Effect of Changing the COOH-Terminal Amide Group Present in the Hydrophilic Cluster of Oxytocin to Dimethylamide[1]

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Oxytocinoic acid dimethylamide was synthesized by stepwise solution techniques as part of an ongoing evaluation of the effects on the biological activity of oxytocin caused by individually changing the groups that comprise the hydrophilic surface of the hormone to more hydrophobic and more bulky groups. The analogue exhibited approximately 3% of the potency of oxytocin in the in vitro uterotonic assay. In addition, it possesses potencies of <0.07, <0.01, and 0.096 unit/mg in the avian vasodepressor, rat pressor, and rat antidiuretic assays, respectively. In the in vitro uterotonic assay, oxytocinoic acid dimethylamide showed a reduced affinity for the oxytocin receptor, a nonparallel dose–response relationship, and most importantly a reduced intrinsic activity as compared to oxytocin. The results suggest that the replacement of the protons of the primary carboxyamide of Gly^9–NH_2 of oxytocin by methyl groups displaces the active elements from the orientation for obtaining maximal intrinsic activity in the isolated rat uterus.

One surface of the 20-membered antiparallel β-pleated sheet structure of oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2, in its proposed biologically active conformation at the uterine receptor, is characterized by a cluster of hydrophilic side chains (Figure 1).[2,3] The cluster is comprised of the hydroxyl group of the tyrosine and the carboxamide groups of asparagine, glutamine, and glycine residues. The asparagine carboxamide and tyrosine hydroxyl groups appear to be the active elements initiating the uterotonic response.[3] Dialkylation of the N^5 of the 4-position glutaminyl carboxamide yields analogues which possess a reduced ability to stimulate maximally uterine contractions in vitro.[4] This result suggests that the proximity of the glutaminyl side-chain carboxamide allows the N^5,N^5-dialkyl groups to interfere sterically with the orientation of one or both active elements, Tyr^2 and Asn^5. On the other hand, dimethylation directly on the asparaginyl N^4-carboxamide active element apparently does not cause a detrimental reorientation of the active elements for interaction with the uterotonic receptor, since [5-(N^4,N^4-dimethylasparagine)]oxytocin has the same intrinsic activity[5] as oxytocin.[6] Deaminooxytocinoic acid dimethylamide has been synthesized,[7] but no dose–response studies in the in vitro uterotonic assay were reported, so that the effect of dimethylation of the carboxamide in position 9 on the intrinsic activity in the uterotonic assay in vitro is not known. In order to study this question we have synthesized oxytocinoic acid dimethylamide and compared some of its pharmacological properties to those of oxytocin.

The protected nonapeptide intermediate Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$ was assembled in a stepwise manner beginning from Z-Pro-Leu-Gly-$N(CH_3)_2$ using *p*-nitrophenyl[8] or *N*-hydroxysuccinimide[9] active esters. Z-Pro-Leu-Gly-$N(CH_3)_2$ was prepared by reaction of dimethylamine with the mixed anhydride of Z-Pro-Leu-Gly-OH.[10] The protecting groups were simultaneously removed from the nonapeptide by treatment with sodium in anhydrous liquid ammonia,[11] and conversion to the cyclic disulfide was achieved by oxidation with 1,2-diiodoethane.[12] The analogue was purified by gel filtration on Sephadex G-15 in 50% HOAc, followed by partition chromatography[13] on Sephadex G-25 and finally by gel filtration on Sephadex G-15 in 0.2 M HOAc.

The dose–response behavior in the in vitro rat uterotonic assay[14] of the analogue compared to oxytocin is shown in Figure 2. A higher concentration of oxytocinoic acid dimethylamide than oxytocin is required to evoke a response. More importantly, oxytocinoic acid dimethylamide has a reduced ability to stimulate maximally uterine contraction ($p < 0.001$). When the dose–response study was performed in media with reduced amounts of calcium, a technique which reveals partial agonistic behavior,[15] no further decrease in the ability of the analogue to evoke maximum contraction of the uterus was seen. The dose–response relationship of the analogue is also characterized by a decrease in slope in the 30–60% response range as compared to oxytocin. As a result of this departure from parallelism with the standard, a rat uterotonic potency could not be reliably estimated by the four-point assay design.[16] From the ED_{50} using standard conditions (Figure 2A) it is apparent that the analogue retains approximately 3% of the potency of oxytocin. In addition, oxytocinoic acid dimethylamide was found to possess potencies of

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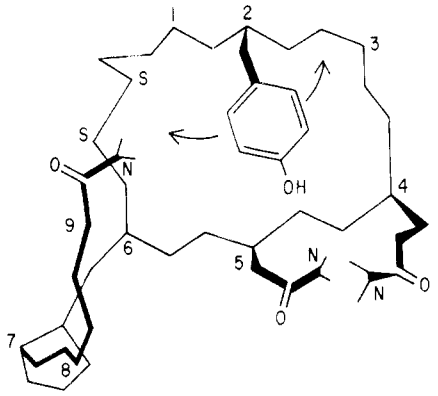

Figure 1. Schematic representation of the conformation of oxytocin thought to be optimal for interaction of the hormone with the uterine smooth-muscle receptor. Numbers indicate residue positions. Featured are the COOH-terminal carboxamide and the side chains comprising the hydrophilic cluster which is above the plane of the molecule formed by the 20-membered disulfide-containing ring.

<0.07, <0.01, and 0.096 ± 0.004 unit/mg in the avian vasodepressor,[17] rat pressor,[18] and rat antidiuretic[19] assays, respectively.

The lack of parallelism between the dose–response relationships of oxytocin and oxytocinoic acid dimethylamide and the reduced ability of the analogue to stimulate maximally uterine contraction suggest that the replacement of the protons of the primary carboxamide of Gly^9-NH_2 by more hydrophobic, bulky methyl groups interferes with the orientation of the active elements in their interaction with the uterotonic receptor. The substitution also reduces the ability of the analogue to bind to the receptor.

Experimental Section

Melting points were determined in open capillary tubes, on a Buchi SMP-20 apparatus, and are reported uncorrected. Thin-layer chromatograms were done on silica gel plates with sample loads of 10 to 25 μg. The following solvent systems were used and allowed to ascend for 12–15 cm: A, 1-butanol–acetic acid–water (4:1:1, v/v); B, 1-butanol–pyridine–acetic acid–water (15:10:3:6); C, 1-butanol–acetic acid–water (4:1:5, upper phase). The compounds were visualized by reaction with ninhydrin or chlorine followed by tolidine[20] solution. Optical rotations were measured in a Carl Zeiss precision polarimeter (0.005°). Elemental analyses were done by the Microanalytical Laboratory at the National Hellenic Research Foundation and data fall within ±0.4% theory. Amino acid analyses were performed on aliquots of samples after hydrolysis in deaerated ampules with 6 M HCl (110 °C, 22 h), on a Durrum D-500 analyzer.[21] Cysteine was determined as cysteic acid.[22]

Z-Pro-Leu-Gly-$N(CH_3)_2$ (1). To a chilled solution of Z-Pro-Leu-Gly-OH[10] (4.19 g, 10 mmol) and triethylamine (1.01 g, 10 mmol) in THF (25 mL) was added ethyl chloroformate (1.1 g, 10 mmol). After 2 min, a solution of dimethylamine hydrochloride (2.43 g, 30 mmol) and triethylamine (3.03 g, 30 mmol)

Table I. Physical Properties of Intermediates in the Synthesis of Oxytocinoic Acid Dimethylamide

no.	compd	coupling agent	% yield	mp, °C	$[\alpha]^{24}_D$, deg (c 1, DMF)	formula	analysis	C	H	N	TLC R_f (A)	R_f (B)	R_f (C)
3	Boc-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$	Boc-Asn-ONp	65	153–155	−80.1	$C_{34}H_{53}N_7O_8S$	calcd:	56.73	7.42	13.62	0.73	0.88	0.67
							found:	56.52	7.35	13.50			
4	Boc-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$	Boc-Gln-ONp	74	197–199	−48.5	$C_{39}H_{61}N_9O_{10}S$	calcd:	55.24	7.25	14.86	0.69	0.84	0.63
							found:	55.06	7.09	14.64			
5	Boc-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$	Boc-Ile-OSu	83	205–207	−60.1	$C_{45}H_{72}N_{10}O_{11}S$	calcd:	56.23	7.55	14.57	0.71	0.87	0.64
							found:	56.55	7.44	14.70			
6	Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$	Boc-Tyr(Bzl)-OSu	82	216–218	−44.5	$C_{61}H_{87}N_{11}O_{13}S$	calcd:	60.33	7.22	12.69	0.76	0.92	0.68
							found:	60.04	7.15	12.44			
7	Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-$N(CH_3)_2$	Z-Cys(Bzl)-OSu	80	243–246	−65.2	$C_{74}H_{96}N_{12}O_{14}S_2$	calcd:	61.65	6.71	11.66	0.79	0.96	0.70
							found:	61.40	6.62	11.55			

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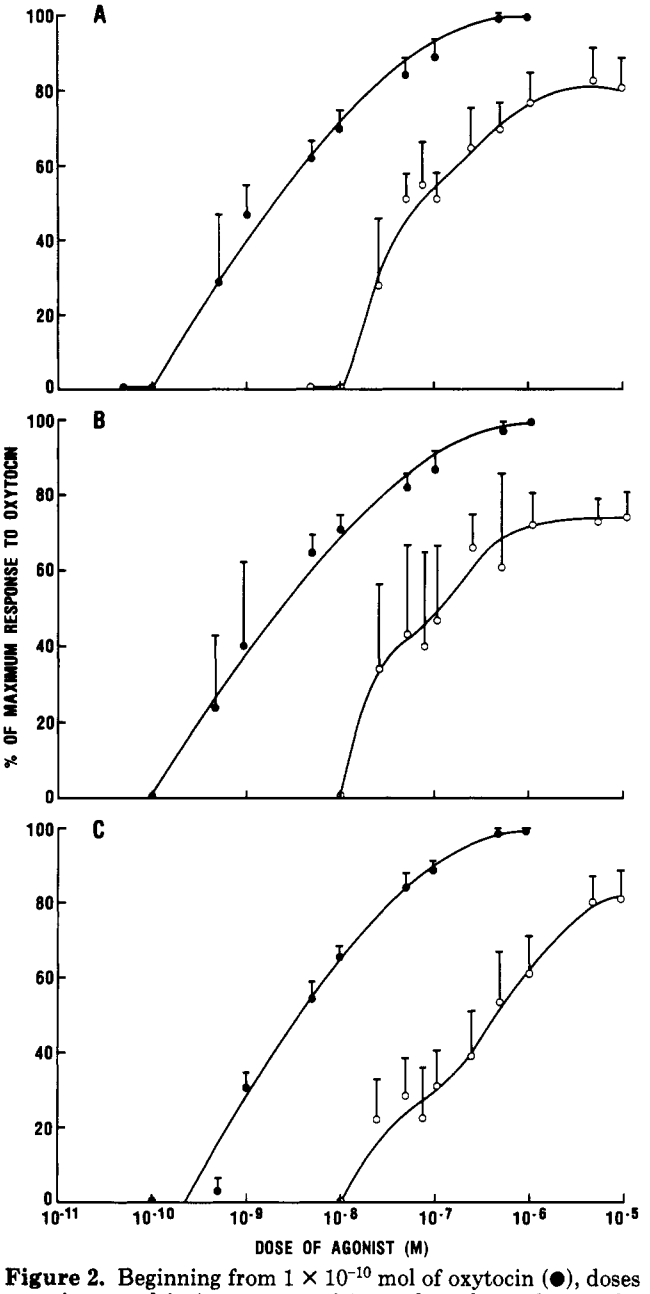

Figure 2. Beginning from 1×10^{-10} mol of oxytocin (●), doses were increased in increments of 0.7 and 0.3 $\log_{10}$ alternately. Beginning from 1×10^{-8} mol of oxytocinoic acid dimethylamide (○), doses were increased in increments of 0.7 and 0.3 $\log_{10}$ alternately; in the dose range producing responses nonparallel to those of oxytocin, intermediate doses of 2.5×10^{-8}, 7.5×10^{-8}, and 2.5×10^{-7} mol were included in order to obtain a clearer picture of the response pattern. Each point represented with its standard deviation represents an average of at least eight experiments on six uterine horns. Panel A is using standard conditions (i.e., 0.5 mM Ca^{2+}); panels B and C are results with 0.3 and 0.15 mM Ca^{2+} in the bathing fluid, respectively.

in 10 mL of THF–H_2O (7:3) was added. After 1 h at room temperature, the solvent was evaporated under vacuum and the residue was taken up in EtOAc. The EtOAc solution was washed with 5% $NaHCO_3$ and H_2O and dried over Na_2SO_4. The solvent was removed by evaporation and the product was crystallized twice from ethyl acetate–petroleum ether: yield 3.43 g (77%); mp 123–124 °C; $[\alpha]^{24}_D$ –63° (c 1, DMF); TLC R_f (A) 0.78, R_f (B) 0.89, R_f (C) 0.73. Anal. Calcd for $C_{23}H_{34}N_4O_5$: C, 61.86; H, 7.67; N, 12.55. Found: C, 61.60; H, 7.50; N, 12.38.

Boc-Cys(Bzl)-Pro-Leu-Gly-N(CH_3)$_2$ (2). A solution of 1 (2.23 g, 5 mmol) in EtOH (150 mL) was subjected to hydrogenation over Pd introduced initially as PdO, 260 mg. After 8 h the solution was filtered and the solvent removed in vacuo to afford an oily product (1.52 g, 98%) that was homogeneous on TLC: R_f (A) 0.62, R_f (B) 0.74, R_f (C) 0.59. Two batches of H-Pro-Leu-Gly-N(CH_3)$_2$ (3.12 g, 10 mmol) were combined, dissolved in DMF (20 mL), and allowed to react with Boc-Cys(Bzl)-OSu[9] (4.49 g, 11 mmol). After 2 days at room temperature, the solvent was removed under reduced pressure and the remaining oily residue was solidified by addition of ethyl acetate–ether (4:6). The product was isolated by filtration, washed several times each with 5% $NaHCO_3$ and water, and dried over P_2O_5. The product was washed with ether–ethyl acetate (6:4) under reflux and finally was precipitated from ethyl acetate–petroleum ether (1:3, v/v): yield 5 g (82%); mp 92–94 °C; $[\alpha]^{24}_D$ –62° (c 1, DMF); TLC R_f (A) 0.82, R_f (B) 0.91, R_f (C) 0.78. Anal. Calcd for $C_{30}H_{47}N_5O_6S$: C, 59.48; H, 7.82; N, 11.56. Found: C, 59.65; H, 7.96; N, 11.70.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-N(CH_3)$_2$ (7). Intermediates 3–7 were synthesized using a stepwise scheme. In brief, the Boc protecting group was removed by treatment with CF_3CO_2H. After precipitation with Et_2O and drying, the trifluoroacetate salt was dissolved in DMF, neutralized with *N*-methylmorpholine, and allowed to react with the N^{α}-protected amino acid active ester of the next residue. Progress of the coupling reaction was followed by TLC and the ninhydrin test. Workup consisted of evaporating the DMF, trituration with Et_2O or EtOAc, washing the precipitate with 5% $NaHCO_3$ and H_2O, and finally precipitation from DMF with Et_2O. Yields and other physical properties of the intermediates are given in Table I. Amino acid ratios of 7 were as follows: Asp, 1.00; Glu, 1.02; Pro, 1.00; Gly, 1.00; Ile, 0.96; Leu, 1.02; Tyr, 0.88; Cys(Bzl), 1.83; NH_3, 2.20.

Oxytocinoic Acid Dimethylamide (8). A sample of 7 (138 mg, 95.7 μmol) was deprotected by sodium in liquid ammonia.[11] Following removal of the ammonia by a N_2 stream, the residue was dissolved in deaerated methanol–water (1:1, 200 mL), and the disulfide was formed by oxidation with 1 equiv of diiodoethane.[12] The product was partially purified by gel filtration on a column of Sephadex G-15 (fine, 117 × 2.5 cm) using 50% acetic acid[23] (elution volume ~148 mL): yield 81 mg. Further purification of the product was attained by partition chromatography[13] on a column of Sephadex G-25 (block polymerizate, 100–200 mesh, 68 × 2.8 cm) using the system 1-butanol–1.5% pyridine in aqueous 3.5% acetic acid (1:1, v/v), R_f 0.35, and by gel filtration on a column of Sephadex G-15 (fine, 90.5 × 2.5 cm) using 0.2 M acetic acid (elution volume ~308 mL): yield following lyphilization 40.8 mg (39%); $[\alpha]^{23}_D$ –5° (c 0.5, glacial acetic acid); TLC R_f (A) 0.21, R_f (B) 0.55. Amino acid analysis: Cys(O_3H), 2.09; Asp, 1.03; Glu, 1.05; Pro, 1.02; Gly, 1.00; Ile, 0.95; Leu, 1.02; Tyr, 0.93; NH_3, 1.97.

Biological Assays. Rat uterotonic assays were performed on isolated horns from virgin rats in natural estrus according to the method of Holton, as modified by Munsick, with the use of Mg^{2+}-free van Dyke–Hastings solution as bathing fluid.[14] For dose–response determinations compared to synthetic oxytocin on the rat uterus in vitro, the conditions were those of above or the Ca^{2+} ion concentration of the bath was varied from the standard 0.5 mM to lower concentrations (0.3 and 0.15 mM). The individual injection method was used with doses being increased until a maximal response was reached. Details of the experimental procedure have been previously published.[24] Avian vasodepressor assays were performed on conscious White Leghorn roosters by the method of Coon as described with modifications.[17] Antidiuretic[19] and pressor[18] assays were performed on anesthetized Sprague–Dawley male rats. The four-point assay design of Schild[16] was attempted whenever measurable activity was detected. USP posterior pituitary reference standard was used as control for the assays.

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