ELSEVIER

European Journal of Pharmacology 355 (1998) 203–210

ejp

Oxytocin receptor binding and uterotonic activity of carbetocin and its metabolites following enzymatic degradation

Thomas Engstrøm [a,*], Tomislav Barth [b], Per Melin [c], Hans Vilhardt [a]

[a] *Department of Medical Physiology, 12.2.39, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark*
[b] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague 6, Czech Republic*
[c] *Ferring Pharmaceuticals, Malmö, Sweden*

Received 6 April 1998; revised 29 June 1998; accepted 7 July 1998

Abstract

Metabolites of the analogue 1-deamino-1-carba-2-tyrosine(*O*-methyl)-oxytocin (carbetocin) following incubation with a rat kidney homogenate were isolated and their pharmacodynamic properties investigated. Apart from the parent compound two metabolites were identified namely desGlyNH_2-carbetocin (carbetocin metabolite I) and desLeuGlyNH_2-carbetocin (carbetocin metabolite II). Both carbetocin, carbetocin metabolite I and carbetocin metabolite II displayed binding affinities to the myometrial oxytocin receptor of a similar magnitude as oxytocin. Carbetocin was found to have agonistic properties on isolated myometrial strips and it was found to exert this effect through generation of inositol phosphates, as is the case for oxytocin. However, maximal contractile effect of carbetocin was approximately 50% lower than that of oxytocin (2.70 ± 0.12 g compared to 5.22 ± 0.26 g) and EC_{50} was approximately ten times higher (48.0 ± 8.20 nM compared to 5.62 ± 1.22 nM). Neither carbetocin metabolite I nor carbetocin metabolite II were able to contract isolated myometrial tissue. All three compounds displayed antagonistic properties against oxytocin in vitro, with carbetocin being the strongest inhibitor ($pA_2 = 8.21$) and carbetocin metabolite II ($pA_2 = 8.01$) being stronger than carbetocin metabolite I ($pA_2 = 7.81$). These results indicate that carbetocin is a partial agonist/antagonist to the oxytocin receptor while the two metabolites carbetocin metabolite I and carbetocin metabolite II are pure antagonists. All three analogues bound to the myometrial vasopressin V_1 receptor, albeit with much lower affinities than to the oxytocin receptor. Carbetocin metabolite II showed the weakest binding affinity of 33.7 ± 7.34 nM compared to 7.24 ± 0.29 nM for carbetocin and 9.89 ± 2.80 nM for carbetocin metabolite I. Only carbetocin bound to the renal vasopressin V_2 receptor though the binding affinity was very low (61.3 ± 14.6 nM).

Keywords: Carbetocin; Oxytocin; Carbetocin metabolite; Uterus; Receptor binding

1. Introduction

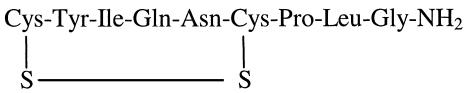
Carbetocin (Fig. 1) is an alkylated carba-analogue of 1-deamino-oxytocin (1-deamino-1-carba-2-tyrosine (*O*-methyl)-oxytocin), which has been shown to have both milk ejecting and uterotonic activity (Barth et al., 1975). Due to its short half-life oxytocin used for induction of labour needs to be infused intravenously to obtain sustained myometrial activity. Carbetocin has a long half-life (85–100 min) (Cort et al., 1981) compared to that of oxytocin (3.4 min) (Gazis, 1978) and vasopressin (1.6–8.0 min) (Forsling et al., 1973; Gazis and Sawyer, 1978) and it is therefore considered being used in human subjects for the control of uterine atony and excessive bleeding after delivery by caesarean section. Indeed in post partum women Hunter et al. (1992) found intramuscular administration of carbetocin to be superior to the intravenously route concerning the duration of uterotonic action. In addition they observed only minimal side effects of the treatment.

In spite of the molecular differences between oxytocin and carbetocin previous studies have shown, that the two peptides bind to myometrial plasma membranes with receptor affinities in the same order of magnitude (Atke and Vilhardt, 1987). The structural modifications made in the N-terminal part of the carbetocin molecule protect the molecule from aminopeptidase and disulphidase cleavage.

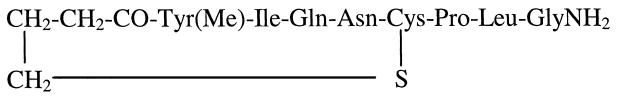
* Corresponding author. Tel.: +45-3532-7531; Fax: +45-3532-7537; E-mail: engstrom@mfi.ku.dk

0014-2999/98/$19.00
PII: S0014-2999(98)00513-5

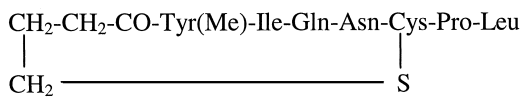
Oxytocin



Carbetocin



DesGlyNH$_2$-Carbetocin (carbetocin metabolite I)



DesLeuGlyNH$_2$-Carbetocin (carbetocin metabolite II)

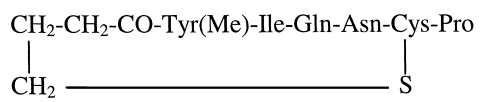


Fig. 1. Chemical structures of oxytocin, carbetocin and carbetocin metabolites I and II.

The living organism does, however, posses a number of enzymes capable of degrading carba-analogues at the C-terminal end of the molecule (Barth et al., 1969; Suska-Brzezinska et al., 1972). In this context the kidneys constitute important sites for peptide metabolism.

The prolonged uterotonic activity of carbetocin is proposed to be a result of an increased stability (Vilhardt et al., 1997). Mitchell et al. (1997) have demonstrated that oxytocin incubated with a cytosolic fraction of uterine homogenates is primarily degraded by post-proline endopeptidase activity, whereas the particulate fraction contained predominantly aminopeptidase activity.

In the present study, the aim was to identify the metabolites of carbetocin following incubation with a kidney homogenate and to describe the pharmacological properties of the compounds as to binding of the metabolites to oxytocin receptors and to vasopressin V_1 and vasopressin V_2 receptors. In addition agonistic and antagonistic effects of the metabolites on myometrial contractility were investigated.

2. Materials and methods

2.1. Materials

Female Wistar rats (250–350 g) used for binding and contraction studies were bred and maintained under controlled conditions in the Panum Institute Animal House. Food and water were freely available. Sprague–Dawley rats used for preparation of kidney homogenates were obtained from Møllegaard (Ejby, Denmark).

Carbetocin, oxytocin, carbetocin metabolite I and carbetocin metabolite II prepared by solid phase synthesis were obtained from Ferring (Malmö, Sweden). [^{3}H]oxytocin and [^{3}H]vasopressin were purchased from New England Nuclear (Boston, MA, USA) and [^{3}H]inositol was from Amersham (North Chicago, IL, USA). All other chemicals were of analytical grade.

2.2. Preparation of rat kidney homogenate

Kidneys from 10 rats were cut in small pieces and homogenized on ice in a 25 mM sodium-phosphate buffer (pH 6.5) containing 1 mM EDTA. Subsequently the homogenate was centrifuged $20\,000 \times g_{av}$ for 30 min at 4°C. The resulting supernatant was aliquoted and store frozen (−20°C).

2.3. Identification of carbetocin metabolites

6.25 ml carbetocin (5 mM) was pre-incubated for 5 min at 37°C in 23.75 ml 25 mM sodium-phosphate buffer, pH 6.5 containing 1 mM EDTA. Thereafter 1.25 ml kidney homogenate was added. Following further 100 min incubation the enzymatic reaction was stopped by addition of 62.5 ml ice-cold 2% $ZnSO_4$ in methanol/water (1:1). The reaction mixture was kept on ice for 10 min. After centrifugation (5 min, $10{,}000 \times g_{av}$, 4°C) the supernatant was evaporated and re-dissolved in acidified water. Pre-purification was made using a Sep-Pak C18 column to remove impurities, which could shorten the lifetime of the semi-preparative column used for separation of the metabolites. The Sep-Pak eluent was analyzed by High Pressure Liquid Chromatography (HPLC) using a Kromasil (C8) column with an acetonitril/triflouroacetic acid gradient to separate metabolites. Amino acid analysis and Fast Atom Bombardment Mass Spectrometry (FAB-MS) was employed to determine the structure of each metabolite.

2.4. Preparation of plasma membranes

Preparation of rat myometrial plasma membranes was performed as described (Engstrøm et al., 1988a). Two days prior to decapitation the animals received an i.m. injection of 75 μg oestradiol-benzoate (Engstrøm et al., 1998).

Myometrial tissue was homogenised followed by centrifugation $100 \times g_{av}$ (10 min, 4°C). The supernatant was re-centrifuged $10\,000 \times g_{av}$ (10 min, 4°C) and the resulting supernatant centrifuged $113\,000 \times g_{av}$ (30 min, 4°C). The sediment from the $113\,000 \times g_{av}$ centrifugation was resuspended in 10% sucrose and placed on top of a 28% sucrose solution. Following centrifugation at $112\,000 \times g_{av}$ (120 min, 4°C) the layer between the two sucrose solutions was carefully pipetted off, washed with distilled water and finally resuspended in distilled water to concentrations of 0.5–1.0 mg/ml. An aliquot was taken for protein determination according to Lowry et al. (1951). Electron microscopy was done to ensure that the preparation consisted of purified plasma membranes (Fig. 2).

Rat kidney plasma membranes were isolated using the technique described by Fitzpatrick et al. (1969). Medullary renal tissue was dissected on ice and minced with a pair of scissors. The tissue was homogenized in 3 volumes of 0.25 M sucrose–1 mM EDTA followed by centrifugation at $1.475 \times g_{av}$ for 10 min at 4°C. The sediment was resuspended in 2 M sucrose, re-homogenized and re-centrifuged at $13{,}300 \times g_{av}$ for 10 min at 4°C. The supernatant of the $13\,300 \times g_{av}$ centrifugation was diluted to isotonicity by the addition of 7-volume cold distilled H_2O and was centrifuged at $35\,000 \times g_{av}$ for 15 min at 4°C. The resulting pellet consisted of an upper pink layer of membranes and a lower brown layer. The upper layer was removed by swirling isotonic sucrose over the pellet. Following homogenization the resulting suspension was centrifuged three times at $35\,000 \times g_{av}$ for 15 min at 4°C with homogenizations in between each. The final pellet was aliquoted and the protein content measured (Lowry et al., 1951).

2.5. Binding assays

Receptor binding to myometrial or kidney plasma membranes was performed as described earlier with some modifications (Engstrøm et al., 1988a). Membranes were incubated for 60 min in 250 μl of a medium containing 50 mM Tris–maleate (pH 7.6), 10 mM $MnCl_2$ with concentrations of [^{3}H]oxytocin varying from 0.42 to 24.20 nM or concentrations of [^{3}H]vasopressin varying from 0.09 to 5.40 nM. The assay was performed at room temperature and was initiated by the addition of plasma membranes. At the end of the incubation period bound ligand was separated from unbound by filtration. Specific binding of ^{3}H-ligand was calculated by subtraction of binding in the presence of a 1000-fold excess of unlabelled hormone and was finally related to protein concentration. Linear regressions of double reciprocal plots were used to calculate dissociation constants of the receptor binding of [^{3}H]oxytocin and [^{3}H]vasopressin. R^2-values of these re-

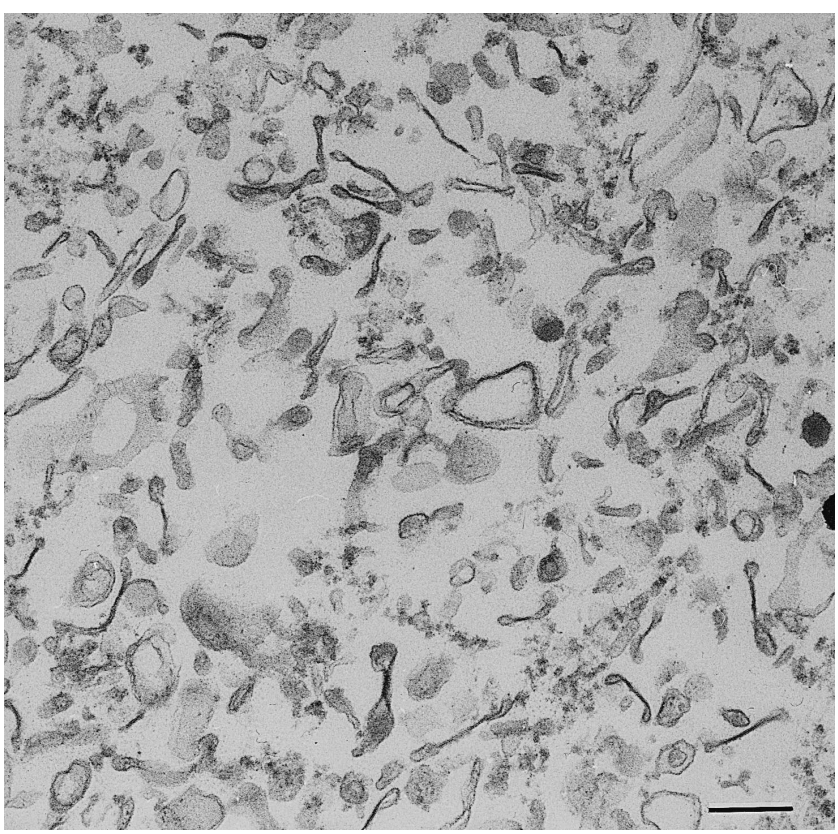


Fig. 2. Electron microscopy of isolated myometrial plasma membranes. Bar represents 1 μM.

gressions varied from 0.92 to 0.97. Scatchard plots were alternatively drawn. These gave K_d-values comparable to those obtained from double reciprocal plots (data not shown). Receptor binding of carbetocin, carbetocin metabolite I and carbetocin metabolite II was measured as displacement of specifically bound [^{3}H]vasopressin or [^{3}H]oxytocin. Concentrations of unlabelled ligand over ranges of 0.8 nM–1.0 μM (carbetocin), 8.0 nM–1.0 μM (carbetocin metabolite I) or 0.8 nM–1.0 μM (carbetocin metabolite II) were incubated in the presence of 1.56 nM [^{3}H]oxytocin or 1.08 nM [^{3}H]vasopressin. The resulting binding of labelled peptide was expressed as a percentage of the binding in the absence of unlabelled peptide and was plotted against the logarithm of the concentration of unlabelled peptide. According to Levitzki (1980), dissociation constants of the unlabelled peptides could subsequently be calculated from the concentrations needed to displace 50% of receptor bound [^{3}H]peptide:

$$K_{d(\text{peptide})} = \frac{EC50_{([^3H]\text{peptide})}}{\frac{[^3H]\text{peptide}}{K_{d([^3H]\text{peptide})}} + 1}.$$

2.6. In vitro contractility of isolated uterine strips

In vitro contractility of isolated uterine strips was measured basically as described previously (Engstrøm et al., 1988b). Two days prior to decapitation the animals received an i.m. injection of 75 μg oestradiol-benzoate. One uterine horn was opened longitudinally and a middle segment measuring 5 mm was mounted in an isometric myograph connected to a Grass force transducer. The resting tension was adjusted to 1.0 g. The strip was placed in an organ bath containing 8 ml of Munsick's buffer, pH 7.4 (Munsick, 1960) and allowed to rest for 30 min. The temperature was kept at 30°C and the solution constantly aerated with 5% CO_2 in O_2. Agonistic effects on the myometrium of oxytocin, carbetocin, carbetocin metabolite I and carbetocin metabolite II were evaluated from individual dose–response curves. Oxytocin was added at concentrations over the range of 0.25×10^{-9}–0.25×10^{-6} M. Carbetocin, carbetocin metabolite I and carbetocin metabolite II were added over concentration ranges of 0.13×10^{-9}–14.0×10^{-6} M. Upon addition of each dose the amplitude of the contractile response was recorded. Three washings with buffer followed each stimulation.

The antagonistic effects of carbetocin, carbetocin metabolite I and carbetocin metabolite II were evaluated by obtaining cumulative dose–response curves for oxytocin in the presence of the peptides at concentrations of 1 and 10 nM (carbetocin) or 10, 20, 50 and 100 nM (carbetocin metabolite I and carbetocin metabolite II). pA_2-values were calculated according to Eggena et al. (1968):

$$pA_2 = -\log \frac{[B]}{\frac{[A_{50B}]}{[A_{50}]} - 1}$$

[B]: molar concentration of inhibitor, $[A_{50}]$: molar concentration of oxytocin revealing 50% of E_{max}, $[A_{50B}]$: molar concentration of oxytocin revealing 50% of E_{max} in the presence of [B].

2.7. Myometrial inositol phosphate production

Rats received 75 μg oestradiol-benzoate two days prior to decapitation. Myometrial tissue pieces (1 × 1 mm) were incubated for 3 h in Krebs–Ringer Buffer, pH 7.4, 37°C containing 10 μCi [^{3}H]myoinositol/ml and 0.1% BSA. Thereafter the tissue was rinsed four times with 5 ml of Krebs–Ringer buffer with 0.1% BSA and was allowed to rest in this buffer for 10 min at 37°C. The tissue was then incubated for 10 min at 37°C in Krebs–Ringer buffer containing 0.1% BSA and 10 mM LiCl in the absence of [^{3}H]myoinositol. Subsequently the tissue was stimulated with 20 μM oxytocin or 20 μM carbetocin for 10 min at 37°C in Krebs–Ringer buffer containing 10 mM LiCl and 0.1% BSA. The reactions were stopped by the addition of an equal volume of ice-cold 10% (V/V) perchloric acid. The samples were placed on ice for 20 min and were intensively whirled every 5 min to liberate inositol phosphates. The resulting suspension was centrifuged $2000 \times g_{av}$ for 5 min, 4°C. Ten mM EDTA was added to the supernatant and inositol phosphates were extracted using a tri-*n*-octylamin/freon (1:1) gradient. Inositol phosphates were subsequently separated from remaining [^{3}H]inositol and quantitated by HPLC on an anion-exchange column (Mono Q) using a combined ammonium formate/pH gradient.

2.8. Data analysis

Myometrial contractions were recorded by means of a Data Acquisition System (Biopac Systems, WPI, USA). A computer program (Fig. P., Biosoft, Cambridge, UK) was used for analysis of binding assays and contractile experiments. One-way Analysis of Variance was used to compare means of multiple groups. Pair-wise comparisons of means were done by means of Student's *t*-test or The Mann–Whitney test. $P < 0.05$ was considered statistically significant.

3. 3. Results

3.1. Carbetocin metabolites

Incubation of carbetocin with kidney homogenate indicated a fast phase and a slow phase degradation pattern

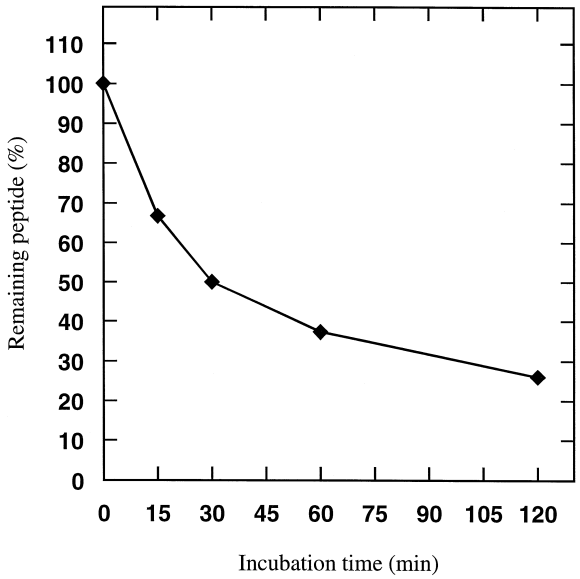


Fig. 3. Incubation of carbetocin with rat kidney homogenate. The amount of non-metabolised carbetocin was determined by preparative HPLC at different times after the start of incubation.

(Fig. 3) which resulted in the formation of two metabolites (carbetocin metabolite I and carbetocin metabolite II). After 100 min of incubation the HPLC chromatogram showed a predominance of metabolite carbetocin metabolite I (Fig. 4). Analytical HPLC of the fractionated compounds revealed purities greater than 97%. Amino acid analysis showed that carbetocin metabolite I was missing Gly–NH_2^9. Carbetocin metabolite II was missing Leu^8 and Gly–NH_2^9. FAB-MS confirmed the results of the amino acid analysis. The structures of the metabolites are shown in Fig. 1.

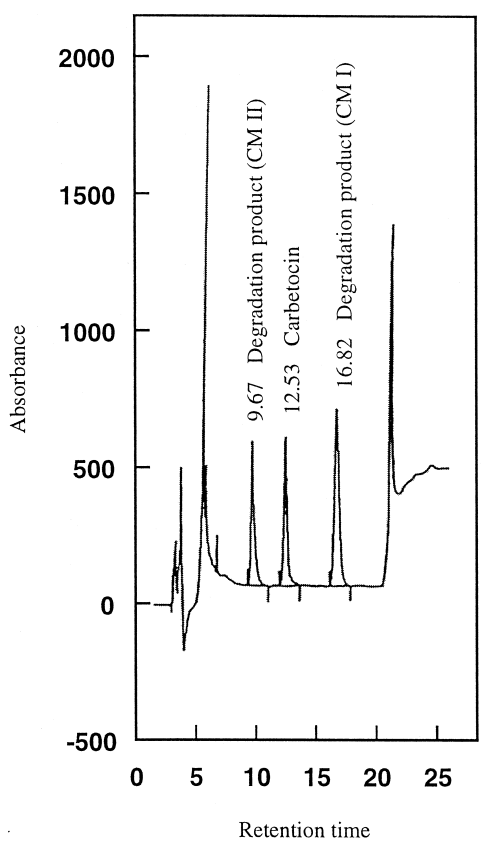


Fig. 4. HPLC chromatogram of carbetocin incubated with rat kidney homogenate for 100 min showing three peaks corresponding to carbetocin and two degradation products (carbetocin metabolite I and carbetocin metabolite II). The retention times are indicated on the top of each peak.

Table 1
Receptor binding of labelled oxytocin and vasopressin

	OT-receptors	V_1-receptors	V_2-receptors
[^{3}H]oxytocin (nM)	1.20 ± 0.45	–	–
[^{3}H] vasopressin (nM)	–	0.49 ± 0.12	0.23 ± 0.12

Dissociation constants (K_d) of the binding of [^{3}H]oxytocin and [^{3}H] vasopressin to myometrial oxytocin receptors (OTR), and to vasopressin V_1- and V_2-receptors.
Values are means $\pm$ S.E.M., $n = 3$, except for [^{3}H]oxytocin to OTR, where $n = 4$.

3.2. Binding assays

Binding of [^{3}H]oxytocin and [^{3}H]vasopressin to myometrial plasma membranes and binding of [^{3}H]vasopressin to kidney membranes followed Michaelis–Menten kinetics. Double reciprocal plots and Scatchard plots revealed only one myometrial binding site for oxytocin and only one binding site for vasopressin to myometrial plasma membranes and kidney membranes respectively. Non-specific binding accounted for approximately 50% of the total binding and consisted primarily of filter binding. K_D values of the receptor binding of [^{3}H]oxytocin and [^{3}H]vasopressin were in the nanomolar range (Table 1). K_d values of carbetocin, carbetocin metabolite I and carbetocin metabolite II were calculated from displacement curves. All three metabolites showed high affinity for the myometrial oxytocin receptor (Table 2). K_d values of the three compounds were not statistically different ($P = 0.252$, One-way ANOVA). Carbetocin, carbetocin metabo-

Table 2
Receptor binding of carbetocin, carbetocin metabolite I and carbetocin metabolite II

	OT-receptors	V_1-receptors	V_2-receptors
Carbetocin (nM)	1.96 ± 0.12	7.24 ± 0.29^a	61.3 ± 14.6
Carbetocin metabolite I (nM)	2.82 ± 0.70	9.89 ± 2.80^a	No affinity
Carbetocin metabolite II (nM)	1.78 ± 0.16	33.7 ± 7.34^b	No affinity

Dissociation constants (K_d) of carbetocin, carbetocin metabolite I and carbetocin metabolite II to oxytocin receptors, and to vasopressin V_1- and V_2-receptors.
Values are means $\pm$ S.E.M., $n = 3$.
Values marked [a] and [b] are statistically different from each other ($P < 0.05$).
Values are calculated from displacement curves.

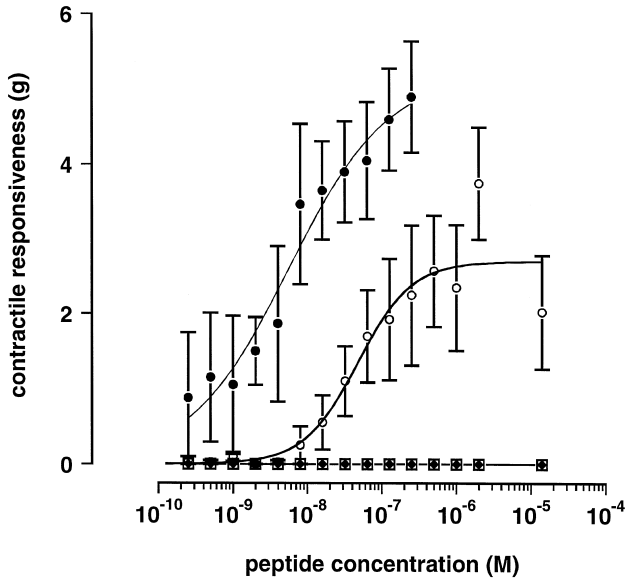


Fig. 5. Agonistic properties of oxytocin (●), carbetocin (○), carbetocin metabolite I (□) and carbetocin metabolite II (♦) on isolated uterine strips. Values are means ± S.E.M., $n = 4$.

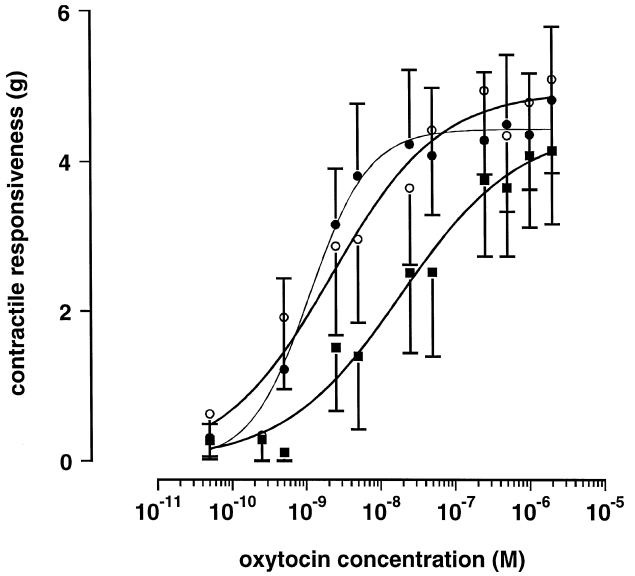


Fig. 6. Antagonistic properties of carbetocin against oxytocin induced myometrial contractility. Dose–response curves for oxytocin were obtained in the absence (●) or presence of 10^{-9} M (○) or 10^{-7} M (■) carbetocin. Values are means ± S.E.M., $n = 4$.

lite I and II were specifically bound to myometrial vasopressin V_1 receptors although carbetocin metabolite II showed a significantly higher K_d than the other two peptides ($P < 0.05$, One-way ANOVA, Table 2). Only carbetocin was bound to renal vasopressin V_2 receptors. K_d was, however, more than a decade higher than those of carbetocin to the oxytocin receptor and the vasopressin V_1 receptor. Carbetocin metabolite I and carbetocin metabolite II did not bind specifically to the vasopressin V_2 receptor.

3.3. *In vitro* contractility

The agonistic effects of oxytocin, carbetocin, carbetocin metabolite I and carbetocin metabolite II on isolated uterine strips are shown in Fig. 5. Maximal contractile responsiveness (E_{max}) of carbetocin was approximately 50% lower than that of oxytocin ($P < 0.05$, Table 3). EC_{50} of carbetocin was a decade higher than that of oxytocin ($P < 0.05$, Table 3). Neither carbetocin metabolite I nor carbetocin metabolite II showed agonistic properties on the myometrium (Fig. 5).

The antagonistic effects of carbetocin, carbetocin metabolite I and carbetocin metabolite II to oxytocin induced contractility of isolated uterine strips are presented in Table 4. As seen the order of rank of antagonism was carbetocin > carbetocin metabolite II > carbetocin metabolite I when evaluated from pA_2 values ($P < 0.05$ between

Table 3
Agonistic effects of carbetocin, carbetocin metabolite I and carbetocin metabolite II as compared to oxytocin

	Oxytocin	Carbetocin	Carbetocin metabolite I	Carbetocin metabolite II
E_{max} (g)	5.22 ± 0.26	2.70 ± 0.12[a]	no effect	no effect
EC_{50} (nM)	5.62 ± 1.22	48.0 ± 8.20[a]	–	–

Agonistic effects of carbetocin, carbetocin metabolite I and carbetocin metabolite II on isolated uterine strips.
Values are means ± S.E.M., $n = 4$.
[a] Indicates statistical difference from oxytocin ($P < 0.05$).

Table 4
Antagonistic properties of carbetocin, carbetocin metabolite I and carbetocin metabolite II towards oxytocin

	Carbetocin	Carbetocin metabolite I	Carbetocin metabolite II
pA_2	8.21	7.81	8.01
delog pA_2 (nM)[a]	6.23 ± 1.85	30.8 ± 6.58	16.3 ± 3.47

Antagonistic activity of carbetocin metabolite I and carbetocin metabolite II to oxytocin induced contractility of isolated uterine strips.
Values are means ± S.E.M., $n = 4$ (carbetocin), $n = 8$ (carbetocin metabolite II) and 16 (carbetocin metabolite I).
[a] $P < 0.05$ between all groups.

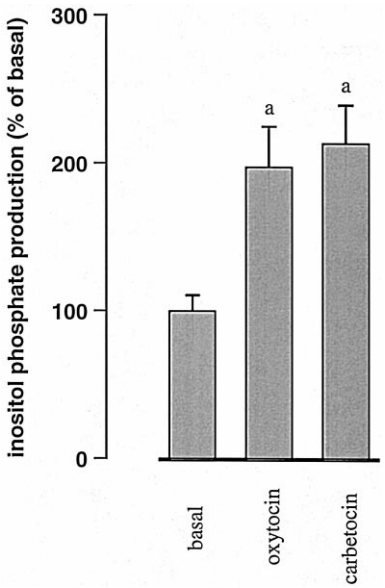


Fig. 7. Inositol phosphate production following stimulation of myometrial tissue with oxytocin and carbetocin. Values significantly different from basal inositol phosphate production are indicated by (a). ($P < 0.05$). Values are means ± S.E.M., $n = 3$.

all groups). The antagonistic effect of carbetocin to oxytocin induced contractility is shown graphically in Fig. 6.

3.4. Inositol phosphate production

Both carbetocin and oxytocin caused a statistically significant increase in the formation of inositol phosphates as compared to baseline values ($P < 0.05$). There was no significant difference between the effect of oxytocin and carbetocin. Means ± S.E.M. were 100 ± 11% for controls, 197 ± 27% for oxytocin and 213 ± 26% for carbetocin (Fig. 7).

4. Discussion

Due to its increased duration of action in vivo (Hunter et al., 1992) and in vitro (Atke and Vilhardt, 1987) carbetocin is intended to be used in human subjects for the control of uterine atony and excessive bleeding after delivery by caesarean section and has already been registered as a drug in the UK and Canada (E. de Witt, Ferring Pharmaceuticals, personal communication). The present study shows that carbetocin in an in vitro model using kidney homogenates is degraded enzymatically by C-terminal cleavage leaving carbetocin metabolite I and by further cleavage of carbetocin metabolite I leaving carbetocin metabolite II. The precise species of enzymes involved in the degradation of carbetocin is not known, except that they are carboxypeptidases, possibly of chymotryptic nature, since Vilhardt et al. (1997) have shown that incubation of carbetocin with that enzyme leads to the formation of carbetocin metabolite I. Oxytocin itself may be a substrate for the enzymes involved in the degradation of carbetocin. However, oxytocin is also degraded in the N-terminal part of the molecule by aminopeptidases (Mitchell et al., 1997) and this fact may partly explain the shorter half-life of oxytocin.

The data show that both carbetocin, carbetocin metabolite I and carbetocin metabolite II display binding affinity to myometrial oxytocin receptors in the same order of magnitude as is known for oxytocin (Atke and Vilhardt, 1987). All three test substances also bound to the vasopressin V_1 receptor, albeit with much lower affinities, carbetocin metabolite II being the weakest ligand. The affinity of carbetocin to vasopressin V_2 receptors was very low and carbetocin metabolite I and carbetocin metabolite II had no affinity at all for these receptors. These findings for oxytocin analogous are not unexpected, since oxytocin itself has low vasopressor (vasopressin V_1 receptors) and low antidiuretic (vasopressin V_2 receptors) activities (Berde and Boissonnas, 1968).

In agreement with earlier observations (Atke and Vilhardt, 1987) the contractile effect of carbetocin on isolated uterine strips was weaker than that of oxytocin both as to E_{max} and EC_{50}. The generation of myometrial inositol phosphates following stimulation confirmed that the two peptides activate the same intracellular second messenger system to approximately the same extent. Nevertheless, the reduced maximal contractile response to carbetocin indicates weaker activation of the oxytocin receptor to this peptide compared to oxytocin in spite of equal binding affinities for the two ligands. Increased EC_{50} values from uterine strips exposed to carbetocin suggest that the coupling between receptor occupancy and contractile response is also weaker than that for oxytocin. Both carbetocin, carbetocin metabolite I and carbetocin metabolite II displayed antagonistic properties against the contractile effect of oxytocin, with carbetocin being the strongest inhibitor and carbetocin metabolite II being stronger than carbetocin metabolite I. Since carbetocin displays high affinity to the oxytocin receptor it was not unexpected, that this compound should have antagonistic properties against oxytocin. In this context the question could be raised, whether carbetocin through a potential blockade of endogenous oxytocin at its receptor site might be an inexpedient compound in the control of uterine atony. Although a weaker agonist than oxytocin, carbetocin has, however, been reported to effectively induce contractions in the human uterus (Hunter et al., 1992) and it must be kept in mind that the effect of endogenous oxytocin appears inadequate when uterine atony is present suggesting low plasma concentrations of the hormone. Furthermore, the long half-life and few side effects of carbetocin make it a useful uterotonic agent. Desglycinamide-oxytocin is devoid of agonistic action (Barth et al., 1967) and therefore, as shown in the present paper, it was expected that the same held true for carbetocin metabolite I and carbetocin metabolite II.

That carbetocin metabolite I and carbetocin metabolite II possessed rather potent antagonistic properties is in agreement with similar findings for C-terminal shortened analogous of oxytocin (Melin and Trojnar, 1988).

5. Conclusion

In conclusion we have shown, that carbetocin upon incubation with a kidney extract is degraded at the C-terminal leaving metabolites desGlyNH_2-carbetocin and desLeuGlyNH_2-carbetocin, which both show affinity to the myometrial oxytocin receptor with almost identical binding affinities as the genuine ligand itself. Both carbetocin metabolite I and carbetocin metabolite II showed potent antagonistic properties against oxytocin and might therefore be expected to be potential blockers of the agonistic effect of carbetocin. However, clinical experiments (Hunter et al., 1992) demonstrate a marked uterotonic effect of carbetocin, indicating that carbetocin metabolite I and carbetocin metabolite II may be more short-lived than the metabolically stable carbetocin with an in vivo half-life of 85–100 min (Cort et al., 1981) or may be excreted at a higher rate.

Acknowledgements

Professor Morten Møller is thanked for doing the electron microscopy of myometrial plasma membranes. Lone Jørgensen and Jakob Utzon-Frank are thanked for their skilful technical assistance. The Danish Biotechnology Programme, Brødrene Hartmanns Fond, Ferring Pharmaceuticals, Novo Nordisk Foundation, 'Ove Villiam Buhl Olesen og ægtefælle Edith Buhl Olesens Mindelegat', The Beckett Foundation and The Danish Medical Association Research Fund supported this study.

References

Atke, A., Vilhardt, H., 1987. Uterotonic activity and myometrial receptor affinity of 1-deamino-1-carba-2-tyrosine(*O*-methyl)-oxytocin. Acta Endocrinol. 115, 155–160.

Barth, T., Pliska, V., Rychlik, I., 1967. Chymotrypsic and trypsic cleavage of oxytocin and vasopressin. Collection Czechoslov Chem. Commun. 32, 1058–1063.

Barth, T., Hutter, H.J., Pliska, V., Sorm, F., 1969. Inactivation of deamino-oxytocin, deamino-carba-oxytocin, (D-lys)-, and (D-arg)vasopressins by kidney cell particles. Experientia 25, 646–647.

Barth, T., Jost, K., Rychlik, I., 1975. Milk-ejecting and uterotonic activities of oxytocin analogues in rats. Endocrinol. Exp. 9, 35–42.

Berde, B., Biossonnas, R.A., 1968. Basic pharmacological properties of synthetic analogues and homologues of the neurohypophysial hormones. In: Berde, B. (Ed.) Handbook of Experimental Pharmacology. Springer Verlag, New York, pp. 802–870.

Cort, N., Einarsson, S., Schams, D., Vilhardt, H., 1981. Blood concentrations of oxytocin equivalents after single injections of deamino-1-monocarba-[2-*O*-methyltyrosine]-oxytocin in lactating sows. Am. J. Vet. Res. 42, 1804–1806.

Eggena, P., Schwartz, I.L., Walter, R., 1968. A sensitive hydroosmotic toad bladder assay. Affinity and intrinsic activity of neurohypophyseal peptides. J. Gen. Physiol. 52, 465–481.

Engstrøm, T., Atke, A., Vilhardt, H., 1988a. Oxytocin receptors and contractile response of the myometrium after long term infusion of prostaglandin F2 alpha, indomethacin, oxytocin and an oxytocin antagonist in rats. Regul. Pept. 20, 65–72.

Engstrøm, T., Atke, A., Vilhardt, H., 1988b. Receptor-binding characteristics and contractile responsiveness of the myometrium following prolonged infusion of bradykinin and oxytocin in rats. J. Endocrinol. 118, 81–85.

Engstrøm, T., Bratholm, P., Vilhardt, H., Christensen, N.J., 1998. β_2-Desensitization in non-pregnant estrogen primed rat myometrium involves modulation of oxytocin receptor gene expression. J. Mol. Endocrinol. 20, 261–270.

Fitzpatrick, D.F., Welch, L.T., Landon, E.J., 1969. Characterization of plasma membrane proteins in mammalian kidney: II. The binding of organic mercurials to rat kidney plasma membrane proteins in vivo. J. Biol. Chem. 244, 3570–3574.

Forsling, M.L., Martin, M.J., Sturdy, J.C., Burton, A.M., 1973. Observations on the release and clearance of neurophysin and the neurohypophysial hormones in the rat. J. Endocrinol. 57, 307–315.

Gazis, D., 1978. Plasma half-lives of vasopressin and oxytocin analogs after iv injection in rats. Proc. Soc. Exp. Biol. Med. 158, 663–665.

Gazis, D., Sawyer, W.H., 1978. Elimination of infused arginine–vasopressin and its long-acting deaminated analogue in rats. J. Endocrinol. 78, 179–186.

Hunter, D.J., Schulz, P., Wassenaar, W., 1992. Effect of carbetocin, a long-acting oxytocin analog on the postpartum uterus. Clin. Pharmacol. Ther. 52, 60–67.

Levitzki, A., 1980. Quantitative aspects of ligand binding to receptors. In: Schulster, D., Levitzki, A. (Eds.), Cellular Receptors. Wiley, New York, pp. 9–28.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Melin, P., Trojnar, J., 1988. Antagonism of oxytocin analogues on the human uterus and in animal models. In: Yoshida, S., Share, L. (Eds.), Recent Progress in Posterior Pituitary Hormone Research. Elsevier Science, Amsterdam, pp. 161–165.

Mitchell, B.F., Fang, X., Wong, S., 1997. Metabolism of oxytocin in rat uterus and placenta in late gestation. Biol. Reprod. 57, 807–812.

Munsick, R.A., 1960. Effect of magnesium ion on the response of the rat uterus to neurohypophysial hormones and analogues. Endocrinology 66, 451–457.

Suska-Brzezinska, E., Fruhaufova, I., Barth, T. et al., 1972. Inactivation of deamino-carba analogues of oxytocin in the rat uterus, liver and kidney. Collection Czechoslov Chem. Commun. 90, 2793.

Vilhardt, H., Atke, A., Barthova, J., Ubik, K., Barth, T., 1997. Interaction of chymotrypsin with carbetocin ([1-deamino-1-monocarba-2-*O*-methyltyrosine]-oxytocin). Pharmacol. Toxicol. 81, 147–150.