0026-895X/01/5905-1235–1242$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:1235–1242, 2001

Vol. 59, No. 5
333/901113
Printed in U.S.A.

Use of Antisense Oligonucleotides to Verify the Role of the α_{1A}-Adrenergic Receptor in the Contractility of the Rat Uterus Post Partum

E. DUCZA, R. GÁSPÁR, A. MÁRKI, P. GYULA, S. BOTTKA, and G. FALKAY

Department of Pharmacodynamics and Biopharmacy, University of Szeged, Szeged, Hungary (E.D., R.G., A.M. G.F.); and Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary (P.G., S.B.)

Received June 5, 2000; accepted February 6, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The adrenergic system plays a major role in the regulation of the contractility of the uterus during pregnancy. This study investigated the role of the α_{1A}-adrenergic receptor (AR) in this regulation. The use of partial phosphorothioate antisense oligodeoxynucleotides (AONs) permitted the sequence-selective inhibition of AR gene expression. AONs were injected together with a cationic liposomal carrier agent into the post partum rat uterus. Incubation for 12 or 24 h with the most effective AON (480-AON) caused a 58.7 or 53.0% inhibition, respectively, of the expression of the α_{1A}-AR density, whereas incubation for 36 or 48 h resulted in only a 38.8 or 26.7% inhibition, respectively. The decrease of the α_{1A}-AR density by 480-AON was demonstrated by Western blot analysis and a radioreceptor binding assay on rat uterus preparations 24 h after delivery. The changes in the contractility of the uterus after AON treatment were measured on isolated rat uterine tissue by electric field stimulation. The significant decrease in the ability of the uterus to contract was indicated by the area under the curve method. The electric field studies revealed that the specific α_{1A}-blockers 5-methylurapidil and WB 4101 inhibited the rhythmic contraction by about 74 and 70% in the control uteri and by 25 and 20% in 480-AON–treated uteri, respectively. The curves for the β-mimetic (terbutaline) and α_{1D}-antagonist (BMY7370) inhibitors were unchanged after 480-AON treatment of the uteri. These results suggest the importance of the α_{1A}-AR in the tocolytic effect exerted by the α_1-antagonist, although high concentrations of antagonists can not exclude the role of α_{1D}-ARs, too. Additionally, these prove that the knockdown transformation by AONs offers a useful animal model for the investigation of receptors controlling the function of uterine tissue.

The extent of the contribution of the α_{1A}-adrenergic receptors (α_{1A}-ARs) in the regulation of uterine contractility remains an open question. Our research was motivated by the problem of tocolysis (inhibition of uterine contraction during labor), which has not been adequately solved. The adrenergic part of the autonomic nervous system plays an important role in the regulation of the motor activity of the uterus (Borda et al., 1997). The spontaneous contractility of the uterine muscle is controlled by the α-ARs, which mediate uterine contraction (Vallieres et al., 1978; Hoffman et al., 1981; Rexroad, 1981), and by the β-ARs, which mediate uterine relaxation (Levin et al., 1980; Tanfin-Tougui et al., 1981).

It has been proved that α-adrenergic antagonists induce a significant decrease in the uterine activity of the rat, both in vitro and in vivo (Zupkó et al., 1997; Gáspár et al., 1998), similar to the effects of β-agonists. Additionally, it has been found that at the end of pregnancy, the α_1/β-adrenoceptor ratio of the rat uterus is increased in parallel with the increase in contractility (Zupkó et al. 1998).

It is known that the number of α_{1A}-ARs is significantly increased in the rat myometrium at term (Legrand et al., 1987; Limon-Boulez et al., 1997). The extent of their contribution to the regulation of uterine contractility remains an open question. Before the synthesis of uterus-selective α_{1A}-AR antagonists, the role of these receptors in uterine contractility should be investigated. The antisense oligodeoxynucleotides (AONs) provide an excellent possibility for such studies.

The antisense effect (i.e., the selective inhibition of gene expression by antisense RNA, DNA or oligonucleotides) opened up new possibilities for the selective manipulation of living functions. A series of techniques have been developed to exploit their uses. The direct injection of antisense RNA or transiently expressed DNA vectors into the cytoplasm provides the most straightforward approach, but has several drawbacks and limitations. The procedure for generating stable transgenic species, carrying inversely fused genes,

This work was supported by Hungarian Research Grants FKFP 0618/1999 and OTKA T033126.

ABBREVIATIONS: AR, adrenergic receptor; AON, antisense oligodeoxynucleotides; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; PBS, phosphate-buffered saline; AUC, area under the curve.

controlled by tissue-specific or inducible promoters is a solution to these problems, but demands appreciable time and labor. The use of AONs led to a real alternative to these methods. Because of their obvious potential and attractive simplicity, huge efforts have been made to develop effective AONs, but several barriers must be overcome before their successful therapeutic application in human diseases. The use of AONs as a nucleotide sequence-selective research tool involves far fewer problems, and has, in many instances, proved valuable for the elucidation of complex gene functions.

The aim of the study was to develop an α_{1A}-AR "knockdown" transformed animal model, involving the use of AONs, to study the role of the α_{1A}-ARs in the regulation of the contractility of the rat uterus in pregnancy. The efficiency of α_{1A}-AR antisense inhibition was indicated by a radioligand receptor binding assay and Western blot analysis. Electric field stimulation was applied to test the pharmacological reactivity of the post partum-isolated rat uterus.

Materials and Methods

Synthesis of AONs. AONs were synthesized on an Expedite 8909 synthesizer (Perkin-Elmer Biosystems Inc., Norwalk, CT), using solid-supported standard β-cyanoethyl phosphoramidite chemistry at a nominal scale of 1 μmol. Phosphorothioate linkages were generated individually at chosen points of the sequence after the coupling step with tetraethyldithiuram treatment for 15 min. In these cases, the iodine oxidation step was omitted. Purification was performed on Poly PAK (Glen Research, Sterling, VA) cartridges according to the procedure supplied by the manufacturer. The purified product was converted into the sodium salt on Dowex 50X8 [Na^+] resin. Purity was controlled by anion exchange high-performance liquid chromatography, and proved to be 97 to 98% by absorbance measurements at 260 nm. None of the impurities exceeded 1% of the total absorbance. High-performance liquid chromatography was carried out on a Vydac 301 VHP column (Vydak, Hesperia, CA) with a gradient of 0 to 0.3 M NaCl in 10 mM Tris-HCl, pH 8.0. The phosphodiester/phosphorothioate ratio in 480-AON was checked by ^{31}P-NMR spectroscopic experiments with a Bruker WM-250 instrument (101.2 MHz, external H_3PO_4 standard; Bruker, Newark, DE) in D_2O and found to be 11:8. Undesired phosphodiester contamination was below the detectable limit (3%).

Treatment of Animals with AONs. The AONs were mixed with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Roche Molecular Biochemicals, Mannheim, Germany) and 20% F127 Pluronic gel (Sigma-Aldrich, Budapest, Hungary). The solution was maintained in liquid form at 4°C before injection. Postpartum Sprague-Dawley rats (91 rats with weights in the range 180–200 g) under urethane anesthesia (1 g/kg, i.p.) were treated with AONs 2 to 3 h after labor. An incision was made in the lower abdomen and AON solution was injected from prechilled syringes into the luminal space of each uterine horn. Each AON sample was administered in two steps at two discrete locations along the length of the horn. The incision was then closed, and the animals were returned to their cages. Uteri were used for further investigation 24 h after delivery.

Membrane Preparation. The uteri were removed and homogenized in 20 volumes (w/v) of ice-cold buffer (10 mM Tris-HCl, 1 mM EDTA, 0.6 mM $MgCl_2$, and 0.25 M sucrose, pH 7.4) with an Ultra Turret T25 (Janke & Kunkel, Staufen, Germany) homogenizer and the suspension was then filtered on four layers of gauze and centrifuged (40,000*g*, 4°C, 20 min). After centrifugation, the pellet was resuspended in a 5-fold volume of buffer. The membranes were stored at −70°C. For Western blot assay, the resulting pellet was resuspended in lysate buffer (50 mM Tris-HCl, pH 8.0, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin) and stored at −70°C. Protein concentration was determined by the method of Bradford (1976).

Radioreceptor Binding Assays. The frozen membranes were thawed at room temperature. Radioligand-binding experiments were carried out on membrane preparations of rat uterus in 50 mM Tris-HCl buffer, pH 7.4, containing 3 mM $MgCl_2$ and 1 mM ascorbic acid in a final volume of 300 μl. The concentration of tritiated prazosin in saturation experiments was varied between 0.1 and 2.5 nM. The reaction mixture contained 100 μl of [^{3}H]prazosin (PerkinElmer Life Science Products, Hounslow, UK), 100 μl of membrane preparation and 100 μl of 10^{-5} M unlabeled phentolamine (Sigma-Aldrich) for nonspecific binding, or 100 μl of buffer for total binding. The concentration of tritiated prazosin was 1.5 nM in displacement studies. The concentrations of unlabeled ligand (prazosin, WB4101) were varied in the range 10^{-5} to 10^{-12} M. Incubations began with the addition of the membrane suspension and continued in a shaking water-bath until a steady state was achieved (30°C, 30 min). The reaction was terminated by rapid filtration on a Brandel M24R cell harvester (BioMedical Research & Development Laboratories Inc., Gaithersburg, MD) through Whatman GF/C filters, and the residue was washed with three applications of 5 ml of ice-cold Tris-HCl buffer, pH 7.4. The bound radioactivity was determined in a HighSafe (Wallac Oy, Turku, Finland) scintillation cocktail in a Wallac 1409 liquid scintillation counter (Wallac Oy). The total binding was defined as that measured in the absence of a competing agent. Nonspecific binding was determined in the presence of 10 μM unlabeled phentolamine and was about 15% of the total binding. All assays were carried out at least three times in duplicate, and values are given as means ± S.E.M. The binding capacities were analyzed using the computer program Prism 2.01 (GraphPad Software, San Diego, CA) with a nonlinear least-squares fitting algorithm. Statistical analyses were performed with the Newman-Keuls analysis of variance test to assay significant differences in AON-treated uteri.

Western Blot Assay. Protein (20–30 μg per well) was subjected to electrophoresis on 12.5% SDS polyacrylamide gels in Series Standard Dual Cooled Units (Bio-Rad, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), using a semidry blotting technique (BioRad). The membranes were blocked with phosphate-buffered saline (PBS), pH 7.4, containing 1% nonfat dry milk, 0.1% Tween 20, and 1% polyvinylpyrrolidone overnight at 4°C. After washing, the blots were incubated for 1 to 3 h, at room temperature, on a shaker with anti-α_{1A}-AR, α_{1B}-AR, α_{1D}-AR polyclonal antibody (diluted 1:400, Research Diagnostics, Flanders, NJ) in PBS with 1% nonfat dry milk, 0.1% Tween 20, and 1% polyvinylpyrrolidone. The blots were washed and incubated with 1:1000 peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) in PBS with 0.1% Tween 20. The antibody binding was detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and exposed on Kodak X-Omat film (Sigma-Aldrich). Anti-β-actin monoclonal antibody (Sigma-Aldrich) was used as a control.

Electric Field Stimulation. Pretreated uteri were removed from rats 24 h after delivery. Muscle rings 0.5 cm long were sliced from the uterine horns and mounted vertically between two platinum electrodes in an organ bath containing 10 ml of de Jongh solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM 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. The initial tension was set at 1.25 g, which was relaxed to 0.5 g at the end of equilibration. After the incubation period, rhythmic contractions were elicited by a digital, programmable stimulator (ST-02; Experimetria Ltd., Budapest, Hungary), using square pulses with a duration of 200 ms every 60 s at 40 V. The tension of the myometrial rings was measured with a strain gage transducer (SG-02; Experimetria, Ltd.) and recorded by

an ISOSYS Data Acquisition System (Experimetria, Ltd.). Noncumulative concentration-response curves to the selective α_{1A}-receptor antagonist 5-methylurapidil and WB 4101 (RBI, USA), the α_{1D}-antagonist BMY7378 (RBI/Sigma, Natick, MA) and terbutaline (Sigma-Aldrich) were constructed in each experiment. Areas under the curves (AUCs) were evaluated and were analyzed statistically with the computer program Prism 2.01.

Results

The selection of target sites for AONs was based on theoretical modeling and careful optimization. The most probable secondary structures of α_{1A}-AR mRNA (GenBank accession no. M60654) were first computed by free energy minimization. For this purpose, the computer software *mfold* (version 3.0) of Zuker and Turner was applied through an Internet connection (Zuker et al., 1999). With the RNA base-sequence as input, the computation provided a series of thermodynamically optimal and suboptimal foldings of α_{1A}-AR mRNA. Single-stranded regions were listed as potential target sites. The chosen fragments were further screened on the basis of the general criteria of primer planning and the free energy of duplex formation. Finally, a set of four AONs was chosen together with the appropriate control sequences. The AONs were directed toward different functional regions of the mRNA: 214-AON against the 5′-untranslated region, 480-AON against the translation-initiation site, and 1834-AON against the coding region.

To stabilize the oligonucleotides against nucleolytic decomposition inside the cells, phosphorothioate internucleotide linkages were incorporated in a sequence-specific manner according to the principle of minimal modification (Peyman and Uhlmann, 1996). Two thioate substituents were built in, on both the 5′ and 3′ termini, and one was positioned in the vicinity of each pyrimidine nucleotide on either the 5′ or the 3′ side. The structures and properties of the AONs used in the experiments are listed in Table 1. The free energy of hybridization (i.e., the duplex-forming abilities of all the examined oligomers) fell into a relatively narrow range. We have tabulated the melting temperatures (T_m) of the oligonucleotides, which were calculated with a fully complementary opposite strand and disregarding phosphorothioate substitutions, as an indication of their comparable hybridizational energy. With the use of the dose-response analysis, the effective dose was determined as 50 nmol/200 μl (Fig. 1).

The changes in the receptor density caused by the AONs were investigated by radioreceptor binding assays 12, 24, 36, and 48 h after delivery to establish the optimum time of AON treatment. In the saturation experiments, the maximum number of α_{1A} binding sites (B_{max}) in the AON-treated rat uterus was determined. Of the synthesized AONs, 480-AON reduced the α_{1A}-AR density in a time-dependent fashion compared with the untreated group "C" (B_{max} = 29.05 ± 1.27) and the random oligonucleotide-treated control group "R" (B_{max} = 29.13 ± 1.12) (Fig. 2). The most significant changes were observed 12 and 24 h after delivery ($p < 0.001$ in comparison with the control). Incubation with 480-AON for 12 or 24 h caused a 58.7 or 53.0% inhibition and the B_{max} values under these conditions were 12.04 ± 0.86 and 13.69 ± 0.42 fmol/mg, respectively. Thirty-six or 48 h after the delivery, 38.8% ($p < 0.001$) or 26.7% ($p < 0.001$) inhibition was detected, and the α_{1A}-AR density was 17.99 ± 0.43 and 21.40 ± 0.51 fmol/mg, respectively. No significant changes in receptor density were found for the other AONs.

The α_{1A}-selective WB4101 identified two α_1-adrenergic binding sites in the nontreated rat uterus, with K_d values of 0.011 and 15.05 nM, whereas in the treated animals, it bound to only one binding site (K_i = 36.12 nM) (Fig. 3). In Western blot analysis, the α_{1A}-AR density was only decreased by 480-AON (Figs. 4 and 5). Representative contraction curves of control, operated control, DOTAP-treated, 480-mAON–treated, and 480-sAON–treated uteri display different responses to electric field stimulation (Fig. 6).

The AUCs of the uterine contractility indicated the significantly decreased contractility of the 480-AON-treated uterus as compared with those of the control, the operated control, the DOTAP-treated control, the 480-mAON–treated control, and the 480-sAON–treated control (Fig. 7). Additionally, the EFS studies revealed that the specific α_{1A}-blockers 5-methylurapidil and WB 4101 inhibited the rhythmic contractions by about 75 and 70% in the control uteri and 25 and 20% in the 480-AON–treated uteri, respectively. The IC_{50} values for 5-methylurapidil and WB 4101 in the control uteri were 3.8×10^{-5} and 9.5×10^{-6}, respectively. In the event of a mismatched sequence, inhibitions can be experienced only with high doses, whereas with the scrambled control the maximum inhibitions corresponded to the untreated control (Fig. 8, b and c). The inhibition curves of terbutaline and BMY 7378 remained unchanged for the 480-AON-treated uteri compared with those of the control (Fig. 8a).

TABLE 1

Oligodeoxynucleotides

Melting temperature was calculated according to the nearest neighbor method at 50 mmol salt concentration (Breslauer et al., 1986) with a fully complemented opposite strand and disregarding the destabilization effect of phosphorothioate substituents. Subscript "s" in the base sequence denotes phosphorothioate linkage.

Target Region/Type	Sequence	T_m
		°C
Antisense Oligodeoxynucleotides		
14-233/5′-UTR	5′-$G_sA_sGT_sC_sAC_sAAGGA_sAAGAAG_sG_sG$-3′	50.0
80-499/START	5′-$A_sG_sGA_sTGT_sCGC_sGGAAAG_sTC_sA_sT$-3′	55.7
834-1853/CDR	5′-$T_sG_sTGC_sAGT_sGAGGGC_sTAGA_sG_sG$-3′	55.0
Control Oligodeoxynucleotides		
Random	5′-$T_sG_sAA_sGGC_sGAC_sGAG_sAAGGT_sG_sT$-3′	56.8
Random	5′-$A_sT_sCGT_sCAAT_sAC_sTAT_sCAAT_sC_sG$-3′	43.8
14-233/scrambled	5′-$G_sA_sGAGAA_sAGGAG_sC_sGAGA_sC_sA_sT$-3′	49.8
80-499/scrambled	5′-$A_sG_sCAAT_sGGC_sAGGAT_sAC_sGG_sT_sT$-3′	55.7
14-233/mismatched	5′-$G_sA_sGT_sC_sAC_sAAGA_sG_sGAAGAAG_sG_sG$-3′	47.4
80-499/mismatched	5′-$A_sG_sGA_sTGT_sCGG_sCGAAAG_sTC_sA_sT$-3′	55.7

Discussion

Cloning and pharmacological data have demonstrated that α_1-ARs can be classified into three subtypes: α_{1A}-, α_{1B}-, and α_{1D}-ARs. The genes for each of the subtypes are expressed in discrete, tissue-specific patterns (Perez et al., 1994; Price et al., 1994; Scofield et al., 1995). A major problem as concerns the determination of the function of each of the α_1-AR subtypes is the paucity of pharmacological tools available to distinguish among them. To address this question, AONs were used for the selective blocking of the receptor protein expression. This may provide an alternative means of studying individual α_1-AR subtype functions (Pedro et al., 1998).

AONs are complementary nucleic acid fragments that hybridize to the target sequence within RNA to form a DNA-RNA duplex, resulting in a blockade of the translation of mRNA into protein (Akhtar and Agrawal, 1997). One of the major problems in the application of AONs, for either research or therapeutic purposes, is the selection of the target sites on the chosen mRNA. To date, most of the effective molecules have been found on a trial and error basis. This approach requires much time and effort and involves only a moderate and varying chance of success (5–15%, which is effective enough for further evaluation). An often used, reliable method is to choose functionally important sites (e.g., ribosome-binding, translational-initiation, or splicing sites) of the RNA. Among the studies that examined one or only a few AONs, a strong majority targeted the AUG translation-initiation codon (Tu et al., 1998). The chance of success is higher at these sites, but targets that are more vulnerable can be found throughout the RNA. Of 14 AONs that reached the clinical trial phase, only two are targeted against the AUG initiation regions (Hogrefe, 2000). The simple strategy of choosing the thermodynamically most stable duplex-forming sites failed.

Better results were obtained with another approach, which was based on the secondary structure of the mRNA. A recent study provided the first clear-cut evidence that the antisense efficiency depends mainly upon the secondary structure and accessibility of RNA (Vickers et al., 2000). To improve our chances of finding effective AONs, we chose the latter approach. The secondary structure of the mRNA was predicted by computer modeling, using improved thermodynamic parameters (Mathews et al., 1999). As potential antisense target sites, single-stranded loop and hairpin structures were considered (Vickers et al., 2000; Lima et al., 1992). As further criteria, the strength of duplex formation and the lack of strong secondary structures were used. AONs with too low T_m values ($< 35°C$) were rejected. Among the oligonucleotides chosen, 480-AON conformed to both the functional and the secondary structure selection strategy, because it covered the translation initiation site. This AON was the only one that displayed a significant inhibitory effect. This result confirms the accessibility of the translation initiation site as a potential antisense target, but the fact that the effective AON met both criteria, and one very small number of molecules tested does not allow us to draw any conclusion concerning the selection strategy.

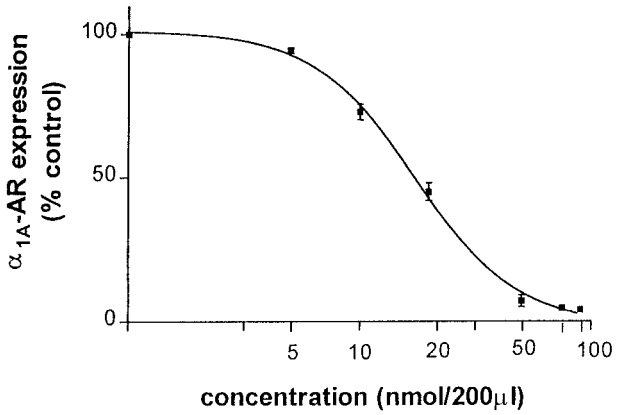

Fig. 1. Dose-dependent inhibition response. The rat uteri were treated with 5, 10, 20, 50, 75, and 90 nmol/200 μl 480-AON. The levels of the α_{1A}-ARs protein expression were normalized to the levels of β-actin. The resulting values were plotted as a percentage of levels obtained from untreated samples. The error bars represent the S.E.M.

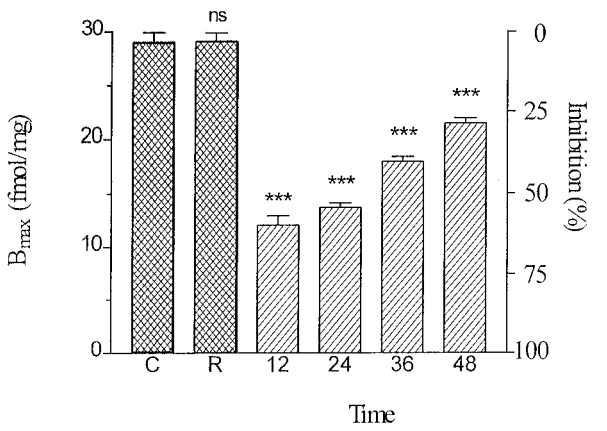

Fig. 2. Rate of change of density of maximum number of α_1-AR binding sites (B_{max}) and percentage of inhibition, 12, 24, 36, and 48 h after delivery, compared with untreated (C) and random oligonucleotide-treated (R) control uteri. Densities were measured as B_{max} values in saturation binding assays with [H^3]prazosin. Fifty nanomoles of both 480-AON and the random oligodeoxynucleotide (R) were used, which significantly (**$p < 0.01$; ***$p < 0.001$ versus control, analysis of variance Newman-Keuls test) decreased the α_1- AR density 12 to 48 h after delivery. (12 versus 24, $p > 0.05$; 24 versus 36, $p < 0.001$; 36 versus 48, $p < 0.01$).

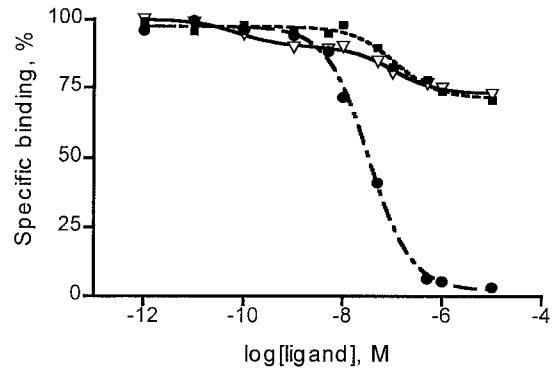

Fig. 3. The 480-AON induced change in the receptor density investigated by radioligand receptor binding assay. The concentration of [^{3}H]prazosin was 1.5 nM and the concentration of the unlabeled ligand was varied between 10^{-5} and 10^{-12} M. The displacement curves demonstrate the lack of the α_{1A}-AR subtypes in 480-AON–treated rat uteri. The WB4101 (α_{1A}-AR subtype-selective) binds to a single class of binding sites in the 480-AON–treated rat uterus membrane preparation, whereas it recognizes two binding sites in the nontreated rat uterus preparation; the high-affinity binding site is identical with the α_{1A}-AR subtype. ●, [^{3}H]prazosin-prazosin displacement (AON-treated rat); ■, [^{3}H]prazosin-WB 4101 displacement (AON-treated rat); ▽, [^{3}H]prazosin-WB 4101 displacement (untreated rat).

Phosphodiester oligonucleotides were found to be rapidly degraded inside the cell. Phosphorothioate oligomers with one sulfur substitution for phosphorus in the nonbridging position have become a common solution to achieve a longer biological life-span of the oligos. However, phosphorothioate oligonucleotides often have nonspecific effects, especially at higher concentrations (Stein and Krieg, 1998). We applied the principle of minimal modification, a combination of end-capping and pyrimidine protection. The end-capping phosphorothioate linkages protected the oligomer against exonucleolytic cleavage, whereas the internal phosphorothioate substitutions in the vicinity of the pyridine nucleotides inhibited the endonucleolytic degradation (Uhlmann et al., 1997). The smallest possible number of modifications was made and appropriate stabilization was expected. Recent studies revealed that a higher specificity can be achieved with partially thioated oligomers than with fully thioated ones. The liposomal cationic carrier used for functional delivery of the oligonucleotides prolonged the antisense effect substantially, by delaying nucleolytic degradation (Galderisi et al., 1999).

Three different oligomer controls were used in our experiments to verify the specificity of the AON: 1) a random control with an arbitrarily chosen nucleotide sequence and composition, 2) a scrambled control with a conserved base composition and an altered sequence, and 3) a mismatched control, in which the base sequence was changed arbitrarily at a single point. Moreover, untreated and excipient-treated animals were used as controls.

The effects of AONs in the down-regulation of α_{1A}-AR were tested at two levels. We studied the decrease in receptor protein density via Western blot analysis and a radioligand binding technique and detected the changes in function at a physiological level with the electric field stimulation technique (Elmer et al., 1980; Taneike et al., 1999).

The results of the receptor binding studies indicated that the antisense effect is most expressed 12 and 24 h after delivery. The density of α_{1A}-ARs in the postpartum rat uterus reaches a plateau 24 h after delivery. The optimum duration of treatment was set to 24 h, thereby coinciding with the maximum effect of AON. The α_{1A}-AR density was decreased most effectively by one (480-AON) of the four synthesized α_{1A}-AONs, which was in keeping with the literature data.

The α_{1A}-AR subtype-selective WB4101 binds to high-affinity (α_{1A}-ARs) and low-affinity binding sites in the nontreated rat uterus membrane preparation. On the other hand, in the 480-AON-treated rat uterus, WB4101 recognizes only a single class of binding site, which has the same K_i value as the inhibition constant of the low-affinity binding site in the nontreated rat uterus.

The results of the molecular biological studies were supported by the physiological and pharmacological investigations, which presented further evidence of the selectivity of 480-AON and proved the importance of the α_{1A}-ARs in the regulation of the rat uterine contractility.

In the electric field studies, we used direct muscle stimulation, because a denervation process takes place in the rat uterus during pregnancy (Mustafa, 1988). However, the inhibition of the α_{1A}-ARs can be investigated through a blockade of the nonsynaptic receptors in the uterus (Gáspár et al., 1998).

The contractilities of the treated and untreated uteri were

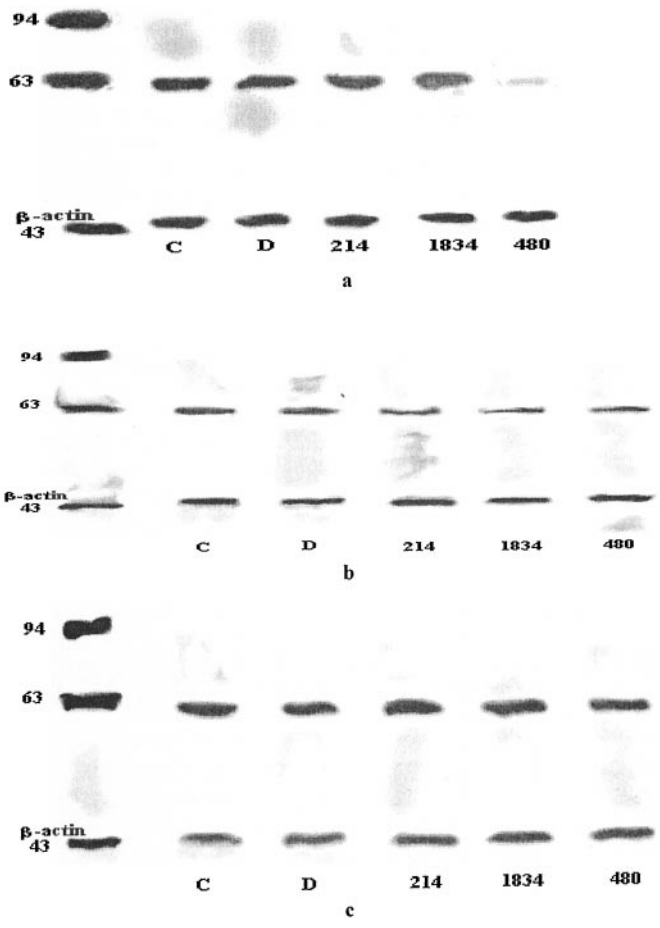

Fig. 4. Changes in α_{1A}-AR (a), α_{1B}-AR (b), and α_{1D}-AR (c) protein expression of the AON-treated (214, 1834, and 480-AON), postpartum uteri illustrated by Western blot analysis, as compared with DOTAP-treated (D) and nontreated (C) uteri. The sequence of oligonucleotides is shown in Table 1. In this analysis, the density of the α_{1A}-ARs was only significantly decreased by 480-AON. Samples (50 μg/lane) were subjected to gel electrophoresis on 12.5% polyacrylamide gel. The blot was stripped and reprobed for β-actin, and a similar intensity was observed in each lane.

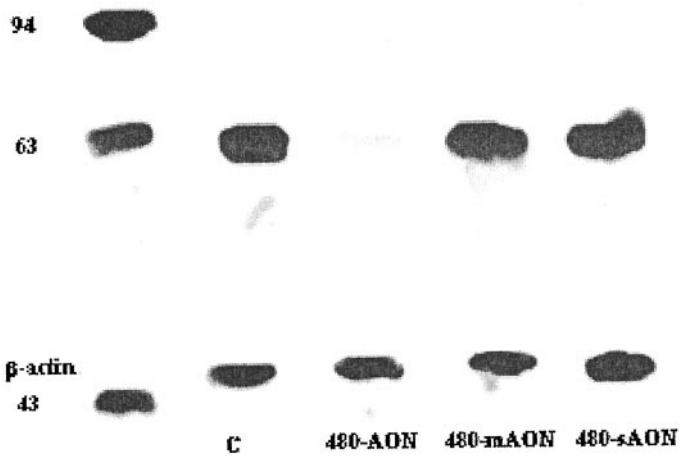

Fig. 5. Two different oligomer controls (480-sAON and 480-mAON) and an untreated control (C) were used to verify the specificity of 480-AON. 480-AON significantly decreased the α_{1A}-AR density. β-actin was used as a control. 480-sAON, the AON base composition was retained but the base sequence was mixed; 480-mAON, the AON base sequence contained an by arbitrary change at one point.

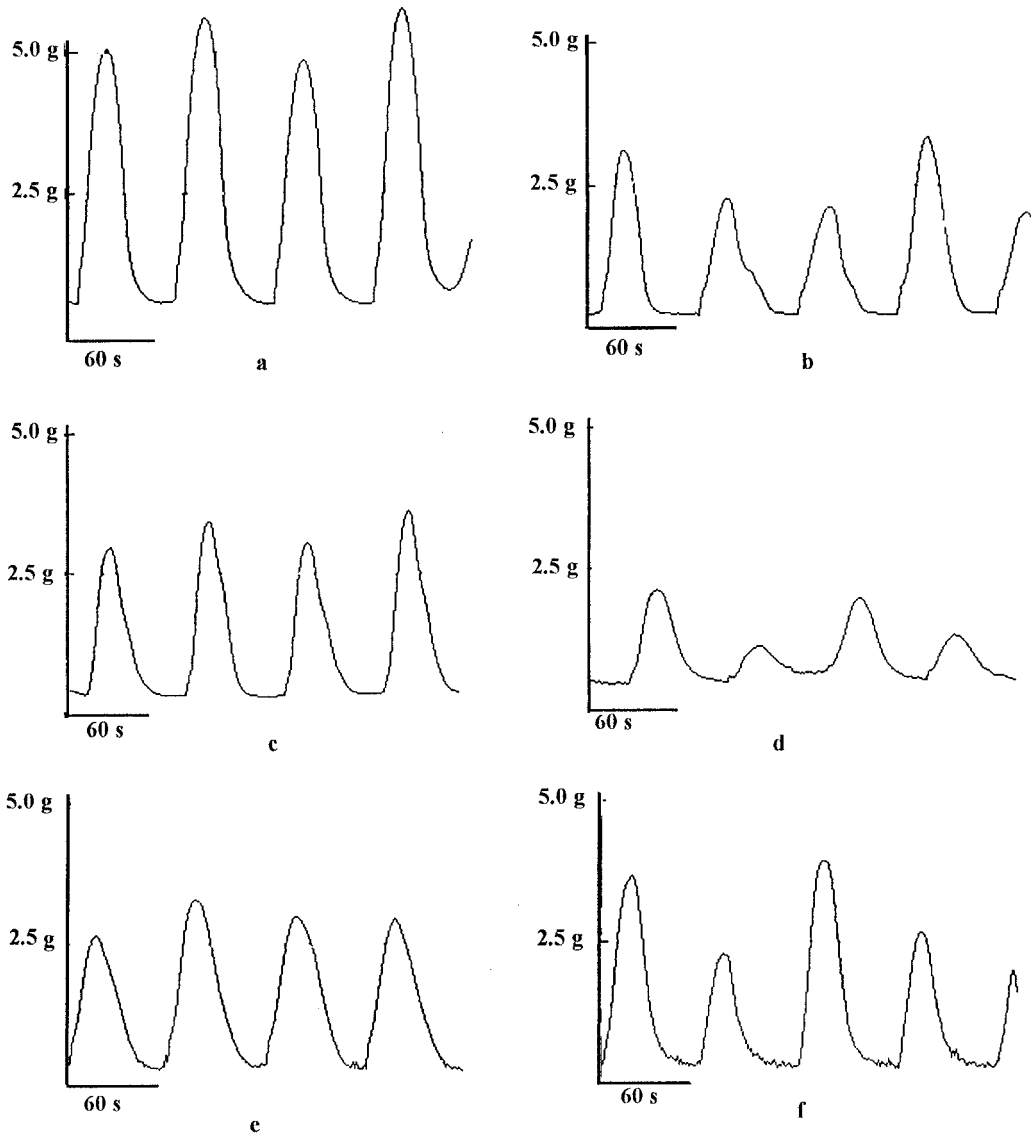

Fig. 6. Representative curves of control (a) operated control (b), DOTAP-treated control (c), 480-AON– (d), 480-mAON– (e) and 480-sAON–treated post partum uteri contracted by electric field stimulation.

deduced from their responses to stimuli. The abilities of the uteri to contract were compared via the AUCs (because of the lack of an in vivo model) and basic conclusions were drawn as to the physiological activity of pregnant uterine contractility. The surgical operation reduced the average of contractions AUCs from 0.35 to 0.25, whereas the antisense treatment decreased the average from 0.25 to 0.15. If the changes are expressed in percentages, it can be said that the surgery caused a ~28% reduction. However, the antisense treatment elicited a further 40% decrease in the contractility. It is thought that urethane anesthesia is responsible for the decrease in contractility after surgery because the untreated controls were not involved in any type of anesthesia. These values indicate that the α_{1A}-ARs have a significant, although obviously nonabsolute, role in the contractility.

In pharmacological experiments, we found that the contraction inhibitory effects of the specific α_{1A}-AR competitive antagonists 5-methylurapidil (Gross et al., 1988) and WB 4101 (Michel M et al., 1995) were significantly decreased in the 480-AON–treated uteri, but the unchanged effects of an α_{1D}-sympatholytic (BMY 7378) and β-mimetic (terbutaline) demonstrated that the AON treatment did not affect the

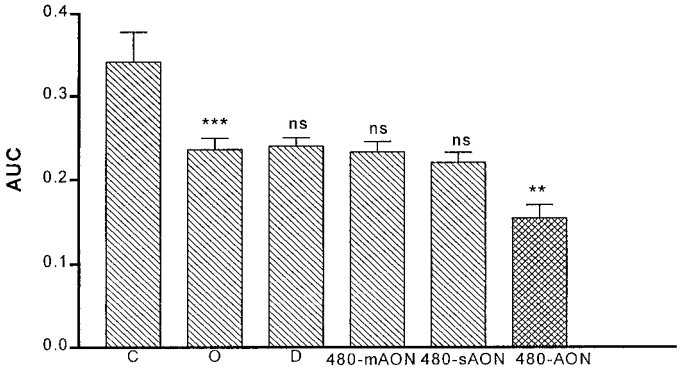

Fig. 7. Changes in values of AUC for the contractions stimulated by an electric field. The differences in AUCs between the untreated control (C) and the operated control (O), the DOTAP-treated control (D), the 480-mAON–treated control and the 480-sAON–treated control were statistically significant. The AUC value for the 480-AON–treated uterus was significantly different (**$p < 0.01$) from those for the DOTAP-treated control (D), the operated control (O), the 480-mAON–treated control, and the 480-sAON–treated control. There was no significant difference between the operated control (O), the DOTAP-treated control (D), the 480-mAON–treated control, and the 480-sAON–treated control.

function of the α_{1D}-ARs and β-ARs, suggesting the selectivity of 480-AON. It seems impossible that the subtype-selective α_1-blockers remain selective even at such a high concentration, but if the molecules lost their selectivity to the α_1-receptor subtypes, 5-MU should not lose its effectiveness after the antisense treatment. In parallel, the inhibitory effect of BMY 7378 should also be changed in the case of nonselective binding, because the α_{1A}-component of its effect should be eliminated. Additionally, in our system, the receptor affinity of WB 4101 is four times higher than that of 5-MU, this result coinciding with the literature data by Yamamoto and Koike (1999), Barbieri et al. (1998), and Arias-Montano et al. (1996). These results show that the difference in affinity between WB 4101 and 5-MU remains at such a high dose.

The decrease in the effects of 5-methylurapidil and WB 4101 in the 480-AON–treated uteri suggests "negative" pharmacological evidence of the significance of the inhibition of uterine contractility through the α_{1A}-ARs. The local use of AONs in the uterus, in an attempt to clarify the role of the receptors, which influence the uterine contractility, is a new method in pharmacology. This method was used to develop an α_{1A}-AR knock-down transformed animal model, which provided experimental opportunities that were unavailable with the previously existing pharmacological methods.

In light of these facts, we suppose that the α_{1A}-ARs have an important role in the contractility, although high concentrations of subtype-selective antagonists were required in contractility studies. However, the role of α_{1D}-ARs can not be excluded in the control of pregnant uterine contraction.

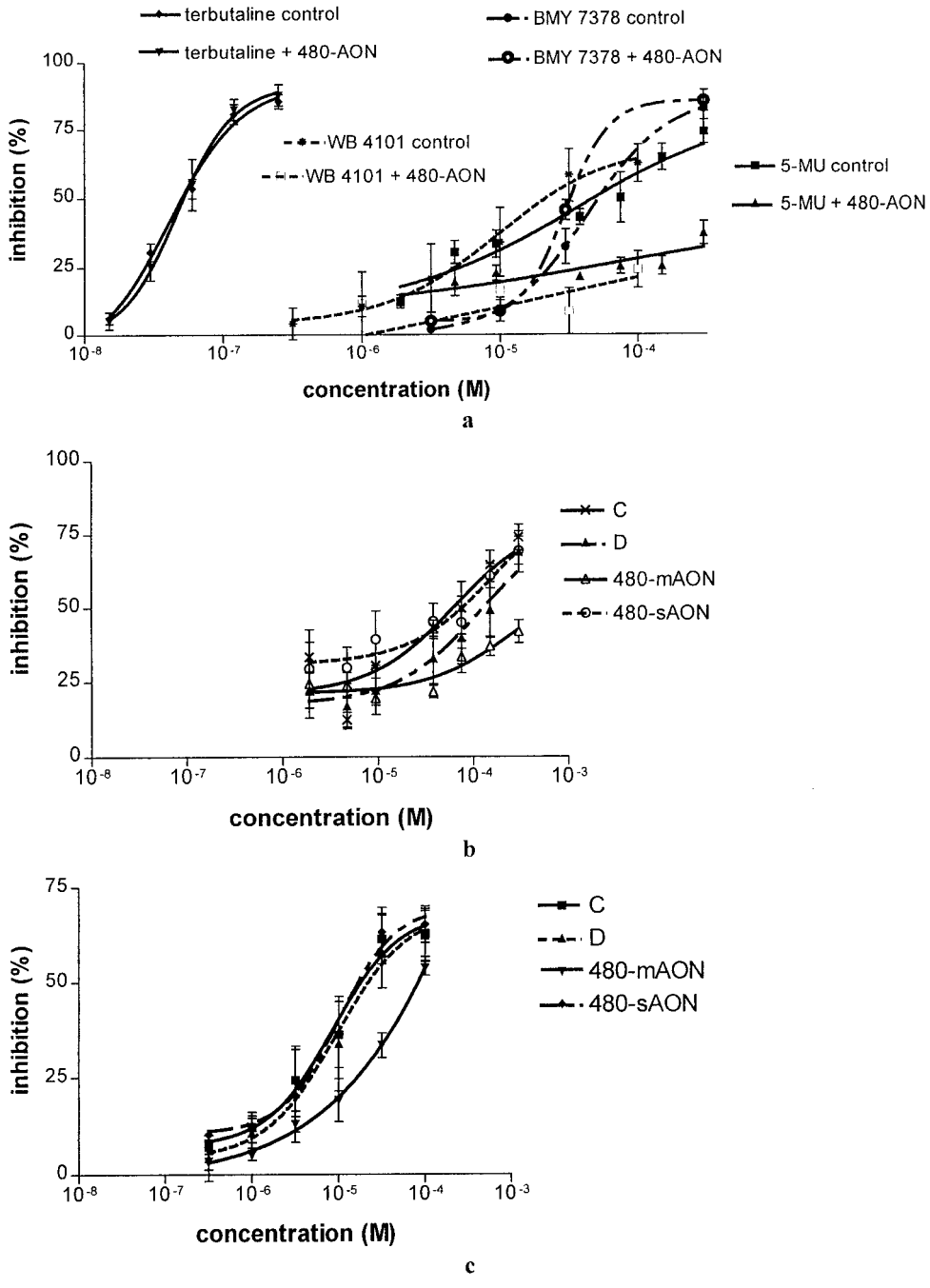

Fig. 8. Effects of 5-methylurapidil, WB 4101, BMY7378, and terbutaline on the EFS-stimulated uterine contraction in the presence of 480-AON (a). Effects of 5-methylurapidil (b) and WB 4101 (c) on the EFS-stimulated uterine contraction for the control (C), the DOTAP-treated control (D), the 480-mAON-treated control, and the 480-sAON-treated control. The data are the averages of the results of six experiments ± S.E.M.

References

Akhtar S and Agrawal S (1997) In vivo studies with antisense oligonucleotides. *Trends Pharmacol Sci* **18:**12–18.

Arias-Montano JA, Aceves J and Nunez A (1996) Noradrenaline-induced inositol phosphate formation in rat striatum is mediated by alpha 1A-adrenoceptors. *Europharmacology* **35:**1605–13.

Barbieri A, Santagostino-Barbone MG, Zonta F and Luchelli A (1998) Pharmacological characterization of alpha-adrenoceptors that mediate contraction in splenic artery strips from the pig. *Naunyn Schmiedeberg's Arch Pharmacol* **357:**654–61.

Borda E, Sauvage J, Stein-Borda L, Gimeno MF and Gimeno AL (1997) Adrenoceptors involved in the contractile activity of isolated pregnant rat uterus. *Eur J Pharmacol* 56:61–67.

Bradford 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.

Breslauer KJ, Frank R, Blocker H and Marky LA (1986) Predicting DNA duplex stabilit from the base sequence. *Proc Natl Acad Sci USA* **83:**3746–3750.

Elmer M, Alm P and Thorbert G (1980) Electrical field stimulation of myometrial strips from non-pregnant and pregnant guinea-pigs. *Acta Physiol Scand* **108:**209–213.

Galderisi U, Di Bernardo G, Melone MAB, Galano G, Cascino A, Giordano A and Cipollaro M (1999) Antisense inhibitory effect: A comparison between 3′-Partial and full phosphorothioate antisense oligonucleotides. *J Cell Biochem* **74:**31–37.

Gáspár R, Márki A, Zupkó I and Falkay G (1998) Evidence of non-synaptic regulation of post-partum uterine contractility in the rat. *Life Sci* **62:**1119–1124.

Gross G, Collius G and Rugevics C (1988) 5-methyl-urapidil discriminates between subtypes of the α_1-adrenoceptor. *Eur J Pharmacol* **151:**333–335.

Hoffman BB, Lavin TN, Lefkowitz RJ and Ruffolo RR (1981) Alpha-adrenergic receptor subtypes in rabbit uterus: mediation of myometrial contraction and regulation by estrogens. *J Pharmacol Exp Ther* **219:**290–298.

Hogrefe RI (2000) An antisense oligonucleotide. Available from: http://www.trilinkbiotech.com/Technical%20Information/Antisense%20primer.html

Legrand C, Maltier JP and Benghan-Eyene Y (1987) Rat myometrial adrenergic receptor in late pregnancy *Biol Reprod* **37:**641–650.

Levin LC, Korenman SG and Krall JF (1980) Agonist-dependent desensitization of myometrial β-adrenergic catecholamine-sensitive adenylate cyclase. *Biol Reprod* **22:**493–499.

Lima WF, Monia BP, Ecker DJ and Freier SM (1992) Implication of RNA structure on antisense oligonucleotide hybridization kinetics. *Biochemistry* **31:**12055–12061.

Limon-Boulez I, Mhaouty-Kodja S, Coundouel N, Benoit de Coignac A, Legrand C and Maltier JP (1997) The alpha$_{1B}$-adrenergic receptor subtype activates the phospholipase C signaling pathway in rat myometrium at parturition. *Biol Reprod* **57:**1175–1182.

Mathews DH, Sabina J, Zuker M and Turner DH (1999) Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. *J Mol Biol* **288:**911–940.

Michel MC, Kenny B and Schwinn DA (1995) Classification of alpha1-adrenoceptors subtypes. *Naunyn Schmiedeberg's Arch Pharmacol* **352:**1–10.

Mustafa FA (1988) Changes in cholinergic and noradrenegic nerves in the pregnant and postpartum uterus of the albino rat and guinea pig. *Acta Anat (Basel)* **132:**310–316.

Pedro J, Gonzalez-Cabrera PJ, Iversen PL, Liu MF, Scofield MA and Jeffries WB (1998) Selective inhibition of α_{1B}-adrenergic receptor expression and function using a phosphorothioate antisense oligodeoxynucleotide. *Mol Pharmacol* **53:**1034–1039.

Perez DM, Piascik MT, Malik N, Gavin R and Graham RM (1994) Cloning, expression and tissue distribution of the rat homolog of the bovine α_{1C}-adrenergic receptor provide evidence for its classification as the α_{1A}-subtype. *Mol Pharmacol* **46:**823–831.

Peyman A and Uhlmann E (1996) Minimally modified oligonucleotides—combination of end-capping and pyrimidine-protection. *Biol Chem Hoppe Seyer* **377:**67–70.

Price DT, Chari RS, Berkowitz DE, Meyers WC and Schwinn DA (1994) Expression of α_{1C}-adrenergic receptor subtype mRNA in rat tissues and human SK-N-MC neuronal cells: implications for α_1-adrenergic receptor subtype classification. *Mol Pharmacol* **46:**221–226.

Rexroad CE (1981) Binding of dihydroalprenolol and dihydroergocryptine to sheep myometrium. *Biol Reprod* **24:**831–842.

Scofield MA, Liu F, Abel PW and Jeffries WB (1995) Quantitation of steady state expression of mRNA for alpha-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. *J Pharmacol Exp Ther* **275:**1035–1042.

Stein CA and Krieg M (1998) *Applied Antisense Oligonucleotide Technology*. Springer Verlag, New York.

Taneike T, Kitazawa T, Funakura H, Asanuma K, Tsuji S, Yamada M, Teraoka H and Ohga A (1999) Smooth muscle layer-specific variation in the autonomic innervation of bovine myometrium *Gen Pharmacol* **32:**91–100.

Tanfin-Tougui Z, Do-Khac L and Harbon S (1981) Agonist-induced densensitisation of adrenergic-β receptors in rat myometrium. *FEBS Lett* **135:**31–39.

Tu G, Cao Q, Zhou F and Israel Y (1998) Tetranucleotide GGGA motif in primary RNA transcripts. *J Biol Chem* **273:**25125–25131.

Uhlmann E, Ryte A and Peyman A (1997) Studies on the mechanism of stabilization of partially phosphorothioated oligonucleotides against nucleolytic degradation. *Antisense Nucleic Acid Drug Dev* **7:**345–350.

Vallieres J, Fortier M and Bukowiecki L (1978) Alpha- and beta-adrenergic receptors in isolated rabbit uterine membranes: Study of receptor alteration by pregnancy. *Biol Reprod* **19:**318–324.

Vickers TJ, Wyatt RW and Freier M (2000) Effect of RNA secondary structure on cellular antisense activity. *Nucleic Acids Res* **28:**1340–1347.

Yamamoto Y and Koike K (1999) Alpha1-adrenoceptors in the guinea pig thoracic aorta. *Smooth Muscle Res* **35:**181–192.

Zuker M, Matherws DH and Turner DH (1999) Algorithms and thermodynamics for RNA secondary structure precipitation, in *A Practical Guide in RNA Biochemistry and Biotechnology* (Barciszewski J, Clark BFC eds) pp 11–43, NATO ASI Series, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Zupkó I, Gáspár R, Kovács L and Falkay G (1997) Are α-adrenergic antagonists potent tocolytics? In vivo experiments on postpartum rats. *Life Sci* **61:**159–163.

Zupkó I, Márki A, Gáspár R and Falkay G (1998) Correletion between α_1/β-adrenoceptor ratio and spontaneous uterine motor activity postpartum rat. *Mol Hum Reprod* **4:**921–924.

Send reprint requests to: Dr. George Falkay, Dept of Pharmacodynamics and Biopharmacy, University of Szeged, Eötvös u. 6, H-6720 Szeged, Hungary. E-mail: falkay@pharma.szote.u-szeged.hu