Antagonists for the human oxytocin receptor: an *in vitro* study

M. Maggi¹, G. Fantoni¹, E. Baldi¹, A. Cioni¹, S. Rossi¹,
G. B. Vannelli², P. Melin³, M. Åkerlund⁴ and M. Serio¹

¹Department of Clinical Physiopathology, Endocrinology Unit; and ²Department of Human Anatomy and Histology, University of Florence, 50134 Florence, Italy; ³Research Department, Ferring Pharmaceuticals, Malmö, Sweden; and ⁴Department of Obstetrics and Gynecology, University Hospital, Lund, Sweden

The oxytocin antagonist [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin has been successfully used for treating premature labour. The interactions of this antagonist with neurohypophysial hormone receptors in the human myometrium were investigated. Competition curves among [³H]oxytocin, [³H]arginine vasopressin, [³H][1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)2-(O-methyl)-tyrosine, 8-arginine] vasopressin, the corresponding unlabelled peptides and a series of oxytocin antagonists including [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin were constructed from results taken from the myometrium of pregnant women and rabbits, and were analysed simultaneously using the computer program LIGAND. The biological activity of [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin in the human uterus was investigated by studying its effect on oxytocin-induced intracellular Ca²⁺ mobilization in human myometrial cells in culture that were expressing high concentrations of oxytocin receptors. The results indicate that [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin and related antagonists are selective for the oxytocin receptor in the myometrium of pregnant rabbits but not of pregnant women. In women, they bind with high affinity to the V1 vasopressin receptor. In myometrial cells [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin inhibits the oxytocin-induced increase in intracellular Ca²⁺ concentration in a dose-dependent fashion, with an IC₅₀ value of 5 nmol l⁻¹. The uterine relaxant effect of this antagonist might result not only from the block of the oxytocin receptor, but also from interaction with the V1 vasopressin receptor.

Introduction

In pregnant females at term, oxytocin signalling in the uterus is upregulated. The increase in oxytocin-mediated events is believed to be responsible for the onset of coordinated and forceful contractions that lead to the expulsion of the conceptus. However, the molecular mechanisms underlying oxytocin-induced uterine hyperactivity are still unclear. An increase in the circulating concentration of oxytocin, uterine responsiveness to oxytocin (Fuchs and Fuchs, 1984; Casey and MacDonald, 1988; Leake, 1990; Steer, 1990) and the synthesis of oxytocin in the uterus (Ciarochi *et al.*, 1985; Lefebvre *et al.*, 1992) have been suggested to be involved.

In any case, the activation of the uterine oxytocin receptor is an obligate step for oxytocin-induced activation of cells in the uterus. Hence, the more rational approach to treating premature labour is the downregulation or blockade of the oxytocin receptor. From animal studies, the only factor known to downregulate the uterine expression of the oxytocin receptor is progesterone (Soloff, 1975; Nissenson *et al.*, 1978; Hixon and Flint, 1987). However, this steroid has been shown to be ineffective in preventing premature labour in humans (Fuchs and Stakemann, 1960). Furthermore, proges-

terone apparently does not inhibit the expression of the oxytocin receptor in humans (Maggi *et al.*, 1992). Recent studies have, therefore, focused on the development of oxytocin receptor antagonists, a whole series of which have been synthesized during the past 20 years (Sawyer and Manning, 1989).

One of these antagonists, [Mpa¹, D-Tyr(Et)², Thr⁴, Orn⁸]-oxytocin (dETVT; also known as ORF 22164, RWJ 22164 or Atosiban), has been used successfully in clinical pilot studies to treat pre-term labour in women (Åkerlund *et al.*, 1987; Andersen *et al.*, 1987); this antagonist inhibited uterine contractions in all (Åkerlund *et al.*, 1987) or in the majority (Andersen *et al.*, 1987) of the patients studied. Although dETVT binds with high affinity (Fuchs *et al.*, 1987) and selectivity (Pettibone *et al.*, 1992) to the oxytocin receptor in the rodent uterus, it has 10 times less affinity for the oxytocin receptor than has oxytocin in the human myometrium (Fuchs *et al.*, 1987). Furthermore, in the human uterus and liver, dETVT binds with high affinity to the V1 vasopressin receptor (Pettibone *et al.*, 1992).

Since both oxytocin receptors and V1 vasopressin receptors are present in the rabbit (Maggi *et al.*, 1988b, 1991a) and human (Maggi *et al.*, 1990, 1992) uterus, the ligand selectivity of dETVT was compared in membranes prepared from the myometrium of pregnant rabbits and women at term. In addition, the antagonistic properties of dETVT were

investigated on oxytocin-induced Ca^{2+} mobilization in oxytocin-receptor-positive human myometrial cells.

Materials and Methods

Chemicals

$\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2, \text{Thr}^4, \text{Orn}^8, [^{125}\text{I}]\text{Tyr}^9\text{-NH}_2]$ -vasotocin ($[^{125}\text{I}]$ OTA; 2200 Ci mmol^{-1}), $[^3\text{H}]$ oxytocin ($[^3\text{H}]\text{OT}$; 36 Ci mmol^{-1}), $[^3\text{H}]$ arginine vasopressin ($[^3\text{H}]\text{AVP}$; 70 Ci mmol^{-1}) and $[^3\text{H}]$ [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid)2-(O-methyl)-tyrosine, 8-arginine]vasopressin ($[^3\text{H}]\text{d}(\text{CH}_2)_5\text{Tyr MeAVP}$; 50 Ci mmol^{-1}) were purchased from New England Nuclear (Boston, MA). The tritiated ligands were divided into aliquots in plastic tubes, sealed under nitrogen, and frozen at -80°C . Arginine vasopressin, oxytocin and the rabbit polyclonal antibodies to human desmin were purchased from Sigma Chemical Co. (St Louis, MO). OTA and $\text{d}(\text{CH}_2)_5\text{Tyr MeAVP}$ were obtained from Peninsula Laboratories, Inc (San Carlos, CA). dETVT , $\text{desGly}^9[\text{Mpa}^1, \text{D-Tyr}(\text{Et})^2, \text{Thr}^4, \text{Orn}^8]$ -oxytocin (F123) and $\text{desGly}^9[\text{D-Tyr}(\text{Et})^2, \text{Thr}^4, \text{Orn}^8]$ - dC6 -oxytocin (F327) were generously provided by Ferring Pharmaceuticals, Malmö. The monoclonal antibody to human vimentin (clone V9) was purchased from Dako Corporation (Carpinteria, CA).

Cells

Hs 805.Ut (corpus uteri, normal, human) cells were obtained from the American Type Culture Collection (Rockville, MD; ATCC CRL 7795) and maintained in culture in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and antibiotics.

Uteri in the early follicular or late luteal phase of the menstrual cycle were taken from two women with a normal menstrual cycle (but who were undergoing hysterectomy for gynaecological reasons), since high densities of oxytocin receptors are present at these times (Maggi *et al.*, 1992) [patient 1 (M1): 40 years old, early follicular phase; patient 2 (M2): 49 years old, late luteal phase].

Myometrial cells were prepared as previously described (Maggi *et al.*, 1991b). Briefly, 1 mg collagenase solution ml^{-1} (Type IV) was added to the finely minced stripped uterine tissue for 12 h at 37°C . Digested fragments were then washed twice in Hanks' balanced salt solution, mechanically dispersed and plated in 25 cm^3 flasks in Coon's Modified Ham's F12 medium containing 10% fetal calf serum and antibiotics. Cultures were maintained in 5% CO_2 at 37°C , refed three times a week, subcultured at confluence and used for up to 10 passages. Every serial passage of the sister cultures was frozen in culture medium supplemented with 10% dimethyl sulfoxide. The smooth muscle origin of the myometrial cells in culture was verified by immunofluorescence (see below) using anti-desmin and anti-vimentin antibodies according to Leoni *et al.* (1990), and by binding studies with the oxytocin antagonist (see below). [Desmin is a Type III intermediate filament, characteristic of muscle cells and absent in connective tissue, which has recently been shown to be extensively

present in human uterine smooth muscle cells (Leoni *et al.*, 1990).] Very low contamination ($<10\%$) with fibroblasts (desmin-negative; vimentin-positive) was observed.

In order to purify myometrial cells further, the cell culture from M1 was dilution-cloned at the third doubling. Cells were diluted (2.5 cells ml^{-1} , $200\text{ }\mu\text{l well}^{-1}$) directly into microwells in the presence of 10^5 human peripheral mononuclear cells that had previously been irradiated with 6000 rad (feeder cells). The clones were selected using immunofluorescence and binding studies. Strains found to be desmin-positive, vimentin-positive and oxytocin-receptor-positive were obtained, maintained as previously described and used for experiments up to 10 passages. The clone used in this study was called D6.

For immunofluorescence studies, human myometrial cells were grown on plastic coverslips (Aclar, Allied Engineered Plastic, Pottsville, PA) and fixed in 3.7% paraformaldehyde in PBS, pH 7.4, for 10 min and for an additional 10 min in 0.1% Triton X-100. Afterwards, coverslips were washed in PBS and incubated for 90 min with anti-desmin and anti-vimentin antibodies (1:100). Incubation with the primary antibody was followed by incubation with fluorescein-coupled goat anti-rabbit IgG at a 1:200 dilution. The coverslips were examined and photographed using a Nikon MICROPHOT-FX microscope equipped with fluorescence filters (Nikon, Kogaku, Tokyo).

Membrane preparation

Human uterine specimens were collected at the time of caesarean section from ten patients who were delivered at an average time of gestation of 38 ± 1.1 weeks. Patients undergoing caesarean section were informed of the study protocol and the procedures used to obtain samples. Uterine horns were also obtained from two pregnant New Zealand White rabbits on day 29 of gestation.

Membranes were prepared as described by Maggi *et al.* (1988b, 1990). Myometrial tissues were suspended in buffer 1 (10 mmol Tris-HCl l^{-1} , pH 7.4, containing 1.5 mmol EDTA l^{-1} , 0.5 mmol dithiothreitol l^{-1} , 1 mmol benzamidine l^{-1} , 0.01% bacitracin and 0.002% soybean trypsin inhibitor) and homogenized. The homogenate was centrifuged at 1000 g for 10 min at 4°C . The supernatant containing the crude membrane fraction was then centrifuged at 160 000 g for 30 min at 4°C . The resulting pellets were washed once in buffer 2 (50 mmol Tris-maleate l^{-1} , pH 7.6, 10 mmol MgSO_4 l^{-1} , 1 mmol benzamidine l^{-1} , 0.01% bacitracin, 0.002% soybean trypsin inhibitor) and centrifuged again at 160 000 g for 30 min at 4°C . The final pellets were then resuspended in buffer 2 and divided into small aliquots (0.2 ml). Membrane preparations were frozen in solid CO_2 and stored at -80°C until assayed. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich).

Binding assays

Binding studies for $[^3\text{H}]$ oxytocin, $[^3\text{H}]\text{AVP}$, and $[^3\text{H}]\text{d}(\text{CH}_2)_5\text{Tyr MeAVP}$ to human and rabbit myometrial membranes were performed as described by Maggi *et al.* (1988b, 1990). According to these studies, human myometrial membranes

(0.3 mg ml⁻¹) were incubated with ligands in buffer 2 in the presence of 0.1% BSA at 22°C for 90 min for tubes containing [³H]AVP, for 60 min for tubes containing [³H]d(CH₂)₅TyrMeAVP, and for 180 min for tubes containing [³H]oxytocin. Rabbit myometrial membranes were incubated as for human membranes but for different durations: 120 min for tubes containing [³H]AVP, 90 min for tubes containing [³H]d(CH₂)₅TyrMeAVP, and 300 min for tubes containing [³H]oxytocin. [³H]AVP, [³H]d(CH₂)₅TyrMeAVP and [³H]oxytocin were present at a concentration of 0.7 nmol l⁻¹ in tubes containing increasing concentrations (0.1–10 000 nmol l⁻¹) of unlabelled peptides and at 0.03–0.7 nmol l⁻¹ in tubes without unlabelled ligands (final volume: 0.25 and 0.5 ml for human and rabbit membranes, respectively). All measurements were obtained in triplicate.

After incubation, membranes were filtered through Whatman GF/B filters (Clifton, NJ), which had been presoaked in ice-cold 50 mmol Tris l⁻¹, pH 7.4, in 0.1% BSA, using the Brandel M-48R 48-well cell harvester (Gaithersburg, MD) or the Millipore 1225 Sampling Manifold (Bedford, MA). Filters were washed twice with 3 ml ice-cold 50 mmol Tris l⁻¹, pH 7.4, and placed in liquid scintillation vials. Filter-retained radioactivity was measured in a liquid scintillation counter, after overnight incubation in 10 ml scintillation fluid (Instagel, Packard Instruments Company Inc, Downers Grove, IL).

Confluent (20–70 × 10³) myometrial cells were washed twice with Dulbecco's modified Eagle's medium, 20 mmol Hepes l⁻¹, 10 mmol MgSO₄ l⁻¹, 0.5% BSA, pH 7.4, and incubated in 200 µl of the same binding medium at 22°C for 60 min with fixed concentrations (15–50 pmol l⁻¹) of [¹²⁵I]OTA in the presence or absence of increasing concentrations of unlabelled ligands (10⁻¹¹–10⁻⁷ mol l⁻¹). After incubation, cells were extensively washed with ice-cold PBS, 0.1% BSA, solubilized in 0.5 mol NaOH l⁻¹ and the cell-bound radioactivity was determined. Measurements were obtained in triplicate. Cell counts routinely varied by less than 10% between wells.

Measurement of the intracellular [Ca²⁺]

Myometrial cells were grown to confluence on plastic coverslips (Aclar, Allied Engineered Plastic). Twenty-four hours before the experiments began, cells were maintained in serum-free Coon's Modified Ham's F12 medium. The intracellular free Ca²⁺ concentration ([Ca²⁺]_i) was determined using the Ca²⁺ sensitive dye fura-2, as reported by Baldi and Dunn (1991). Cells were loaded with 1 µmol fura-2 acetoxymethylester l⁻¹ for 40 min at 37°C in serum-free Coon's Modified Ham's F12 medium. The permeable acetoxymethylester of fura-2 is hydrolysed by cellular esterases on entering the cells, and the fura-2 formed is relatively impermeable and becomes trapped inside the cells.

The monolayers of myometrial cells were then washed and incubated for a further 20 min in fura-2-free medium at 37°C. Coverslips were mounted in a quartz cuvette with 2 ml Krebs-Henseleit Hepes buffer (KHH), containing 130 mmol NaCl l⁻¹, 5 mmol KCl l⁻¹, 1.25 mmol CaCl₂ l⁻¹, 0.8 mmol MgSO₄ l⁻¹, 5.5 mmol glucose l⁻¹, 20 mmol Hepes l⁻¹ (pH 7.4) and 0.1% fatty-acid-free BSA (pH 7.4), and maintained at 37°C.

Fluorescence was measured using a single wavelength spectrofluorometer (University of Pennsylvania Biomedical Group, PA) set at 340 nm excitation with emission at 510 nm. Fluorescence measurements were converted to values of [Ca²⁺]_i by determining maximal fluorescence (F_{max}) with 60 µmol ionomycin l⁻¹ followed by minimal fluorescence (F_{min}) with 7.5 mmol EGTA l⁻¹ and 50 mmol Tris l⁻¹, pH 10.5. [Ca²⁺]_i was calculated according to the method of Grynkiewicz *et al.* (1985) using the 340:380 nm. Autofluorescence by the cells or peptides and leakage of fura-2 outside the cells were negligible.

Analysis of experimental results

The binding data were evaluated quantitatively with non-linear least squares curve fitting using the computer program LIGAND (Munson and Rodbard, 1980). Weighting was used based on the assumption of constant percentage error in bound ligand concentrations. This analysis provides optimal estimates of 'binding parameters' (such as affinity constants, binding capacities and nonspecific binding) for any number of ligands reacting simultaneously with any number of classes of site. The program provides objective measures of goodness of fit, in terms of both magnitude and randomness of residuals. It provides objective criteria for distinguishing between models of different complexity, using an F test based on the 'extra-sum-of squares' principle (Munson and Rodbard, 1980).

The computer program ALLFIT (De Lean *et al.*, 1978) was used for the analysis of sigmoidal dose-response curves obtained in binding studies and in measurements of [Ca²⁺]_i. This program uses the constrained four-parameter logistic model to obtain estimates of ED₅₀ and IC₅₀ values, the logit-log slope ('pseudo-Hill coefficient') and relative potencies. Each data point represents the mean ± SE.

Results

Binding studies of oxytocin antagonists

The neurohypophysial hormone receptors in the uteri of pregnant humans and rabbits were identified pharmacologically by constructing complete sets of self- and cross-displacement curves among [³H]oxytocin, [³H]AVP, [³H]d(CH₂)₅TyrMeAVP, the corresponding unlabelled peptides and the oxytocin antagonists. Experiments were conducted once in pooled membranes from the myometrium of pregnant women, and once in pooled membranes from the myometrium of pregnant rabbits. Computer modelling of experimental results was performed as described by Maggi *et al.* (1988b, 1990, 1991b, 1992). Quantitative analysis using the LIGAND program (Munson and Rodbard, 1980) indicated that a model consisting of only one binding site class was insufficient in both the human and rabbit uterus. Instead, a model with two binding site classes was required to give a satisfactory fit in rabbit myometrium (*P* < 0.0001), while a model with three binding site classes was needed to fit results from the human myometrium (*P* < 0.001).

A complete set of competition curves in the myometrium from pregnant women is shown in Fig. 1. Tables 1 and 2

Fig. 1. Families of self- and cross-displacement curves between oxytocin ($\blacktriangle$), arginine vasopressin ($\circ$), $d(\text{CH}_2)_5\text{TyrMeAVP}$ ($\triangle$), $[\text{Mpa}^1, \text{D-Tyr}(\text{Et})^2, \text{Thr}^4, \text{Orn}^8]\text{-oxytocin}$ ($\blacksquare$), $\text{desGly}^9[\text{Mpa}^1, \text{D-Tyr}(\text{Et})^2, \text{Thr}^4, \text{Orn}^8]\text{-oxytocin}$ ($*$), $\text{desGly}^9[\text{D-Tyr}(\text{Et})^2, \text{Thr}^4, \text{Orn}^8]\text{-dC6-oxytocin}$ ($\bullet$) and $d(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2, \text{Thr}^4, \text{Orn}^8, \text{Tyr}^9\text{-NH}_2]\text{-vasotocin}$ ($\square$). The results shown in this figure are derived from one experiment using pooled membranes prepared from the myometrium of pregnant women. These curves were generated using the computer program LIGAND. The ratio of bound ligand:total ligand for (a) $[^3\text{H}]\text{oxytocin}$, (b) $[^3\text{H}]\text{arginine vasopressin}$ and (c) $[^3\text{H}]\text{d}(\text{CH}_2)_5\text{TyrMeAVP}$ is plotted against the total concentration of the varying ligand [ligand]; that is, labelled plus unlabelled ligand for homologous displacement curves, or unlabelled ligand alone for the heterologous competition curves. In homologous competition curves, the concentrations of the tracers were progressively reduced to give the optimum characterization of the high-affinity region of the binding curves. In the heterologous competition curves, fixed (0.7 nmol l^{-1}) concentrations of tracer were displaced by increasing concentrations of unlabelled ligands. Smooth curves show the predicted relationships for the three-site model shown in Table 1.

present binding parameters of ligands in the myometrium of pregnant humans and rabbits, respectively. In human myometrium, oxytocin binds with high affinity ($0.18\text{--}0.79 \text{ nmol l}^{-1}$) to an apparent heterogeneity of sites (R_1 sites, which can be divided into R_{1a} and R_{1b} sites). R_{1b} sites bind 10 times more oxytocin than do R_{1a} sites and show selectivity for oxytocin. However, the R_{1a} site is not selective for oxytocin but also binds arginine vasopressin with high affinity, as does the oxytocin receptor identified in rabbits. At present, the functional significance of this heterogeneity of oxytocin receptors in the human myometrium is unclear. However, these results add support to those of Kimura *et al.* (1992), who reported multiple hybridizing bands when human myometrium at term was probed with a cDNA specific for the oxytocin receptor.

In the myometrium of pregnant rabbits and humans another distinct subtype of neurohypophysial hormone receptor was identified that probably corresponds to the V1 vasopressin receptor described in the uterus (Maggi *et al.*, 1988b, 1990, 1991a, 1992). This site (R_2) binds arginine vasopressin and the V1 antagonist $d(\text{CH}_2)_5\text{TyrMeAVP}$ with relative selectivity.

The oxytocin antagonists tested, including OTA, DETVT, F123 and F327, are relatively selective for the oxytocin receptor in rabbit myometrium but not in human myometrium. In humans DETVT, F123 and F327 bind with higher affinity to the V1 vasopressin receptor than to the oxytocin receptor, with affinity constants similar to those obtained for the V1 antagonist $d(\text{CH}_2)_5\text{TyrMeAVP}$. This finding is in agreement with a previous observation of Pettibone *et al.* (1992) that although these antagonists are selective for the oxytocin receptor in the rabbit uterus, they have higher affinities for the V1 vasopressin receptor in humans.

To test the hypothesis that the oxytocin antagonists counteract the biological effect of oxytocin in the human uterus, we studied human myometrial cells obtained from the American Type Culture Collection (Hs 805.Ut), and prepared from normal cyclic women undergoing hysterectomy in the late luteal or early follicular phase of the menstrual cycle. These cells bind $[^{125}\text{I}]\text{OTA}$ as a function of time. The maximal specific binding of $0.1 \text{ nmol } [^{125}\text{I}]\text{OTA l}^{-1}$ was obtained after 60 min at 22°C (data not shown). Under these experimental conditions myometrial cells express high concentrations of the oxytocin receptor (Table 3). To purify myometrial cells further from eventual fibroblast contamination, we selected oxytocin-receptor-positive and desmin-positive clones.

Mathematical modelling of $[^{125}\text{I}]\text{OTA}$ binding data indicates that a single class of binding site is present in human myometrial cells in culture, which is similar to the R_{1b} subtype of oxytocin receptor binding site present in myometrium of pregnant women (Fig. 2 and Table 3). In contrast, the presence of V1 vasopressin receptors in human myometrial cells could not be verified in this experiment. This finding could be related to the loss of specialized properties by isolated cells grown *in vitro* owing to the absence of appropriate inducers or to the presence of inhibitors. Williams *et al.* (1992) reported that in rat aortic vascular smooth muscle cells glucose specifically inhibits the expression of both angiotensin II and V1 vasopressin receptors. We could not detect any specific binding of angiotensin II in our preparation of myometrial cells. However, since we were particularly interested in the oxytocin receptor and

Table 1. Concentration of receptors ($B_{\max}$) and affinity constants (K_d) for oxytocin (OT), arginine vasopressin (AVP), $d(CH_2)_5TyrMeAVP$, $d(CH_2)_5[Tyr(Me)^2, Thr^4, Orn^8, Tyr^9-NH_2]$ -vasotocin (OTA), $[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]$ -oxytocin (dETVT), $desGly^9[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]$ -oxytocin (F123) and $desGly^9[D-Tyr(Et)^2, Thr^4, Orn^8]$ -dC6-oxytocin (F327) in the myometrium of pregnant women

Parameter	Binding site		
	R_{1a}	R_{1b}	R_2
$B_{\max}$ (fmol mg ⁻¹)	25.6 ± 8.4	200.4 ± 22.04	110 ± 5.5
K_d (nmol l ⁻¹)			
OT	0.18 ± 0.18	0.79 ± 0.22	35 ± 8.4
AVP	1.8 ± 1.4	13.7 ± 3.3	0.26 ± 0.034
$d(CH_2)_5TyrMeAVP$	621 ± 714	14.2 ± 3.55	0.31 ± 0.037
OTA	6.05 ± 7.55	0.66 ± 0.24	2.6 ± 0.86
dETVT	110 ± 124	41 ± 12	0.4 ± 0.1
F123	66 ± 79.2	34 ± 10	0.5 ± 0.1
F327	1400 ± 1624	40 ± 12	0.6 ± 0.2

Binding parameters and SEs were obtained from computer modelling of a self- and cross-competition study among [³H]OT, [³H]AVP, [³H] $d(CH_2)_5TyrMeAVP$, the corresponding unlabelled peptides and the oxytocin antagonists.

Table 2. Concentration of receptors ($B_{\max}$) and affinity constants (K_d) for oxytocin (OT), arginine vasopressin (AVP), $d(CH_2)_5TyrMeAVP$, $d(CH_2)_5[Tyr(Me)^2, Thr^4, Orn^8, Tyr^9-NH_2]$ -vasotocin (OTA), $[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]$ -oxytocin (dETVT), $desGly^9[Mpa^1, D-Tyr(Et)^2, Thr^4, Orn^8]$ -oxytocin (F123) and $desGly^9[D-Tyr(Et)^2, Thr^4, Orn^8]$ -dC6-oxytocin (F327) in the myometrium of pregnant rabbits

Parameter	Binding site	
	R_1	R_2
$B_{\max}$ (fmol mg ⁻¹)	180 ± 9.0	357 ± 18
K_d (nmol l ⁻¹)		
OT	0.41 ± 0.053	103.5 ± 16
AVP	1.05 ± 0.20	0.51 ± 0.061
$d(CH_2)_5TyrMeAVP$	9.77 ± 8.4	0.13 ± 0.016
OTA	0.05 ± 0.02	8.7 ± 1.7
dETVT	6 ± 1.5	42.5 ± 6.4
F123	4.5 ± 1.1	30 ± 4.5
F327	7 ± 1.7	23 ± 3.45

Binding parameters and SEs were obtained from computer modelling of a self- and cross-competition study among [³H]OT, [³H]AVP, [³H] $d(CH_2)_5Tyr Me AVP$, the corresponding unlabelled peptides and the oxytocin antagonists.

not in the V1 vasopressin receptor, the loss of V1 vasopressin receptors in these myometrial cells was not investigated further.

High concentrations of oxytocin receptors were found in the myometrial cells (500 fmol per 10⁶ cells). The density of oxytocin receptors was further enriched in the cloned myometrial cells (2940 fmol per 10⁶ cells; Table 3). In the cloned cells, more than 90% of the total [¹²⁵I]OTA added was specifically bound, while nonspecific binding was negligible (2%). Hence, this cloned preparation of human myometrial cells

in culture represents an ideal tool to investigate the function of the oxytocin receptor. The activity of dETVT on the oxytocin-induced increase in [Ca^{2+}]_i in human myometrial cell preparations was therefore studied. Since the ligand specificity of the oxytocin receptor present in different preparations of myometrial cells was very similar, results obtained in different cell cultures were pooled.

Effect of oxytocin antagonists on [Ca^{2+}]_i mobilization

The basal [Ca^{2+}]_i in fura-2-loaded myometrial cells was 102.2 ± 3.8 nmol l⁻¹ ($n = 68$); addition of oxytocin induced an immediate initial transient increase of [Ca^{2+}]_i, reaching a peak within 30 s. Figure 3a shows the result of a typical dose-response experiment. The effect of oxytocin was dose dependent with an EC_{50} value of 2.5 ± 0.5 nmol l⁻¹ ($n = 3$). Thus, to test the antagonistic properties of dETVT on oxytocin-induced [Ca^{2+}]_i mobilization, cells were stimulated with 3 nmol oxytocin l⁻¹. This concentration of oxytocin stimulated an increase of [Ca^{2+}]_i to 350.7 ± 48 nmol l⁻¹ ($n = 9$). As shown in Fig. 3b, pretreatment with an increasing concentration of dETVT progressively blunted the effect of 3 nmol oxytocin l⁻¹. The analysis using the program ALLFIT (De Lean *et al.*, 1978) of results from five separate experiments indicates a mean IC_{50} value of 4.9 ± 1.1 nmol l⁻¹ and a slope factor not significantly different from unity, again suggesting the interaction of dETVT with a homogeneous class of receptor in myometrial cells.

Discussion

These results add support to previous evidence that the ligand specificity of neurohypophysial hormone receptors shows consistent interspecies variability. For example, dVDVP is a selective agonist for the rat (Manning *et al.*, 1973) and human (Maggi *et al.*, 1989) V2 vasopressin receptors, but shows low

Table 3. Concentration of receptors ($B_{\max}$) and affinity constants (K_d) for oxytocin (OT), arginine vasopressin (AVP), $d(CH_2)_5TyrMeAVP$, $d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8,Tyr^9-NH_2]$ -vasotocin (OTA), $[Mpa^1,D-Tyr(Et)^2,Thr^4,Orn^8]$ -oxytocin (dETVT), desGly 9 $[Mpa^1,D-Tyr(Et)^2,Thr^4,Orn^8]$ -oxytocin (F123) and desGly 9 $[D-Tyr(Et)^2,Thr^4,Orn^8]$ -dC6-oxytocin (F327) in different preparations of human myometrial cells

Parameter	M ₁	Hs805.Ut	M ₂	D ₆	Mean
$B_{\max}$ (fmol per 10 ⁶ cells)	532	592	360	2940	
K_d (nmol l ⁻¹)					
OT	0.55	0.68	0.39	—	0.54 ± 0.069
AVP	25.5	10.7	11.5	—	15.9 ± 3.9
$d(CH_2)_5TyrMeAVP$	9.4	8.4	—	5.8	7.9 ± 0.86
OTA	0.34	0.32	0.2	0.2	0.26 ± 0.032
dETVT	17.52	15.5	7.8	7.3	12 ± 2.25
F123	—	—	—	7.1	7.1
F327	—	—	—	7.7	7.1

Binding parameters were obtained from computer modelling of homologous and heterologous competition curves between [¹²⁵I]OTA and several neurohypophyseal hormones and analogues, using the program LIGAND (Munson and Rodbard, 1980). Values obtained in separate preparations of myometrial cells from two women (M1 and M2), in myometrial HS 805.Ut cells and in the cloned myometrial cells (D6) are shown.

Fig. 2. Family of competition curves between oxytocin (▲), arginine vasopressin (○), $d(CH_2)_5TyrMeAVP$ (△), $[Mpa^1,D-Tyr(Et)^2,Thr^4,Orn^8]$ oxytocin (■) and $d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8,Tyr^9-NH_2]$ -vasotocin (□) in Hs 805.Ut myometrial cells. These curves were generated by the program LIGAND. The ratio of bound $d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]$ -vasotocin ($[^{125}I]OTA$): total ligand is plotted against the total concentration of the varying ligand [ligand]; that is labelled plus unlabelled ligand for homologous displacement curves, or unlabelled ligand alone for the heterologous competition curves. Smooth curves show the predicted relationships for the one-site model shown in Table 3.

affinity for the V2 vasopressin receptors present in porcine renal medulla (Maggi *et al.*, 1986, 1988a). In addition, OTA is a selective antagonist for the rat (Elands *et al.*, 1988) and rabbit (Maggi *et al.*, 1991a) oxytocin receptor, but the same compound is less selective in humans (Maggi *et al.*, 1990).

From this study, dETVT and two related oxytocin antagonists (F123 and F327) are selective for oxytocin receptors in the rabbit uterus, but not in the human uterus. In the human myometrium, these antagonists bind with high affinity to the

Fig. 3. Representative tracing of waveforms of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by different concentrations of (a) oxytocin or (b) $[Mpa^1,D-Tyr(Et)^2,Thr^4,Orn^8]$ -oxytocin (dETVT) in human myometrial cells. Human myometrial cell monolayers grown on Aclar coverslips were loaded with fura-2 and incubated in Krebs-Henseleit HEPES buffer, pH 7.4. Basal $[Ca^{2+}]_i$ was measured fluorometrically after reaching thermal equilibrium at 37°C. In (b) coverslips were incubated for 2 min with the indicated concentration of dETVT and then stimulated with 3 nmol oxytocin l⁻¹. The arrows indicate the addition of the indicated nanomolar concentrations of peptides. Numbers on the ordinate axis indicate $[Ca^{2+}]_i$ (nmol l⁻¹).

V1 vasopressin receptor. This is in agreement with a previous radioligand binding study (Pettibone *et al.*, 1992) and with the finding that dETVT antagonizes *in vivo* the vasopressin-induced uterine contractility in nonpregnant women (Akerlund *et al.*, 1986; Hauksson *et al.*, 1988). Although originally developed for the treatment of primary dysmenorrhoea

(Akerlund, 1987), clinical studies indicate that dETVT is effective in inhibiting uterine activity in patients with uncomplicated preterm labour (Akerlund *et al.*, 1987; Andersen *et al.*, 1987; Akerlund, 1991). This suggests (1) that V1 vasopressin receptors might be involved in stimulating uterine contractions under these conditions, or (2) that at the concentrations used in clinical studies this antagonist effectively blocks oxytocin receptors, thereby inhibiting the effects of oxytocin in the myometrium.

The first hypothesis is in agreement with the previous identification of a V1 vasopressin receptor in the myometrium of nonpregnant and pregnant humans (Maggi *et al.*, 1990, 1992). However, the uterine concentration of this receptor is relatively constant during reproductive life, irrespective of the hormonal milieu or the stage of the pregnancy. Since labour is not affected in patients with diabetes insipidus (Hendricks, 1954) and the circulating concentration of arginine vasopressin does not change during parturition (De Vane and Porter, 1980), it is unlikely that the V1 vasopressin receptor actively participates in the onset or progression of human labour. Nevertheless, the possibility that the V1 vasopressin receptor could play a role in some pathological conditions such as fetal stress (Maggi *et al.*, 1990) cannot be ruled out.

Concerning the second hypothesis, the present study indicates that as the concentration of dETVT increases, it binds to and blocks the activity of the human oxytocin receptor more effectively. Indeed, this antagonist inhibits the oxytocin-induced $[Ca^{2+}]_i$ mobilization in human myometrial cells in culture. Since *in vitro* these cells express a high concentration of oxytocin receptors but not of V1 vasopressin receptors, this effect is apparently mediated by blocking the oxytocin receptor. It is therefore possible that the effect of dETVT in preventing pre-term labour observed in clinical studies is due to its interaction with the oxytocin receptor. When this antagonist is given as a bolus injection i.v. ($10 \text{ nmol l}^{-1} \text{ kg}^{-1}$ body mass) to humans, it reaches high concentrations (85 nmol l^{-1} ; Lundin *et al.*, 1986); in high nanomolar concentrations our *in vitro* study indicates that dETVT completely blocks the oxytocin receptor and the oxytocin-induced $[Ca^{2+}]_i$ mobilization.

In conclusion, these results indicate that dETVT and related oxytocin antagonists preferentially bind to the V1 vasopressin receptor present in the human uterus. However, they also interact with the oxytocin receptor and block oxytocin-induced $[Ca^{2+}]_i$ mobilization. The high affinity of dETVT for the V1 vasopressin uterine receptor may represent an unexpected benefit for the relaxation of the uterus in pregnant and nonpregnant humans in view of the presence of biologically active V1 vasopressin receptors in the myometrium.

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