). In the cAMP studies, the phosphodiesterase inhibitor IBMX was used to block the degradation of the generated intracellular cAMP (Schlageter et al., 1980), while forskolin was added to enhance the activity of adenylyl cyclase (Seamon and Daly, 1986). BRL 44408 blocked the α_{2A}-ARs, and hence noradrenaline could stimulate only the α_{2B}- and α_{2C}-ARs. The simultaneous stimulation of these two receptors mildly increased the contractions and decreased the intracellular cAMP level. In the presence of ARC 239, only the α_{2A}-ARs remained free; the stimulation of this subtype decreased the effect of noradrenaline, with a rise in the myometrial cAMP level. Spiroxatrine blocked the α_{2C}-ARs, and thus the α_{2A}- and α_{2B}-ARs were stimulated by the agonist, and an increase in contraction and a decrease in the cAMP level were found. The combination BRL 44408 + spiroxatrine blocked the α_{2A}- and α_{2C}-ARs; the stimulation of the free α_{2B}-ARs also increased the uterine contractions and decreased the amount of tissue cAMP. The presence of the antagonists did not alter the EC_{50} values of the contracting dose–response curves of noradrenaline, indicating that the compound had the same affinity for each of the α_2-ARs.

These results suggest that in the late-pregnant myometrium α_{2A}-ARs mediate only weak contractions, which can be regarded as relaxation as they are compared with the effect of noradrenaline on all the three receptor subtypes. The α_{2B}-ARs are responsible for strong contractions. The α_{2C}-ARs also seem to decrease the contractions because the contracting effect mediated through the α_{2B}-ARs was significantly increased when α_{2C}-ARs were blocked by spiroxatrine. The extent of the increase in contraction was lower on the simultaneous stimulation of the α_{2B}- and α_{2C}-ARs than in the case of the α_{2B}- and α_{2A}-ARs, which can be explained by the higher density of the α_{2C}-ARs as compared with the α_{2A}-ARs. Although an elevation of the intracellular cAMP level is very unusual after stimulation of an α_2-AR, pregnancy seems to turn the G-protein activation into the opposite direction, as we found in the β-adrenergic system in 22-day-pregnant rats (Gáspár et al., 2005). On the other hand, it has been proved that pregnancy is able to induce a change in the G_i/G_s-activating property of α_2-AR in rats, resulting in a differential regulation of myometrial adenylyl cyclase activity at mid-pregnancy *versus* term (Mhaouty et al., 1995). The different functions of the postsynaptic α_2-AR subtypes inside the same tissue seem to be unique in the pregnant uterus. Although there are some other tissues in which stimulation of the α_2-AR subtypes results in opposite effects, e.g. in the vasculature, the stimulation of α_{2A}-ARs and α_{2C}-ARs causes relaxation and contraction, respectively, but in this case the α_{2A}-ARs are localized presynaptically, while the α_{2B}-ARs are located on the postsynaptic surface (Philipp et al., 2002).

Another question is why the effect of BRL 44408 + spiroxatrine did not exceed the contraction-increasing effect of spiroxatrine alone. Although an increasing tendency in the maximal contractions and a very slight decrease in the tissue cAMP level were observed when the combination was used, these changes were not significant as compared with the effect of spiroxatrine. It is known that the α_2-ARs are also prone to be involved in the processes of homo- and heterodimerization. It has additionally been proved that, when both α_{2A}- and α_{2C}-ARs are expressed, there is a greater likelihood that the two receptors will form heterodimers than homodimers. The α_{2C}-ARs alter α_{2A}-AR signalling by forming oligomers (Hein, 2006; Small et al., 2006). This means that these two receptor subtypes probably function together, although the details of their cooperation are not well known. If the α_{2C}-AR is also able to alter the function of the α_{2A}-ARs in the uterine smooth muscle, this cooperation might give a partial explanation for the similar maximum effects and changes in tissue cAMP levels. Thus, if α_{2C}-AR is blocked, the function of α_{2A}-AR might be modified independently from its blockade.

The uterus-contracting effect of noradrenaline can be explained as a resultant effect mediated by the AR subtypes. Because of the α_{2B}-AR predominance at the end of pregnancy, the contraction is the main resultant effect, which is altered by the other two subtypes, mediating a decrease in the intensity of noradrenaline-induced contractions.

ARC 239 had a marked relaxing effect on the noradrenaline-stimulated uterine contractions; it was therefore tested on the myometrium from the induced labour model. This test was designed to investigate the effects of this compound on overstimulated uterine tissue which is very close to delivery (within 1 h). Its effect on the noradrenaline-evoked contractions was also convincing; it can be promising in the therapy of premature labour. The increased sensitivity of labour-induced uteri to noradrenaline (where the EC_{50} value was 10 times lower than in normal pregnancy) may mean a further advantage for α_2-AR blockers in the inhibition of premature contractions.

We can conclude that the α_2-AR subtypes play different roles in the contractility of the rat uterus. In non-pregnant animals, they are not involved in the control of myometrial contractions. In last-day-pregnant animals, the α_{2B}-ARs predominate and mediate contraction, while the α_{2A}- and α_{2C}-ARs decrease the contractile response to noradrenaline. Theoretically, if these regularities hold in human too, stimulation of the α_{2A}- and α_{2C}-ARs and inhibition of the α_{2B}-ARs open up new targets for drugs against premature contractions in pregnancy.

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References

Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., Hein, L., 1999. Abnormal regulation of the

sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. Mol. Pharmacol. 56, 154–161.

Barrett, J.E., Hoffmann, S.M., Olmstead, S.N., Foust, M.J., Harrod, C., Weissman, B.A., 1989. Behavioral and neurochemical effects of the serotonin (5-HT)1A receptor ligand spiroxatrine. Psychopharmacology (Berlin) 97, 319–325.

Bouet-Alard, R., Mhaouty-Kodja, S., Limon-Boulez, I., Coudouel, N., Maltier, J.P., Legrand, C., 1997. Heterogeneity of alpha 2-adrenoceptors in human and rat myometrium and differential expression during pregnancy. Br. J. Pharmacol. 122, 1732–1738.

Bottari, S.P., Vokaer, A., Kaivez, E., Lescrainier, J.P., Vauquelin, G., 1985. Regulation of alpha- and beta-adrenergic receptor subclasses by gonadal steroids in human myometrium. Acta Physiol. Hung. 65, 335–346.

Bylund, D.B., Eikenberg, D.C., Hieble, J.P., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Molinoff, P.B., Ruffolo Jr., R.R., Trendelenburg, U., 1994. International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol. Rev. 46, 121–136.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Costall, B., Naylor, R.J., 1978. Dopamine antagonistic effects of a series of analogues of oxiperomide and spiroxatrine measured behaviourally in the rodent. J. Pharm. Pharmacol. 30, 693–698.

Gáspár, R., Ducza, E., Mihályi, A., Márki, A., Kolarovszki-Sipiczki, Z., Páldy, E., Benyhe, S., Borsodi, A., Földesi, I., Falkay, G., 2005. Pregnancy-induced decrease in the relaxant effect of terbutaline in the late-pregnant rat myometrium: role of G-protein activation and progesterone. Reproduction 130, 113–122.

Hein, L., 2006. Adrenoceptors and signal transduction in neurons. Cell Tissue Res. 326, 541–551.

Kitazawa, T., Maezono, Y., Taneike, T., 2000. The mechanisms of alpha2-adrenoceptor agonist-induced contraction in longitudinal muscle of the porcine uterus. Eur. J. Pharmacol. 390, 185–195.

Kyozuka, M., Crankshaw, D.J., Crankshaw, J., Berezin, I., Kwan, C.Y., Daniel, E.E., 1988. Alpha-2 adrenoceptors on nerves and muscles of rat uterus. J. Pharmacol. Exp. Ther. 244, 1128–1138.

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.

Link, R.E., Desai, K., Hein, L., Stevens, M.E., Chruscinski, A., Bernstein, D., Barsh, G.S., Kobilka, B.K., 1996. Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. Science 273, 803–805.

Meana, J.J., Callado, L.F., Pazos, A., Grijalba, B., Garcia-Sevilla, J.A., 1996. The subtype-selective alpha 2-adrenoceptor antagonists BRL 44408 and ARC 239 also recognize 5-HT1A receptors in the rat brain. Eur. J. Pharmacol. 312, 385–388.

Mhaouty, S., Cohen-Tannoudji, J., Bouet-Alard, R., Limon-Boulez, I., Maltier, J.P., Legrand, C., 1995. Characteristics of the alpha 2/beta 2-adrenergic receptor-coupled adenylyl cyclase system in rat myometrium during pregnancy. J. Biol. Chem. 270, 11012–11016.

Michelotti, G.A., Price, D.T., Schwinn, D.A., 2000. Alpha-1 adrenergic receptor regulation: basic science and clinical implications. Pharmacol. Ther. 88, 281–309.

Philipp, M., Brede, M., Hein, L., 2002. Physiological significance of alpha-2 adrenergic receptor subtype diversity: one receptor is not enough. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R287–R295.

Pierce, K.L., Premont, R.T., Lefkowitz, R.J., 2002. Seven-transmembrane receptors. Nat. Rev. Mol. Cell Biol. 3, 639–650.

Rechberger, T., Abramson, S.R., Woessner Jr., J.F., 1996. Onapristone and prostaglandin E2 induction of delivery in the rat in late pregnancy: a model for the analysis of cervical softening. Am. J. Obstet. Gynecol. 175, 719–723.

Renouard, A., Widdowson, P.S., Millan, M.J., 1994. Multiple alpha 2 adrenergic receptor subtypes. I. Comparison of [3H]RX821002-labeled rat R alpha-2A adrenergic receptors in cerebral cortex to human H alpha2A adrenergic receptor and other populations of alpha-2 adrenergic subtypes. J. Pharmacol. Exp. Ther. 270, 946–957.

Roberts, R.E., Tomlinson, A.E., Kendall, D.A., Wilson, V.G., 1998. Alpha2-adrenoceptor-mediated contractions of the porcine isolated ear artery: evidence for a cyclic AMP-dependent and a cyclic AMP-independent mechanism. Br. J. Pharmacol. 124, 1107–1114.

Sallinen, J., Haapalinna, A., MacDonald, E., Viitamaa, T., Lahdesmaki, J., Rybnikova, E., Pelto-Huikko, M., Kobilka, B.K., Scheinin, M., 1999. Genetic alteration of the alpha2-adrenoceptor subtype c in mice affects the development of behavioral despair and stress-induced increases in plasma corticosterone levels. Mol. Psychiatry 4, 443–452.

Schramm, N.L., McDonald, M.P., Limbird, L.E., 2001. The alpha2A-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. J. Neurosci. 21, 4875–4882.

Schlageter, N., Janis, R.A., Gualtieri, R.T., Hechter, O., 1980. Effects of oxytocin and methacholine on cyclic nucleotide levels of rabbit myometrium. Can. J. Physiol. Pharmacol. 58, 243–248.

Seamon, K.B., Daly, J.W., 1986. Forskolin: its biological and chemical properties. Adv. Cyclic Nucleotide Protein Phosphorylation Res. 20, 1–150.

Sia, A.T., Kwek, K., Yeo, G.S., 2005. The in vitro effects of clonidine and dexmedetomidine on human myometrium. Int. J. Obstet. Anesth. 14, 104–107.

Small, K.M., Schwarb, M.R., Glinka, C., Theiss, C.T., Brown, K.M., Seman, C.A., Liggett, S.B., 2006. Alpha2A- and alpha2C-adrenergic receptors form homo- and heterodimers: the heterodimeric state impairs agonist-promoted GRK phosphorylation and beta-arrestin recruitment. Biochemistry 45, 4760–4767.

Stiles, G.L., Caron, M.G., Lefkowitz, R.J., 1984. Beta-adrenergic receptors: biochemical mechanisms of physiological regulation. Physiol. Rev. 64, 661–743.

Taneike, T., Narita, T., Kirazawa, T., Bando, S., Teraoka, H., Ohga, A., 1995. Binding and functional characterization of alpha-2 adrenoceptors in isolated swine myometrium. J. Auton. Pharmacol. 15, 93–105.

Tso, J.U., Sun, X.H., Kao, T., Reece, K.S., Wu, R., 1985. Isolation of rat and human glycerinaldehyde-3-phosphate dehydrogenase cDNA: genomic complexity and molecular evolution of the gene. Nucleic Acid Res. 13, 2485–2502.

Uhlen, S., Lindblom, J., Johnson, A., Wikberg, J.E., 1997. Autoradiographic studies of central alpha 2A- and alpha 2C-adrenoceptors in the rat using [3H]MK912 and subtype-selective drugs. Brain Res. 770, 261–266.

Uhlen, S., Xia, Y., Chhajlani, V., Felder, C.C., Wikberg, J.E., 1992. [3H]-MK 912 binding delineates two alpha 2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d alpha 2-adrenoceptor. Br. J. Pharmacol. 106, 986–995.

Zhou, X.B., Wang, G.X., Huneke, B., Wieland, T., Korth, M., 2000. Pregnancy switches adrenergic signal transduction in rat and human uterine myocytes as probed by BKCa channel activity. J. Physiol. 524, 339–352.