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Effects of Structural Changes on the Natriuretic Activity of Oxytocin Analogs in Conscious Rats[1] (40842)

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Numerous investigators have demonstrated that oxytocin can increase sodium excretion in rats (1–3). Studies on synthetic analogs of oxytocin have shown that natriuretic actions are not correlated with antidiuretic and vasopressor activities of these peptides (4–8). The increase in electrolyte and water excretion appears to be mediated by "natriuretic receptors" which have different structural requirements for interacting with peptides than do "antidiuretic receptors" or "vasopressor receptors." If this is so, it should be possible to synthesize more potent and/or selective natriuretic analogs of the neurohypophysial hormones.

Recently several analogs of oxytocin were synthesized with high oxytocic-antidiuretic selectivity (defined as the ratio of the oxytocic/antidiuretic potencies) relative to that of oxytocin (9). These analogs had glycine substituted for proline in the 7 position. Injection of one of these analogs, [7-glycine]-oxytocin, into conscious, fluid-loaded rats not only increased water excretion but sodium and potassium excretion as well (10). As these analogs possessed negligible antidiuretic and vasopressor activities their natriuretic activity relative to that of oxytocin was of great interest.

These analogs were tested for their ability to increase sodium excretion when injected into conscious, saline-loaded rats. The oxytocin analogs tested which had glycine substituted for proline in the 7 position were: [7-glycine]-oxytocin (9,11), [1-(2-hydroxy-3-mercaptopropanoic acid), 4-threonine, 7-glycine]-oxytocin (9) or "1-hydroxy[4-threonine, 7-glycine]-oxytocin," and [7-glycine]-arginine-vasotocin (synthesis unpublished).

Three additional analogs of oxytocin were also tested: [2-phenylalanine]-oxytocin (12), [8-proline]-oxytocin (synthesis unpublished), and des-9-glycinamide-oxytocin (13). We chose the additional analogs because they also had low antidiuretic and vasopressor activities and [2-phenylalanine]-oxytocin has been reported as having diuretic activity (14). These are oxytocin analogs with single amino acid substitutions or deletions. This should allow some speculations concerning the molecular structures responsible for natriuretic activity.

Methods. Female Sherman rats with an initial body weight of approximately 150 g were adapted to the experimental conditions of gastric intubation and the sc introduction of a 23-gauge hypodermic needle prior to the actual experiments to reduce the effects of struggling on the subsequent diuresis. The rats accepted intubation with a minimal amount of protest after a training period of about 3 weeks. Prior to the experiments, the rats were housed in stainless-steel metabolism cages, two per cage, and maintained on standard rat pellet chow and allowed free access to water. The rats had reached body weights of approximately 200 g when the experiments were started.

On the evening before an experiment, food was removed from the cages of the rats to be tested, but water was allowed overnight. The rats were fasted to ensure an empty stomach so the fluid load could be well absorbed. On the morning of an experiment, the rats were weighed to the nearest 0.1 g and received a 2% (v/w) fluid

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load via a stomach tube. The fluid load contained 0.9% NaCl and was administered at room temperature. The rats were then injected sc with the dose of peptide to be tested in a volume of 1 ml/kg. The solvent used for all stock solutions was a solution of 0.05 *M* acetic acid with 0.5% chlorobutanol added as a preservative. The stock solutions were diluted 1:10 with 0.9% NaCl before being injected into the rats. Control injections were of the acetic acid–chlorobutanol solution diluted 1:10 with 0.9% NaCl. Urine initially present in the bladder was expelled by gently pulling the tail or applying suprapubic pressure. The rats were then put into metabolism cages, one per cage, and the urine was collected. At the end of 4 hr, the rats were removed from the metabolism cages and urine remaining in the bladder was expelled by gently pulling the tail or applying suprapubic pressure. The rats were allowed food and water *ad libitum* for 3 days before being tested again. Analogs were tested in three groups of "trained" rats (n = 6, 8, 11). Varying doses of each analog including solvent injections were given using a separate block design for each analog.

Urine volume was determined gravimetrically and urinary sodium and potassium concentrations were determined with an Instrumentation Laboratories flame photometer using an internal lithium standard. Net responses were calculated as differences between experimental and control responses for each rat. Dose–response curves were graphed as net sodium excreted in milliequivalents per kilogram at 2 hr versus the log dose of analog in micrograms per kilogram. The intercepts (responses obtained at a dose of 1 μg/kg; log dose = 0) and slopes of dose–response curves for each rat were calculated using linear regression analysis. Calculation of intercepts allows statistical comparisons of dose–response regressions for significant shifts along the log-dose axis. Differences between treatment means within each group of rats were analyzed statistically using a one-way analysis of variance with linear contrasts. Differences between the slopes and intercepts of the dose–response curves to oxytocin in the three groups of rats were also analyzed using a one-way analysis of variance with linear contrasts.

Des-9-glycinamide-oxytocin was obtained from Dr. R. Walter (University of Illinois at the Medical Center, Chicago, Ill.). [2-Phenylalanine]-oxytocin was prepared by Dr. M. Bodanszky and Dr. V. du Vigneaud in 1959 (9) and was stored as a refrigerated aqueous solution in a sealed ampule. All other peptides used were prepared in Dr. Manning's laboratory at the Medical College of Ohio. Table I shows the amino acid sequence of oxytocin and lists the analogs tested.

Results. Figure 1 shows the dose–response curves for 2 hr of sodium excretion for oxytocin, [7-glycine]-oxytocin, and [7-glycine]-arginine-vasotocin in Group I rats. These dose–response curves were qualitatively and quantitatively similar to those obtained at 4 hr indicating that the increase in sodium excretion occurs principally during the first 2 hr. The natriuretic responses to 10 and 50 μg/kg of [7-glycine]-oxytocin were similar to those of 0.5 and 2.0 μg/kg oxytocin. Thus [7-glycine]-oxytocin produced natriuretic responses comparable to those of oxytocin at doses which were 20–25 times greater. [7-Glycine]-arginine-vasotocin was found to have natriuretic activity similar to that of [7-glycine]-oxytocin. Table II shows the slopes and intercepts for these dose–response curves obtained from linear regression analysis. The slopes did not differ significantly. However, the intercepts for [7-glycine]-oxytocin and [7-glycine]-arginine-vasotocin were significantly less than that for oxytocin. The intercepts for the two 7-glycine-substituted analogs did not differ significantly from each other.

Oxytocin was given to all three groups of rats. A dose of 0.10 μg/kg oxytocin consistently produced significant natriuretic responses in all groups. Oxytocin produced a dose-related increase in sodium excretion which was consistent in the different groups of rats tested. The slopes and intercepts of these dose–response curves did not differ significantly.

The natriuretic activity of the analogs tested is shown in Table III. The natriuretic activity of each analog relative to oxytocin

TABLE I. AMINO ACID SEQUENCE OF OXYTOCIN

Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly(NH_2)
1	2	3	4	5	6	7	8	9

Oxytocin Analogs Assayed for Natriuretic Activity	
I. Analogs Having a 7-Glycine Substitution	
A. [7-Glycine]-oxytocin	[Gly^7]-OT
B. [7-Glycine]-arginine-vasotocin	[Gly^7]-AVT
C. [1-(L-2-Hydroxy-3-mercaptopropanoic acid), 4-threonine, 7-glycine]-oxytocin	OH[Thr^4, Gly^7]-OT
II. Analogs Having Other Single Amino Acid Substitutions and a Deletion	
A. [2-Phenylalanine]-oxytocin	[Phe^2]-OT
B. [8-Proline]-oxytocin	[Pro^8]-OT
C. Des-9-glycinamide-oxytocin	Des-9-Gly-NH_2-OT

within each group was determined by dividing the dose of oxytocin by the dose of analog necessary to increase sodium excretion by 1 meq/kg at 2 hr and multiplying by 100. The 7-glycine substituted analogs and [8-proline]-oxytocin all possessed similar natriuretic activities: "1-hydroxy-[4-threonine, 7-glycine]-oxytocin," 1.6%; [7-glycine]-oxytocin, 3.2%; [7-glycine]-arginine-vasotocin, 3.2%; and [8-proline]-oxytocin, 7.6%. [2-Phenylalanine]-oxytocin, however, possessed 26.1% of the natriuretic activity of oxytocin. The intercept of the dose–response curve of this analog was greater than that of [8-proline]-oxytocin or des-9-glycinamide-oxytocin but was significantly less than that of oxytocin (Table II). The slopes for these analogs, which were all given to the same group of rats, did not differ significantly from one another except for des-9-glycinamide-oxytocin which retained only about 0.2% of the natriuretic activity of oxytocin (Table III).

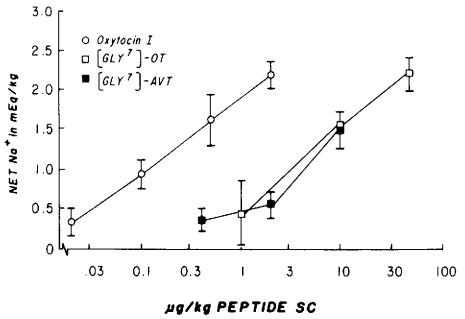

FIG. 1. Net sodium excreted at 2 hr versus the log dose of oxytocin, [7-glycine]-oxytocin and [7-glycine]-arginine-vasotocin in Group I rats. This figure shows the mean net increase in sodium excreted at 2 hr (± SE) versus the log dose of oxytocin, [7-glycine]-oxytocin and [7-glycine]-arginine-vasotocin in Group I rats. The rats received an oral saline load equivalent to 2% of the body weight immediately prior to the sc administration of the peptides. Control responses to solvent injections were subtracted from the responses to the peptides to obtain net responses. The mean control sodium excretion was 0.56 meq/kg in these rats.

Discussion. Oxytocin was found to be a potent natriuretic agent in saline-loaded rats. The low dose of 0.1 μg/kg sc, which is equivalent to about 25 ng/rat, produced natriuretic responses when compared with the control treatment. The slopes of the dose–response curves for all the analogs except des-9-glycinamide-oxytocin did not differ significantly, suggesting that they interact with similar receptors. The intercepts for some of the analogs tested differed significantly indicating significant shifts in the dose–response curves. Only the highest dose of des-9-glycinamode-oxytocin tested, 50 μg/kg, produced a significant natriuretic response. This is probably why the slope of the dose–response curve was low.

[7-Glycine]-oxytocin retained about 3.2% of the natriuretic activity of oxytocin. [8-Proline]-oxytocin retained as much if not more natriuretic activity as did [7-glycine]-oxytocin. These substitutions in position 7 or 8 of oxytocin may have decreased the efficacy of the molecule or its ability to bind to "natriuretic receptors." It is not known if this is due to alterations in the amino acid side chains or an alteration in the conformation of the three amino acid "tail." The natriuretic activity of des-9-glycinamide-

TABLE II. SLOPES AND INTERCEPTS (± SE) OBTAINED FROM LINEAR REGRESSION ANALYSIS OF DOSE–RESPONSE CURVES (NET SODIUM EXCRETED VS LOG DOSE PEPTIDE)

	Slopes[a] (meq Na^+/kg/log dose)	Intercepts (meq Na^+/kg)
Group I (n = 6)		
Oxytocin (I)	0.93 ± 0.05	1.90 ± 0.18
[Gly^7]-OT	1.04 ± 0.15	0.45 ± 0.37[b]
[Gly^7]-AVT	0.84 ± 0.15	0.56 ± 0.29[b,c]
Group II (n = 8)		
Oxytocin (II)	1.06 ± 0.16	1.95 ± 0.18
OH[Thr^4, Gly^7]-OT	0.94 ± 0.19	0.16 ± 0.16[b]
Group III (n = 11)		
Oxytocin (III)	0.86 ± 0.07	1.61 ± 0.15
[Pro^8]-OT	0.82 ± 0.07	0.67 ± 0.06[b]
[Phe^2]-OT	1.00 ± 0.13	1.13 ± 0.13[d,f]
Des-9-Gly-NH_2-OT	0.62 ± 0.12[g]	−0.24 ± 0.09[b,d,e]

[a] Not statistically different for comparisons within groups.
[b] $p < 0.001$ compared to oxytocin.
[c] Not statistically different compared to [Gly^7]-OT.
[d] $p < 0.005$ compared to [Pro^8]-OT.
[e] $p < 0.001$ compared to [Phe^2]-OT.
[f] $p < 0.005$ compared to oxytocin.
[g] $p < 0.002$ compared to [Phe^2]-OT.

oxytocin, which has the same amino acid sequence as oxytocin through position 8, was very weak compared to that of oxytocin. The low activity of this analog indicates that the terminal glycinamide of oxytocin is important, but not absolutely essential, in conferring natriuretic activity to oxytocin.

The natriuretic activity of des-9-glycinamide-oxytocin was of particular interest since this analog may be one of the principal metabolites of oxytocin (15). Des-9-glycinamide-oxytocin, 10 μg/kg, increased sodium excretion 0.12 meq/kg while the same dose of oxytocin produced an increase of 2.57 meq/kg at 2 hr. Assuming total conversion of the 10 μg/kg dose of

TABLE III. NATRIURETIC, OXYTOCIC, AND ANTIDIURETIC ACTIVITIES OF THE ANALOGS ASSAYED RELATIVE TO OXYTOCIN (100%)

Peptide assayed	Dose[a]	Natriuretic activity (%)	Oxytocic activity[b] (no Mg^{2+}) (%)	Oxytocic activity[b] (0.5 mM Mg^{2+}) (%)	ADH activity (%)
Group I					
Oxytocin (I)	0.11	100	100	100	100
[Gly^7]-OT	3.35	3.2	18	200	<1
[Gly^7]-AVT	3.32	3.2	6	40	32
Group II					
Oxytocin (II)	0.13	100	100	100	100
OH[Thr^4, Gly^7]-OT	7.92	1.6	42	206	<1
Group III					
Oxytocin (III)	0.19	100	100	100	100
[Pro^8]-OT	2.53	7.6	3	4	<3
[Phe^2]-OT	0.74	26.1	10	48	11
Des-9-Gly-NH_2-OT	101	0.2	<1[c]	—	4[c]

[a] Dose to increase sodium excretion 1 meq/kg at 2 hr in μg/kg.
[b] Rat uterus *in vitro*.
[c] Calculated from data of Walter *et al.* (13).

oxytocin to des-9-glycinamide-oxytocin, the contribution of this metabolite to the total natriuretic response would be small. The possibility that some other metabolite of oxytocin may have natriuretic activity cannot be ruled out. It is more likely, however, that the intact oxytocin molecule produces most of the natriuretic response.

Substitution of arginine for leucine in position 8 of [7-glycine]-oxytocin did not alter natriuretic activity (Fig. 1). Arginine in position 8 of neurohypophysial hormones appears to contribute to their natriuretic activity in conscious dogs (16). This does not appear to be true in rats with this pair of analogs. This may be due to a species difference or be a property of these particular analogs. Cort *et al.* (17) compared the natriuretic activities of [4-leucine]-oxytocin and [4-leucine]-arginine-vasotocin in chloralose-anesthetized cats. They found that the natriuretic response to [4-leucine]-arginine-vasotocin was far greater in magnitude and duration. These authors also tested the effect of [4-leucine]-arginine-vasotocin on electrolyte excretion in rats given 2% of their body weights in isotonic saline. [4-Leucine]-arginine-vasotocin, 10 μg/kg sc, increased sodium excretion 8% at 5 hr while in our experiments the same dose of [7-glycine]-arginine-vasotocin increased sodium excretion 162% above control levels at 4 hr. Control sodium excretions were comparable. Thus, it appears that [7-glycine]-arginine-vasotocin is a more potent natriuretic analog than [4-leucine]-arginine-vasotocin in the saline-loaded rat, although it was no more potent than [7-glycine]-oxytocin.

[2-Phenylalanine]-oxytocin was the most potent natriuretic oxytocin analog tested. This analog had 32 U/mg oxytocic activity when assayed on the rat uterus in 1959 by Dr. H. B. van Dyke and 15 U/mg when we reassayed it in 1977. Assuming that one-half of the natriuretic activity was also lost, [2-phenylalanine]-oxytocin may actually have 50% of the natriuretic activity of oxytocin. Although the natriuretic activity of [2-phenylalanine]-oxytocin may be less than that of oxytocin, 2-phenylalanine substitution in combination with other substitutions may well be useful in designing more potent and/or selective natriuretic analogs in the future.

The peptides tested exhibited several marked discrepancies in their relative activities. Table III shows the natriuretic, oxytocic and antidiuretic activities for the analogs assayed as percentages of those for oxytocin. Relative natriuretic activities were calculated from doses corresponding to a net sodium excretion of 1 meq/kg at 2 hr. Relative oxytocic and antidiuretic activities were calculated using data obtained from our assays. Oxytocic activities from assays done with Mg^{2+} in the medium were included as these are probably a better approximation of activities *in vivo* (18).

[2-Phenylalanine]-oxytocin and [8-proline]-oxytocin retained more natriuretic activity than antidiuretic activity relative to oxytocin (Table III). [7-Glycine]-oxytocin and "1-hydroxy-[4-threonine, 7-glycine]-oxytocin" had negligible antidiuretic activities relative to oxytocin but retained considerable natriuretic activities. [7-Glycine]-arginine-vasotocin had one-third as much antidiuretic activity as oxytocin but had natriuretic activity similar to that of the 7-glycine-substituted oxytocins. A poor linear correlation ($r = 0.03$) existed between the natriuretic and antidiuretic activities of the analogs tested. Thus, the "natriuretic receptors" appear to be distinct from the receptors which mediate the antidiuretic response. This supports earlier data also indicating that oxytocin and its analogs have a natriuretic action which is independent of antidiuretic activity (4).

"1-Hydroxy-[4-threonine, 7-glycine]-oxytocin" had the least natriuretic activity but the most oxytocic activity (both with and without Mg^{2+}) of the analogs assayed. [7-Glycine]-oxytocin and "1-hydroxy-[4-threonine, 7-glycine]-oxytocin" had greater oxytocic activities than oxytocin when assayed on the rat uterus *in vitro* (with Mg^{2+}). These analogs, however, retained only 1.6 to 3.2% of the natriuretic activity of oxytocin. [2-Phenylalanine]-oxytocin and [8-proline]-oxytocin retained more natriuretic activity than the 7-glycine-substituted oxytocins but had much weaker oxytocic activities. Linear regression analysis of the data obtained with these analogs tends to

indicate a negative correlation ($r = -0.45$ with Mg^{2+}, $r = -0.26$ without Mg^{2+}) or inverse order of potency for the natriuretic and oxytocic activities. The lack of correlation suggests that the "natriuretic receptors" are structurally distinct from the uterine receptors for oxytocin.

Summary. The natriuretic activities of several synthetic analogs of oxytocin having a 7-glycine substitution and some analogs having a different single amino acid substitution or a deletion were tested in conscious, saline-loaded rats. The 7-glycine-substituted analogs and [8-proline]-oxytocin all possessed similar natriuretic activities, 2–8% of the natriuretic activity of oxytocin. [2-Phenylalanine]-oxytocin, however, possessed 26% of the natriuretic activity of oxytocin. Des-9-glycinamide-oxytocin retained about 0.2% of the natriuretic activity of oxytocin. The natriuretic and oxytocic or antidiuretic activities of these analogs were not linearly correlated. The natriuretic response to oxytocin analogs thus appears to be mediated by receptors which are different from the oxytocic and antidiuretic receptors for these peptides.

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