Journal of Peptide Science
J. Peptide Sci. **7**: 413–424 (2001)
DOI: 10.1002/psc.334

Magnesium and Biological Activity of Oxytocin Analogues Modified on Aromatic Ring of Amino Acid in Position 2[1]

JIŘINA SLANINOVÁ[a,*], LENKA MALETÍNSKÁ[a], JIŘÍ VONDRÁŠEK[b] and ZDENKO PROCHÁZKA[c]

[a] Department of Peptide Biochemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[b] Center for Complex Molecular Systems and Biomolecules, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[c] Department of Peptide Synthesis, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received 20 December 2000
Accepted 25 March 2001

Abstract: For the purpose of evaluating substitution effects in the *ortho*, *meta* or *para* positions of the aromatic ring of tyrosine or phenylalanine in position 2 of oxytocin on uterotonic activity *in vitro* in the presence and absence of magnesium ions, six new analogues of oxytocin ([D- and L-*m*-methylphenylalanine[2]]oxytocin, [D- and L-*m*-methoxyphenylalanine[2]]oxytocin and [D- and L-*o*-methyltyrosine[2]]-oxytocin) were synthesized and several previously described analogues resynthesized. For the phenylalanine series, it is found that, in the absence of magnesium ions, substitution of the *ortho* and *meta* positions leads to loss of intrinsic activity (the analogues are antagonists) in contrast to the *para* position. In the tyrosine series, only methyl substitution in the *meta* position has this effect (substitution of *ortho* position only attenuates the agonistic biological activity). Addition of Mg ions restores to a certain degree the agonistic activity in the case of the *o*-methylphenylalanine analogue and enhances the agonistic activity of *o*-methyltyrosine oxytocin. All other analogues keep the original qualities as in the absence of Mg. Molecular modelling calculations of the structure of the above analogues was carried out to help explain these findings of the molecular level. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aromatic ring; biological activity; magnesium; oxytocin analogues

INTRODUCTION

Magnesium is one of the most abundant ions present in living cells and its plasma concentration is remarkably constant in healthy subjects. Activity of many enzymes is dependent on its presence. Intracellular magnesium concentrations have also been shown to be effective in modulating hormone actions, including those of the neurohypophysial hormones oxytocin and vasopressin. The physicochemical mechanism of magnesium action, the exact place of binding and its role in signal transfer is however unknown.

The role of magnesium and other divalent cations in oxytocin actions has been extensively investigated [1–8]. The role of magnesium in the action of oxytocin was studied at the level of isolated organs (uterus, mammary gland, arteries) and at the level of receptors. It has been shown that magnesium strongly influences the biological activities of oxytocin analogues, the agonistic activity being usually enhanced, the antagonistic activity decreased [1–6]. In this aspect, analogues modified in the *para* position of the amino acid in position 2[1] are interesting [2,3] because of changing agonistic, partial agonistic and antagonistic qualities according to the presence or absence of magnesium. We were interested to see what will be the effect of blocking other positions of the aromatic moiety (*ortho* or *meta*) using methyl or methoxy substituents on the biological activity in the presence or absence of

* Correspondence to: Department of Peptide Biochemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic.
[1] Preliminary results were presented in *Peptide Science—Present and Future, Proceedings of the 1st International Peptide Symposium*, Shimonishi Y (ed.). Kluwer Academic Publishers, Dordrecht, 1999; 185–187.

magnesium. Knowledge of the activities of these analogues will also contribute to mapping the hydrophobic pocket of the oxytocin receptor binding site which we have investigated for several years [10–13].

The series of analogues containing *p*-substituted phenylalanine (D- and L-4-methylphenylalanine [3,14], D- and L-4-ethylphenylalanine [3,15], D- and L-4-methoxyphenylalanine [14,16–18], D- and L-4-ethoxyphenylalanine [3,14,19]) and *o*-substituted phenylalanine (D- and L-2-methylphenylalanine [13]) described in the literature was supplemented by the synthesis and activity determination of *m*-substituted phenylalanine analogues (D- and L-3-methylphenylalanine and D- and L-3-methoxyphenylalanine). In the case of analogues having 2-methyl substituted L-tyrosine [14] in position 2, we performed a new synthesis of both diastereoisomers. Thus, the synthesis and biological properties of six new analogues of oxytocin (**I**–**VI**) are described in this paper. In addition, uterotonic activities in the presence of magnesium ions of several previously published analogues were determined. Some of the analogues were resynthesized for the purpose of this study, e.g. D- and L-2-methylphenylalanine.

H-Cys-X-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

	X
OT	L-Tyr
I	L-Tyr(*o*-Me)
II	D-Tyr(*o*-Me)
III	L-Phe(*m*-Me)
IV	D-Phe(*m*-Me)
V	L-Phe(*m*-OMe)
VI	D-Phe(*m*-OMe)

EXPERIMENTAL

General Methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czech Republic) in the following system: 2-butanol:90% formic acid:water (75:13.5:13.5) (SBA) and 1-butanol:acetic acid:water (4:1:1) (BA). Samples for amino acid analysis were hydrolysed with 6 M HCl at 105°C for 20 h and analysed on amino acid analyser T 339 (Mikrotechna Praha, Czech Republic). Optical rotations were determined on a Perkin-Elmer instrument type 141 MCA (Norwalk, VA, USA). Fast atom bombardment mass spectra (FAB MS) were obtained on ZAB-EQ spectrometer (VG Analytical Ltd., Manchester, UK), with xenon at 8 kV as the bombarding gas. High-performance liquid chromatography (HPLC) was carried out on an SP-8800 instrument equipped with a Spectra 100 UV-Vis detector and SP-4400 integrator (all from Spectra Physics, Santa Clara, USA). Preparative HPLC was carried out using a Vydac 218TP-510 (5 μm, 250 × 10 mm) column. Purity of the products was determined on a Vydac 218TP54 column.

Preparation of Amino Acids

D,L-2-Methylphenylalanine was prepared from L-1,2,3,4-tetrahydroisoquinolin-3-carboxylic acid [13]. D,L-2-Methyltyrosine was prepared from 3,5-dibromotyrosine [20] via 6,8-dibromo-7-hydroxy-1,2,3,4-tetrahydroisoquinolin-3-carboxylic acid and via 7-hydroxy-1,2,3,4-tetrahydroisoquinolin-3-carboxylic acid [13]; D,L-3-methylphenylalanine and D,L-3-methoxyphenylalanine were prepared via the modified acetamidomalonate method starting from 3-methylbenzyl chloride and 3-methoxybenzyl chloride (see below). All amino acids were protected by the fluorenylmethoxycarbonyl group. For the side chain protection of cysteine the 4-methylbenzyl group was used.

D,L-3-Methylphenylalanine. Sodium (2.53 g, 110 mmol) was dissolved in boiling absolute ethanol (170 ml) and after 20 min diethyl acetamidomalonate (23.9 g, 110 mmol) and 3-methylbenzyl chloride (13.2 ml, 110 mmol) were added. The reaction mixture was refluxed for 10 h, the precipitate was filtered hot and the filtrate was evaporated. Petroleum ether was added to the residue. After overnight refrigeration, the crystalline product, i.e. diethyl 3-methylbenzylacetamidomalonate, was filtered and washed with water. The crude product (21.6 g) was recrystallized from 30% aqueous ethanol (800 ml). Yield 19.1 g (59%) of pure crystalline product, m.p. 109–109.5°C. HPLC: k 4.24, (methanol:0.05% trifluoroacetic acid, 1:1). For $C_{17}H_{23}NO_5$ (321.4) calculated: 63.54% C, 7.21% H, 4.36% N; found: 63.34% C, 7.23% H, 4.47% N. FAB MS (m/z): 322.0 ($M + H^+$).

This compound (19.0 g, 60 mmol) was refluxed for 2.5 h in 2 N NaOH (90 ml, 180 mmol). To the reaction mixture was added 6 N HCl (120 ml) and refluxed for 3.5 h, cooled in a refrigerator and crystalline product (hydrochloride) was filtered off. The D,L-3-methylphenylalanine hydrochloride was suspended in water. The pH was adjusted using

aqueous ammonia to 7.0. Overnight refrigeration produced crystals that were collected and washed with water, ethanol and ether. Yield: 6.15 g (35%). HPLC: *k* 1.93, (methanol–0.05% trifluoroacetic acid, 1:4). R_F 0.23 (SBA), 0.13 (BA). For $C_{10}H_{13}NO_2$ (179.2) calculated: 67.02% C, 7.31% H, 7.82% N; found: 66.76% C, 7.20% H, 7.72% N. FAB MS (*m*/*z*): 180.0 ($M + H^+$).

Fluorenylmethoxycarbonyl-D,L-3-methylphenylalanine. A suspension of D,L-3-methylphenylalanine (1.8 g, 10 mmol) in a mixture of acetonitrile (15 ml), water (15 ml) and ethyldiisopropylamine (1.7 ml) was treated with Fmoc-ONSu (4.1 g, 12 mmol) with stirring at pH 8 for 3 h. Acetonitrile was evaporated, the aqueous residue was acidified with 1 N HCl (pH 2–3) and the product was extracted with ethyl acetate. Ethyl acetate extracts were washed with water, dried over sodium sulphate, evaporated and triturated with petroleum ether. The oily product, which solidified over night in a refrigerator, was crushed, filtered off and quickly washed with ether. Yield: 3.9 g (97%) of pure crystalline product, m.p. 150–153°C. HPLC: *k* 3.26 (methanol–0.05% trifluoroacetic acid, 7:3). R_F 0.69 (SBA), 0.92 (BA). For $C_{25}H_{23}NO_4$ (401.5) calculated: 74.80% C, 5.77% H, 3.49% N; found: 73.27% C, 5.64% H, 3.56% N. FAB MS (*m*/*z*): 402.1 ($M + H^+$).

D,L-3-Methoxyphenylalanine and fluorenylmethoxycarbonyl-D,L-3-methoxyphenylalanine. The title compounds were prepared from 3-methoxybenzylchloride (5 g, 31.9 mmol) and D,L-3-methoxyphenylalanine (1.95 g, 10 mmol) analogously as in the case of D,L-3-methylphenylalanine and fluorenylmethoxycarbonyl-D,L-3-methylphenylalanine, respectively. Their yields were: 2.55 g of D,L-3-methoxyphenylalanine (41%; HPLC: *k* 1.24 in methanol–0.05% trifluoroacetic acid, 1:4; R_F 0.17 (SBA), 0.09 (BA); for $C_{10}H_{13}NO_3$ (195.2) calculated: 61.53% C, 6.71% H, 7.17% N; found: 61.25% C, 6.54% H, 7.14% N. FAB MS (*m*/*z*): 196.2 ($M + H^+$), 218.2 ($M + Na^+$)) and 3.8 g of fluorenylmethoxycarbonyl-D,L-3-methoxyphenylalanine (91%), pure crystalline product, m.p. 137–141°C; HPLC: *k* 2.26 in methanol:0.05% trifluoroacetic acid, 7:3; R_F 0.65 (SBA), 0.88 (BA); for $C_{25}H_{23}NO_5$ (417.5) calculated: 71.93% C, 5.55% H, 3.36% N; found: 71.74% C, 5.45% H, 3.47% N. FAB MS (*m*/*z*): 418.2 ($M + H^+$).

Fmoc-oxytocin nonapeptide resin. Methylbenzhydrylamine resin (Peptides International, 0.92 meq/g, 2.0 g) was suspended in dichloromethane. After washing with 5% ethyldiisopropylamine in dichloromethane and with dimethylformamide, the resin was coupled with 2 molar excess of Fmoc-Gly-OH in the presence of *N*-hydroxybenzotriazole and diisopropylcarbodiimide in dimethylformamide. The coupling reaction was interrupted after 2 h, and the resin substitution (0.39 mmol/g) was determined by optical-density measurement [21]. The polymer was then acetylated (5 ml of acetic anhydride, 2 ml of triethylamine in 50 ml of dichloromethane). Incorporation of each amino acid residue into the growing peptide chain consisted of the following cycles: (1) cleaving the Fmoc group by 20% piperidine in dimethylformamide; (2) washing with dimethylformamide; (3) adding the Fmoc-protected amino acid derivative in dimethylformamide followed by *N*-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylcarbodiimide (DIC); (4) washing with dimethylformamide. The synthesis was monitored using the bromophenol blue method [22]. All reagents were used in 3 molar excess if not stated otherwise. The protected derivatives were used in the following order: Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Cys(*p*-Me-Bzl)-OH, Fmoc-Asn-OH, Fmoc-Gln-OH and Fmoc-Ile-OH. After this step the resin was divided into four parts, which were then coupled with 1.1 equivalents of either Fmoc-D,L-Phe(*o*-Me)-OH or Fmoc-D,L-Tyr(*o*-Me)-OH or Fmoc-D,L-Phe(*m*-Me)-OH or Fmoc-D,L-Phe(*m*-OMe)-OH. In the last step Fmoc-Cys(*p*-Me-Bzl)-OH was coupled.

Cleavage of the peptide from the resin, oxidation and purification of analogues. After deprotection of the Fmoc group, the nonapeptide resin was treated with liquid hydrogen fluoride (5 ml, 60 min, 0°C) in the presence of anisole (0.5 ml). After the evaporation of hydrogen fluoride, the nonapeptide together with the resin was triturated with ethylacetate, filtered, and washed with ethylacetate. The free peptide was successively extracted with acetic acid, 50% acetic acid, and water, and lyophilized. The residue was dissolved in water (150 ml) and the pH adjusted with 0.1 M NaOH to 7.0. Potassium ferricyanide (0.01 M solution) was added to this solution until a stable yellow colour persisted. During the oxidation (20 min), the pH was maintained at 7.2 by adding 0.1 M NaOH and then adjusted with acetic acid to 4.5. The solution was then put on a column of Amberlite CG-50I (7 ml), which was washed with 0.25% acetic acid and the product eluted with 50% acetic acid (35 ml). After freeze-drying, the crude product was purified by HPLC on a Vydac 218TP510 column in a slow gradient running from 40% to 60% methanol in 0.05% trifluoroacetic acid for 60 min (for **III**, **IV**), from 30% to 40% methanol

in 0.05% trifluoroacetic acid for 60 min (for **I**, **II**), from 30% to 50% methanol in 0.05% trifluoroacetic acid for 60 min (for **V**, **VI**) and lyophilized. Because of easy separability of the diastereoisomeric peptides by means of RP HPLC [23–30], the syntheses of analogues **I**–**VI** were thus performed using racemic amino acids. Peptides containing the appropriate diastereoisomers were separated at the end of the preparation. Formation of both diastereoisomers was accomplished using only 1.1 equivalents of protected racemic amino acid (see Reference [26]). If a higher equivalent was used a considerably higher portion of L-diastereoisomer in comparison to D-one was formed (~ 5:1). Chirality of the amino acid in the pure peptide was determined in hydrolysates by digestion using L-amino acid oxidase [31,32] (digestion time 100 h). In RP HPLC the determined *k* value was always lower for the L-diastereoisomer, which is consistent with previous findings [23,24,26–30]. Values of *k* (the capacity factor [23]), FAB MS and amino acid analyses are given in Table 1.

Biological Testing

Peptides were tested for uterotonic activity *in vitro* according to Holton [33] in Munsick solution [34] either in the absence of magnesium ions or in the presence of 1 mM magnesium. The cumulative dose–response (doses of oxytocin or analogue are added successively to the bath fluid without the fluid being changed; their concentration is increased logarithmically by doubling the dose at 1 min intervals until maximum response is obtained, i.e. until with increasing dose the height of contraction is not increasing) [35] or single dose–response (different doses of standard or tested compounds are added to the organ bath in random order and the bath fluid is changed immediately after the response) procedures were employed, preferentially both on one preparation of rat uterus. The value of the contraction corresponding to the highest dose of oxytocin used was taken for 100%. The agonistic activity in IU/mg was calculated by comparing the threshold doses of the standard and the analogue. In the case of the antagonistic activity, the dose of the antagonist was applied to the organ bath 1 min prior to the start of the cumulative dosing of oxytocin. The antagonistic activity was expressed as pA_2, i.e. the negative decadic logarithm of the concentration of the antagonist, which shifted the dose–response curve of the standard to the right for a factor of 0.3 (log 2). Each analogue was tested on uteruses from three to five different rats. For determination of the activity *in vivo*, the procedure described [36] was used. As a standard, synthetic oxytocin (PolyPeptide Laboratories, Prague, Czech Republic) was used. Binding affinity to uterine membranes was performed basically as described in References [37,38] using tritiated oxytocin from NEN Life Sci, Boston, MA, USA. A crude membrane fraction was incubated with ^{3}H-OT (2 nM) and various concentrations of peptides (0.1–10000 nM) for 30 min at 35°C. The total volume of the reaction mixture was 0.25 ml. The buffer used was 50 mM

Table 1 Value of *k* (Capacity Factor), FAB MS and Amino Acid Analysis for Analogues **I**–**VI**

	I	**II**	**III**	**IV**	**V**	**VI**
Asp	1.00	0.96	1.01	1.03	1.05	1.04
Glu	1.00	1.00	1.01	1.01	0.98	1.06
Pro	1.08	1.20	1.02	1.02	1.04	1.03
Gly	1.03	1.05	0.99	0.99	1.10	1.09
Cys	1.26	1.48	1.45	1.38	1.52	1.56
Ile	1.03	0.98	0.95	0.98	0.93	0.94
Leu	1.05	1.02	0.99	1.00	0.99	0.99
Tyr(*o*-Me)	0.96	0.92	–	–	–	–
Phe(*m*-Me)	–	–	1.10	1.10	–	–
Phe(*m*-OMe)	–	–	–	–	0.91	0.85
k	3.61[a]	5.22[a]	2.59[b]	5.10[b]	3.11[c]	7.51[c]
FAB MS	1021.3	1021.3	1005.2	1005.2	1021.3	1021.3

[a] Methanol:0.05% trifluoroacetic acid (35:65).
[b] Methanol:0.05% trifluoroacetic acid (45:55).
[c] Methanol:0.05% trifluoroacetic acid (4:6).

HEPES pH 7.6 containing 10 mM $MnCl_2$ and 1 mg/ml bovine serum albumin. The reaction was terminated by quick filtration on a Brandel cell harvester. Binding affinities were expressed as pK_i, i.e. negative decadic logarithm of K_i calculated according to expression $K_i = IC_{50}/[(c_{3_{H\text{-}OT}}/K_{d_{OT}}) + 1]$, where $c_{3_{H\text{-}OT}}$ is the concentration of radioactive ligand and $K_{d_{OT}}$ (dissociation constant of oxytocin) is taken as 1.8 nM [39].

Molecular Modelling, Semiempirical, Molecular Mechanics and Molecular Dynamics Calculations

The initial structures of oxytocin and all of its analogues were based on crystal structure of deaminooxytocin (PDB code 1xy1). Using the Builder module of Insight II [40] program package (MSI) the initial structure was modified onto seven separate structures and then optimized by molecular mechanics. The steepest descent method was used for optimization of modelled structures by molecular mechanics (MM) with a criterion of convergence 0.1 followed by approximately 5000 steps of the conjugate gradient method with a criterion of convergence 0.001. The models were also independently optimized by a semiempirical program (MOPAC) [41] using the PM3 [42] method and consequently using the ESP procedure for obtaining partial charge distribution. Minimized structures were superimposed and their root mean square (RMS) of C_α carbons was calculated. Fully optimized structures by the MM method were used for molecular dynamics calculation. The models were not soaked, the MD was performed *in vacuo* conditions. The dynamics of compounds were carried out with neutral side chains. Dynamics were run at room temperature for 30 ps with equilibration at 300 K for 2 ps with AMBER [43] force field implemented in the Discover95 [44] module. The calculations were carried out with the side chains in their uncharged states. All non-restrained MD simulations employed the Verlet velocity integration algorithm with a 1 fs time step.

The resulting trajectory was analysed for transition between conformational states of the substituted tyrosine ring. Minimization was performed periodically during the trajectory to characterize the conformational states being sampled. Solvent was not included in the systems because the interest was not in the conformation of compounds in solution, but rather in conformers bound to their receptor. For this purpose only the simulation *in vacuo* was chosen in these cases.

RESULTS

Biological Activity

The results of *in vitro* and *in vivo* testing of uterotonic potency are summarized in Table 2. In Table 2 are given the potency data of the new analogues (**I**–**VI**) as well as new and old (data published in literature) potency data of previously described and resynthesized analogues with substituted phenylalanine or tyrosine in position 2.

Substituted [Phe^2] analogues. As far as the *ortho* position is concerned, the L-analogue is an antagonist in the absence of magnesium, as illustrated in Figures 1 and 2. It is not able to contract the rat uterus but if present in the organ bath it decreases the response of the uterus to oxytocin (see the shift of the dose–response curve to the right). However, in the presence of magnesium it is a partial agonist if tested using the cumulative dose–response method and a full agonist using single dose method, see the shape of the dose–response curves in Figures 2 and 3. The D-analogue is an antagonist both in the presence and absence of magnesium.

Substituting the *meta* position by a methyl group results in antagonists under all testing conditions with both the L- and D-stereoisomer (**III** and **IV**). A methoxy group in the *meta* position (**V** and **VI**) leads to antagonism in the case of both diastereoisomers in the absence of magnesium, however with the L-diastereoisomer (**V**) to low activity partial agonism in the presence of magnesium.

As can be seen, the least effect on the agonistic activity has a modification at the *para* position in phenylalanine. The L-analogue in the absence or presence of magnesium is an agonist; on the other hand, the D-analogue in the absence of magnesium is a partial agonist and a full agonist in the presence of magnesium (Figure 4). Its uterotonic activity *in vivo* corresponds to the activity in the presence of magnesium. It is, however, interesting that the agonistic activity is prolonged.

Substituted [Tyr^2] analogues. In this series we have resynthesized the [Tyr(*o*-Me)^2]OT (**I**) and described newly the synthesis of the D-stereoisomer (**II**). The L-diastereoisomer (**I**) in our hands showed low agonistic activity in the absence of magnesium and about ten times enhanced agonistic activity in the presence of magnesium. The D-stereoisomer (**II**) showed antagonism.

Table 2 Biological Activity of Oxytocin Analogues (IU/mg or pA_2[a]) with Substituted Phenylalanine or Tyrosine in Position 2

Amino acid in position 2		Uterotonic activity							
		L-Stereoisomer				D-Stereoisomer			Reference for L/for D
		In vitro		*In vivo*		*In vitro*		*In vivo*	
		No Mg^{2+}	1 mM Mg^{2+}			No Mg^{2+}	1 mM Mg^{2+}		
Tyr		450	450	450		7.5	$pA_2 = 6.8$	–	[45]/[46]
o-MeTyr	**I**	3.1 ± 0.5	47 ± 5.0	31.5 ± 3.7	**II**	$pA_2 = 7.4 \pm 0.1$	$1.5/pA_2 = 6.2 \pm 0.1$	5.5 ± 1.5	[14]/[b]
m-MeTyr		$pA_2 = 6.8$	–	–		–	–	–	[8]/–
m-OHTyr		23	–	–		–	–	–	[47]/–
m-ITyr		$pA_2 = 7.2$	–	–		–	–	–	[48]/–
m-NO_2Tyr		1.1	–	–		$pA_2 = 6.3$	–	–	[49]/[19]
Phe		25–32	77	168		$pA_2 = 6.0$	2.3 ± 1.0[d]	$2.0/pA_2 = 6.0 \pm 0.3$[d]	[45,50]/[51]
o-MePhe		$pA_2 = 6.7 \pm 0.2$	3.2 ± 0.7[c]	3.6 ± 2.0[c]		$pA_2 = 7.6 \pm 0.3$	$pA_2 = 6.5 \pm 0.2$[c]	$pA_2 \sim 6.0$[c]	[13,14]/[13,14]
m-MePhe	**III**	$pA_2 = 7.7 \pm 0.2$	$pA_2 = 6.7 \pm 0.3$	$pA_2 = 6.0 \pm 0.2$	**IV**	$pA_2 = 8.0 \pm 0.1$	$pA_2 = 7.3 \pm 0.3$	$pA_2 = 6.3 \pm 0.3$	[b]
m-OMePhe	**V**	$pA_2 = 7.4 \pm 0.1$	$1.1/pA_2 = 6.2 \pm 0.1$	2.7 ± 0.5	**VI**	$pA_2 = 7.8 \pm 0.2$	$pA_2 = 6.8 \pm 0.3$	$0.3/pA_2 = 6.5 \pm 0.1$	[b]
p-MePhe		19; 30.3 ± 6.2	70 ± 20[d]	72[d]		$0.5/pA_2 = 7.6 \pm 0.2$[e]	11.2 ± 3.0[d]	2.9 ± 1.0[d]	[3,14]/[14]
p-EtPhe		6.5	18 ± 5[d]	62 ± 20[d]		$pA_2 = 8.15$	$pA_2 = 7.7 \pm 0.2$[d]	$pA_2 = 7.7$	[3]/[15,25]
p-OMePhe		1.5	–	–		$pA_2 = 7.74$		$pA_2 = 6.6$	[16–18]/[14]
p-OEtPhe		0.15	–	–		$pA_2 = 7.36$	$pA_2 = 6.8 \pm 0.3$[d]	–	[3]/[14,19]
p-PhPhe		$pA_2 = 7.0$	$pA_2 = 5.9$	0		$pA_2 = 7.0$	$pA_2 = 6.3 \pm 0.2$	0	[52]/[52]
p-NO_2Phe		0.66							[17]
o-Me,*p*-EtPhe		$pA_2 = 7.6 \pm 0.2$	$7.0/pA_2 \sim 7.00$[d]	5.8 ± 1.5		$pA_2 = 8.0 \pm 0.2$	$pA_2 = 8.0 \pm 0.2$[d]	$7.0/pA_2 = 6.1 \pm 0.2$[d]	[14]/[14]
o,*o*′-diMePhe		$pA_2 = 6.2$	0[d]	0		$pA_2 = 7.4$	$pA_2 = 6.2 \pm 0.2$[d]	$pA_2 = 6.2$	[13]/[13]
pentaMePhe		$pA_2 = 6.3$	0	0		$pA_2 = 6.2$	$pA_2 = 5.8$	0	[52]/[52]

[a] The agonistic activity was calculated in IU/mg by comparing the threshold doses of the compound and standard oxytocin; antagonistic activity was expressed as pA_2, i.e. the negative decadic logarithm of the concentration of the antagonist which shifted the dose–response curve of the standard oxytocin to the right for a factor 0.3 (log 2), in other words which caused the response of a 2*X* dose of oxytocin to decrease to the response of *X* dose.

[b] New analogue synthesized and described in this paper; the values are averages ± S.E.M. from four to five independent experiments.

[c] Activity determined in previously published papers, however, for this research analogue was resynthesized; in the cited papers mostly only the uterotonic activity *in vitro* is given.

[d] Activity determined for the purpose of this paper on originally synthesized sample stored at −20°C as lyophylisate.

[e] Activities in the absence of magnesium corresponded under both [c] and [d] to the originally published values with the exception of this one; the originally published value was higher ($pA_2 = 8.2$).

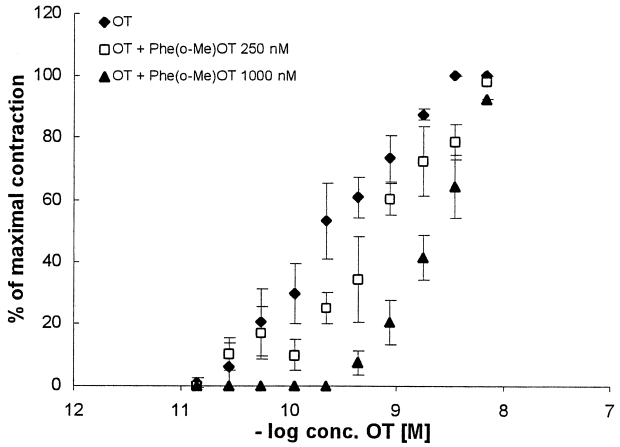

Figure 1 Cumulative dose–response curves of oxytocin in the uterotonic test *in vitro* in the absence of magnesium in the bathing medium in the absence (◆) or in the presence of [L-*o*-MePhe^2]OT (□, 0.25 μM and ▲, 1 μM). The values are averages ± S.E.M. of three independent experiments. The response to the highest dose of oxytocin applied in the absence of an antagonist was taken as 100%.

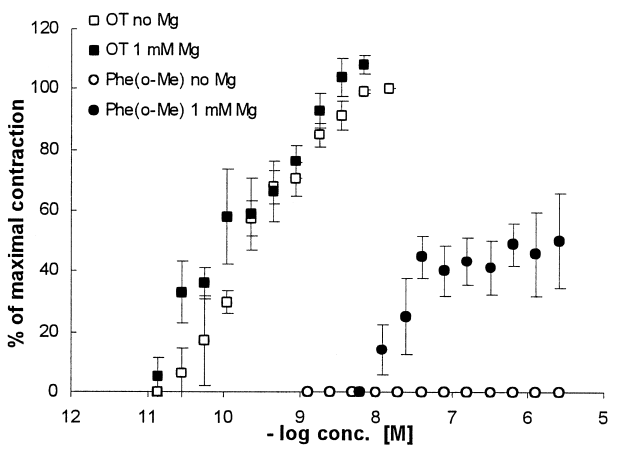

Figure 2 Activity of standard oxytocin and [L-*o*-MePhe^2]OT in the uterotonic test *in vitro* in the absence (■ and ●, respectively) and in the presence (□ and ○, respectively) of 1 mM Mg^{2+} in the bathing medium using cumulative dose–response procedure. The values are averages ± S.E.M. of four independent experiments. The response to the highest dose of oxytocin applied in the absence of Mg^{2+} was taken as 100%.

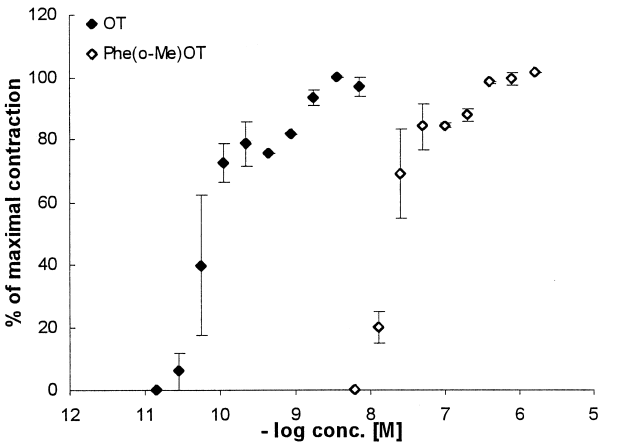

Figure 3 Activity of standard oxytocin (◆) and [L-*o*-MePhe^2]OT (◇) in the uterotonic test *in vitro* in the presence of 1 mM Mg^{2+} in the bathing medium using single dose–response procedure. The values are averages ± S.E.M. of three independent experiments. The response to the highest single dose of oxytocin applied was taken as 100%.

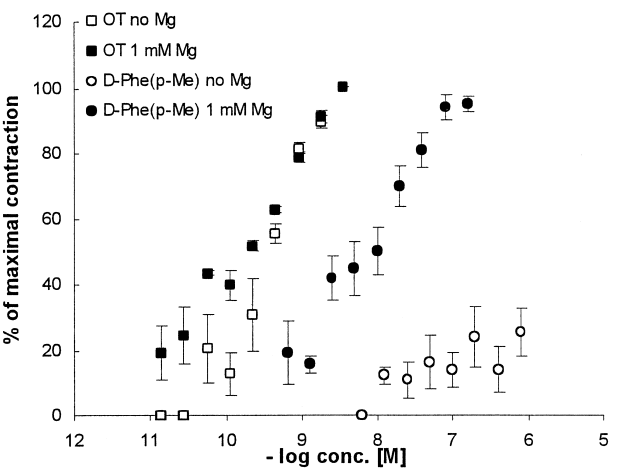

Figure 4 Activity of standard oxytocin and [D-*p*-MePhe^2]OT in the uterotonic test *in vitro* in the absence (□ and ○, respectively) and in the presence (■ and ●, respectively) of 1 mM Mg^{2+} in the bathing medium using cumulative dose–response procedure. The values are averages ± S.E.M. of three independent experiments. The response to the highest dose of oxytocin applied in the absence of Mg^{2+} was taken as 100%.

Binding Affinity

Table 3 summarizes the binding affinities of the analogues to the rat uterine membranes. As can be seen, all the analogues have rather high affinity for the rat uterine oxytocin receptors. The K_i ranges from 1.25 to 80 nM (pK_i 8.9–7.1, respectively) for the most affine analogue [L-Phe(*p*-Et)^2]OT (comparable to oxytocin) and the least affine analogue [L-Phe(*m*-OMe)^2]OT.

Table 3 Affinity of Oxytocin Analogues with Substituted Phenylalanine or Tyrosine in Position 2 to Rat Uterine Oxytocin Receptor[a]

Amino acid in position 2[b]	Binding affinity (pK_i)	
	L-Stereoisomer	D-Stereoisomer
Tyr	8.4	–
Phe	–	7.8
o-Me Phe*	7.4	7.9
o-Me Tyr **I** and **II**	8.2	8.0
m-Me Phe **III** and **IV**	7.6	7.4
p-Me Phe	8.7	8.0
p-Et Phe	8.9	–
o-Me,*p*-Et Phe	8.2	8.5
m-OMe Phe **V** and **VI**	7.1	7.8
p-OMe Phe	–	7.1

[a] Affinity of the analogues was determined as described in 'Experimental' using tritiated oxytocin as tracer and membranes from rat uteri; binding affinities were expressed as pK_i, i.e negative decadic logarithm of K_i in mol/l calculated according to expression $K_i = IC_{50}/[(c_{3_{H\text{-}OT}}/K_{d_{OT}}) + 1]$.
[b] Analogues **I–VI** were described in this paper; analogues marked by * were resynthesized for this project; all other analogues were tested on samples stored as lyophilized powder (their purity was routinely checked on HPLC).

Molecular Modelling

As described in the Experimental section, the structures of oxytocin, [Phe^2]oxytocin, [Phe(*o*-Me)^2]-oxytocin, [Tyr(*o*-Me)^2]oxytocin, [Phe(*m*-Me)^2]oxytocin, [Tyr(*m*-Me)^2]oxytocin and [Phe(*p*-Me)^2]oxytocin were analysed. We visualized the distance change between the *para* carbon atom of the aromatic ring in position 2 and the C_α atom of cysteine in position 1, and a time stability of the aromatic ring in terms of ring plane deviation from its stable (crystalographically determined) position. Because of sterically non-constrained simulation, we have also studied RMS deviation of superimposed C_α atoms of all studied species in their energetically remarkable states.

Two of the agonists, i.e. oxytocin and [Phe^2]oxytocin, show similarity in the pattern of characteristics studied. They both have the *para* C atom of the aromatic ring relatively stable in a distance of about 9 Å from the C_α atom of cysteine in position 1 during the simulation and the dihedral angel describing the aromatic ring plane fluctuating slightly around the value of 180°. The [Phe(*p*-Me)^2]oxytocin which has comparable activity as [Phe^2]oxytocin differs in these studied characteristics substantially (see below). On the other hand, comparison of conformers during the simulation in terms of their RMS value for superimposed C_α atoms shows a very similar pattern as for the superimposed structures of oxytocin and [Phe^2]oxytocin. It could be explained by a placement of the methyl group in *para* position in terms of its sterical requirements mentioned below.

Generally, it is possible to say that the antagonist compounds studied and the Phe(*p*-Me)^2]oxytocin do not show significant preference for the orientation of the substituted aromatic ring. Introducing a methyl group in the *ortho* or *meta* positions of the Tyr residue shows a continuously unstable position of the tip of the aromatic ring. It fluctuates around the distance between the relevant atoms in the range of 4–7.5 Å. The aromatic ring, considering its position in the crystallographically determined structure of deaminooxytocin, is twisted at about + 40° and this twist is relatively stable during the simulation or converged to its stable value.

Modified [Phe^2]oxytocin in *ortho*, *meta* and *para* positions shows very similar characteristics. The orientation of the aromatic ring plane is changed in respect to the Tyr ring in oxytocin with a difference of about 80° and the whole side chain is oriented towards the interior of the oxytocin ring and seems to constitute more tightly packed conformers.

All structures having Me group in positions *ortho* or *meta* have also changed the overall conformation of the S–S bridged oxytocin ring. This can be explained by sterical requirements of the methyl group inducing conformational changes along the backbone and residues adjacent to its position. An interesting feature of the substituted Phe ring was observed during performed minimization and MD stimulation. Even if the methyl group in *ortho* and *meta* positions was placed on both possible positions of the aromatic ring, after the performed minimization, only one stable orientation of the methyl group in respect to the studied backbone was always found.

DISCUSSION

It was found previously that for binding of agonists to uterine membrane preparations magnesium is necessary and may be substituted by manganese. The same applies for membranes of cells having stable expressed OT receptors. For binding of antagonists, however, omission of magnesium has no

effect. Furthermore, we know that testing of the oxytocic activity *in vitro* can be performed in the absence of magnesium in the bathing medium. This would suggest that magnesium is bound intracellularly and causes the receptor complex to have the optimal conformation for binding and for signal transduction. However, low concentrations of magnesium in the bathing medium (0.1–0.5 mM) which do not substantially enhance the magnesium concentration intracellularly influence the activity of oxytocin and its analogues. This would imply that the extracellular magnesium also contributes to the formation of the optimal conformation of the ligand receptor complex for transduction of the signal.

As illustrated in Table 3, all the analogues substituted in various positions of the aromatic ring of Phe or Tyr show high affinity to the receptors in uterine tissue. Thus, the modifications are related to the intrinsic activity, in other words with transduction of the signal. However, alkyl substitution in the *ortho* position of the benzene ring of phenylalanine provides analogues with antagonistic activity. Additional alkyl substituents in *o′*- or *p*-positions have no influence on the quality (i.e. dialkyl-substituted analogues *o*,*p*- and *o*,*o′*- are antagonists). However, disubstituted analogues where one of the substituents is a free hydroxyl group have different activities; [*o*-methyl-tyrosine]oxytocin is an agonist, but [*m*-methyl-tyrosine]-oxytocin (see Table 2) is an inhibitor. We can conclude that the free hydroxyl preserves the agonistic character of the analogues in both L- and D-series. The hydroxyl group in the *para* position has an opposite and superior effect to the methyl substituent in the *ortho* position, however, not to that in the *meta* position. Meanwhile, introduction of *o*-Me into phenylalanine leads to the change of agonism into antagonism, introduction of *o*-Me into tyrosine leads just to a decrease of agonistic activity. Introduction of *m*-Me leads to the change from agonism into antagonism as already in the case of L-tyrosine.

The electronic effect of the hydroxyl group in the *para* position is probably much stronger than the steric effect of the methyl group in the *ortho* position and the resulting analogue with these two substituents has low intrinsic activity. Analogues substituted in the *meta* position of tyrosine are mostly antagonists (see *m*-methyl and *m*-I in Table 1 and *m*,*m′*-diBr analogues [53]) with the exception of *m*-nitro and *m*-hydroxy tyrosine analogues which have intrinsic activity. Unfortunately no data about the biological activity of these kind of *meta*-substituted analogues derived from phenylalanine have been reported.

As far as the D aromatic amino acid in position 2 is concerned, only the analogue with a free hydroxyl in *para* position (D-tyrosine) and D-Phe(*p*-Me) have agonistic activity in the absence of magnesium. In the case of all other substituents in whatever position, the configuration is probably so different that there is no signal transmission and the analogues are antagonists. Addition of magnesium causes enhancement of agonism with D-Phe(*p*-Me) analogue, however, paradoxically it causes antagonism with [D-Tyr]^{2}oxytocin.

The results indicate that the *meta* position of the aromatic ring of the amino acid in position 2 may be the most important for the intrinsic activity. It is crucial for the transfer of the signal — both its position (L or D series) and slightly electronegative character (oxytocin). The extracellularly effective magnesium might optimize the orientation of the aromatic moiety for signal transfer if the *meta* position is not blocked. In case the *meta* position is blocked, the signal transfer is interrupted.

According to the molecular dynamics simulations, the methyl group in *meta* or *para* position cannot change at all the overall conformation of the molecule. Methyl in the *ortho* position enhances slightly the barrier for the rotation of the aromatic ring.

Recently, Liao *et al.* [54] published very interesting results concerning bicyclic conformationally constrained analogues of oxytocin. Using steric constraints in the side chain of amino acid in position 2, they were able to make strong antagonists as well as compounds devoid of any activity. There was a special position of the aromatic ring which enabled binding, i.e. the *gauche* one. In the case of *trans* configuration there was no binding. In other words, the *gauche* conformation means that the Tyr side chain is closer to the α-carbon of the amino acid in position 1, the *trans* conformation means that it is distant from that point. Now if we hypothesize that two conformations are necessary for the full activity of oxytocin, the first for binding, i.e. aromatic ring of tyrosine is close to the α-carbon atom, the second for transfer of the signal that is induced by the interaction of the oxytocin with the receptor, i.e. the aromatic ring of tyrosine is pushed to a distant position from the α-carbon atom of Cys. We could hypothesize that in the conformation for transfer of signal, the compound is not able to form the initial contact for binding. The synthesis of RS or SR mono methyl tyrosine analogues would prove this hypothesis. The biological activity of RS and RR β-methyl phenylalanine analogues support this idea (unpublished results).

The different shape of the analogues dose–response curves using cumulative or single dosing may reflect the fact that in the cumulative dosing procedure, the analogue is in a longer contact with the tissue (approximately 10 min) and thus not only the fraction of the compound having the most favourable conformation may interact with the receptor/receptors, however, also other conformational states of the analogue may play a role here. In the case of the single dose–response procedure, when the interval between the addition of an analogue to the organ bath and its wash-out is only 1 min, we may register only the primary interaction of a small fraction of the peptide with the receptors. The theoretical explanation of several mechanisms leading to partial agonism is analysed by Pliška [55].

The OH group of tyrosine, even if not necessary for activity, causes optimal orientation of the side chain of the tyrosine for the transfer of signal. Substitution of H for Me will give an analogue — under some conditions an antagonist, under other conditions a partial agonist (i.e. agonist with lower activity and lowered maximal effect) character. Similarly with OMe in the positions *o*- or *m*- the analogue is, in the presence of magnesium, an agonist, in the absence of magnesium, an antagonist.

Binding of the hormone to the receptor is by non-covalent bonds only and there is evidence that magnesium, in addition to its effect on stimulus response coupling may potentiate binding at one particular unidentified site of interaction.

According to the present results, one may bear in mind in designing selective oxytocin antagonists, that blocking the *meta* position of the aromatic side chain of amino acid in position 2 should lead to a strong antagonist.

Acknowledgements

The authors are indebted to Mrs M. Plačková for her skilful technical assistance in the pharmacological evaluation and to Mr J. Zbrožek for the amino acid analyses. The elemental analyses were carried out in the Analytical Laboratory of our Institute (Dr V. Pechanec, Head) and mass spectra in the Laboratory of Mass Spectroscopy (Dr K. Ubik, Head). This work was supported by GA of the Academy of Sciences of the Czech Republic, grant no. A 4055602, GA of the Czech Republic, grant no. 204/99/1453, and HŠMTČR, project LN00A032.

NOTES

1. All the chiral amino acids mentioned in this work are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations [9]: Phe(*o*-Me) denotes the 2-methylphenylalanine moiety, Tyr(*o*-Me) denotes the 2-methyltyrosine moiety, Phe(*m*-Me) denotes the 3-methylphenylalanine moiety and Phe(*m*-OMe) denotes the 3-methoxyphenylalanine moiety.

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