THE STRUCTURAL REQUIREMENTS OF OXYTOCIN AND VASSOPRESSIN ANALOGUES FOR THE ACTIVATION OF ADENYLATE CYCLASE IN THE RAT KIDNEY MEDULLARY MEMBRANE SYSTEM

Daniel BUTLEN[a], Tomislav BARTH[b], Bernard CANTAU[a], Gilles GUILLON[a], Serge JARD[a], Michal LEBL[b], František BRTNÍK[b] and Karel JOŠT[b]

[a] *Laboratoire de Physiologie Cellulaire, Collège de France, 11 place M. Berthelot, Paris V, France and*
[b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, ČSSR*

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The binding properties and the activation of adenylate cyclase by the structural analogues of neurohypophysial hormones in the rat kidney membrane system were investigated. Certain structural modifications of the vasopressin molecule, *i.e.* absence of the primary amino group, removal of the carboxy-terminal part and combinations of the modifications lowered the affinity and activating properties of the compounds. A similar effect was observed in the oxytocin series where the absence of the primary amino group decreased the binding and activation of the adenylate cyclase. Two modifications improved the binding affinity: the carba substitution in both the series of deamino-analogues and the introduction of a lipophilic substituent in the *para*-position of the aromatic amino acid in position 2 of the peptide chain. An attempt was made to correlate the binding and activating properties with the biological effects.

Rat kidney medullary membranes were found to be a suitable system for the investigating the interactions between vasopressin analogues and hormonally activated adenylate cyclase[1,2]. The understanding of the character of binding and activation provides the fundamental data on the relation between the structure of an individual peptide and its binding and activating properties. A guideline may thus be obtained for designing new synthetic analogues.

The previous studies on the rat kidney system[1,3,4], where the best possibility exists for the direct correlation of the antidiuretic potencies of the analogues, have already revealed a certain relation between the structure and binding/activating properties (ref.[3–5]). In an effort for a more detailed understanding of the structure–activity relationship, new structural analogues were selected and tested in the abovementioned membrane system.

EXPERIMENTAL

Materials

The following synthetic peptides prepared at the Institute of Organic Chemistry and Biochemistry, Prague were used in the study: [8-lysine]deamino-vasopressin[6,7], [8-arginine]deamino-vasopressin[8], [8-arginine]deamino-1-carba-vasopressin[9], [8-arginine, 9-desglycineamide]deamino-vasopressin[10] (prepared by the tryptic treatment of [8-arginine]deamino-vasopressin), [8-lysine, 9-desglycineamide]vasopressin[11], N^{α}-glycyl-glycyl-glycyl[8-ornithine]vasopressin[12], N^{α}-glycyl-glycyl-glycyl[8-lysine]vasopressin[13,14], N^{α}-glycyl-glycyl-glycyl[7-glycine, 8-ornithine]vasopressin[15], N^{α}-glycyl-glycyl-glycyl[8-lysine, 9-desglycineamide]vasopressin[14], deamino-oxytocin[16], deamino-1-carba-oxytocin[17], deamino-6-carba-oxytocin[18], deamino-dicarba-oxytocin[18], [2-*p*-ethylphenylalanine]oxytocin[19], [2-*p*-ethylphenylalanine]deamino-6-carba-oxytocin[20], [2-*p*-methylphenyl alanine]deamino-6-carba-oxytocin[20], [2-phenylalanine]deamino-6-carba-oxytocin[20], [2-O-ethyltyrosine]deamino-6-carba-oxytocin[20], [2-*p*-aminophenylalanine]deamino-6-carba-oxytocin[20], [2-*p*-nitrophenylalanine]deamino-6-carba-oxytocin[20], [2-methionine]deamino-6-oxytocin[21], [2-O-methyltyrosine]deamino-1-carba-oxytocin[22], [4-glutamic acid]deamino-1-carba-oxytocin[23], [4-isoleucine]deamino-1-carba-oxytocin[24], oxytocin and [8-lysine]vasopressin. A sample of [8-arginine]deamino-vasopressin was kindly provided by Dr M. Flegel. Mono- and diiodo[8-lysine]vasopressin were prepared in the Paris laboratory[25].

Tritiated [8-lysine]vasopressin (^{3}H-LVP) was prepared according to a described procedure[26]. The labelled peptide was purified by affinity chromatography using neurophysin bound to Sepharose 4B (ref.[27]). Its specific radioactivity was 8·1 Ci/mmol (3·14 . 10^{11} Beq/mmol). The biological activity determined in the rat vasopressor assay[28] and pig[29] and rat[30] adenylate cyclase systems was identical with that of the unlabelled material.

Neutral aluminium oxide was obtained from Woelm (Eschewege), Dowex AG 50 × 8 from Biorad Lab; sodium dodecyl sulfate from Serlabo, bovine serum albumin fraction (BSA) from Pentex, Tris, ATP (disodium salt) from Sigma Chem. Comp., cyclic AMP, creatine kinase and phosphocreatine (disodium salt) from Boehringer, EDTA, ouabain and sodium azide from Merck. ^{3}H-cyclic AMP (21 Ci/mmol) from Commisariat à l'Énergie Atomique, Saclay, α-^{32}P-ATP (20 Ci/mmol) from New England Nuclear.

Methods

The membrane fraction was prepared from the medullary portions of Wistar rat kidney, according to the described procedure[2,5]. Adenylate cyclase activity was measured by the rate of conversion of α-^{32}P-ATP to labelled cyclic AMP (ref.[4]). The incubation medium (100 µl final volume) contained Tris-HCl 100 µmol l^{-1} pH 7·4, cyclic AMP 1 mmol l^{-1}, $MgCl_2$ 75 mmol l^{-1}, creatine phosphate 20 mmol l^{-1}, creatine kinase 100 µg per tube (1 mg/ml), ouabain 0·1 mmol l^{-1}, sodium azide 10 mmol l^{-1}, EDTA 0·25 mmol l^{-1}, BSA 0·25 mg/ml and various amounts of [8-lysine]vasopressin or its analogues. Membranes (40—60 µg of protein) were preincubated for 15 min at 30°C in the medium described above. The reaction was initiated by the addition of the substrate ATP (resulting concentration 0·25 mmol l^{-1}) and α-^{32}P-ATP of about 0·65 µCi, the reaction proceeded for 6 min at 30°C and was stopped by the addition of sodium dodecyl sulfate (2% final concentration). Labelled cyclic AMP was separated by the method of Salomon and coworkers[31] with minor modifications. Cyclic AMP recovery was monitored using ^{3}H cyclic AMP added immediately after stopping the reaction. The dose dependence for adenylate cyclase activation by vasopressin and analogues was characterized by K_{act} (concentration of peptide leading to half maximal activation[4], Hill coefficient (n) and by $V_{max} = A_{max}/A_{max LVP\%}$, where A_{max} is the maximal increase in enzyme activity over basal value.

Binding assay. Binding of ^{3}H-LVP was measured under experimental conditions identical with those used for the adenylate cyclase assay, except for the absence α-^{32}P-ATP in the incubation medium. After 15 min incubation of membranes in the presence of ^{3}H-LVP, the reaction was stopped by the addition of a cold solution (2 ml of Tris-HCl 25 mmol l^{-1} pH 7·4, $MgCl_2$ 0·75 mmol l^{-1}). The membranes containing bound ^{3}H-LVP, were separated by filtration on Millipore Filters EAWP 1·0 μm. The filters were washed three times with 10 ml of a cold Tris-HCl and $MgCl_2$ solution. All determinations were corrected for non-specific binding, *i.e.* residual radioactivity measured in the presence of 5 μmol l^{-1} of [8-lysine]vasopressin. The binding curve for [8-lysine]vasopressin was determined using increasing amounts of the labelled peptide. The binding constant (K_{bind}) was calculated as the concentration of the peptide leading to half maximal specific binding. The binding of the peptide was also characterized by B_{max}, maximal binding capacity. The data were obtained from the Scatchard plot of the binding curve[32].

Proteins were estimated by the method of Lowry and coworkers[33].

RESULTS

The system of rat kidney medullary membranes was characterized in each experiment by ^{3}H-LVP binding and activation of adenylate cyclase. The value $K_{bind} = 4·4 \pm 0·5$-nanomoles l^{-1} (6 experiments) and maximal specific binding capacity (B_{max}) was

TABLE I

Activation of adenylate cyclase and binding to the rat kidney membrane system; vasopressin analogues

Peptide	Adenylate cyclase pK$_A$	Adenylate cyclase *n*, Hill	A_{max}/A_{maxLVP}%	Binding pK$_D$
[8-Lysine]vasopressin	8·35 ± 0·19[a]	0·86 ± 0·04	100	8·32 ± 0·05[b]
[8-Lysine]deamino-vasopresssin	8·10	0·73	75	8·46
[8-Lysine, 9-desglycinamide]vasopressin	6·74	0·79	80	6·25
[2-(3-Iodotyrosine), 8-lysine]vasopressin	5·3	0·96	67	5·59
[2-(3,4-Diiodotyrosine), 8-lysine]vasopressin	5·1	0·97	55	4·96
N^{α}-glycyl-glycyl-glycyl-[8-lysine]-vasopressin	5·77	0·97	97	5·28
N^{α}-glycyl-glycyl-glycyl-[8-ornithine]-vasopressin	5·74	0·81	85	5·31
[8-Arginine]deamino-vasopressin	8·68	0·54	95	9·07
[8-Arginine, 9-desglycinamide]deamino-vasopressin	7·96	0·60	69	7·11
[8-Arginine]deamino-1-carba-vasopressin	8·64	0·67	88	8·28

[a] — Means + SE ($n = 6$); [b] — means + SE ($n = 9$). All other values were means of 2 individual experimental determinations

0·4 ± 0·03 picomoles of ^{3}H-LVP bound per mg of protein. Adenylate cyclase activities measured under basal conditions and in the presence of a saturating amount of [8-lysine] vasopressin (5 μ moles l^{-1}) were 94 ± 13 and 406 ± 52 picomoles of cyclic AMP (6 min) mg protein respect. (6 experiments). K_{act} = 4·8 ± 0·5 nanomoles. .l^{-1} (9 experiments). The Hill coefficient for adenylate cyclase activation was 0·86 ± 0·04 (mean ± SE, 6 experiments). Such a system was used for studying the effect of structural modifications of a series of synthetic analogues of vasopressin and oxytocin. In the vasopressin series, the absence of the primary amino group, carba substitution, aminoacylation of the amino-terminal part, elimination of the carboxy-terminal glycinamide and introduction of bulky atoms in the aromatic moiety of tyrosine were the structural features whose effect on the binding and adenylate cyclase activation was studied. In the oxytocin series, the effect was studied of the absence of the primary amino group, different carba modifications, and substitution of the amino acid in position 2 and 4 of oxytocin and its carba analogues. The results are summarized in Table I and II.

The absence of the primary amino group of vasopressin or its acylation with a short peptide chain lowered the affinity to the receptor and the activation of adeny-

TABLE II

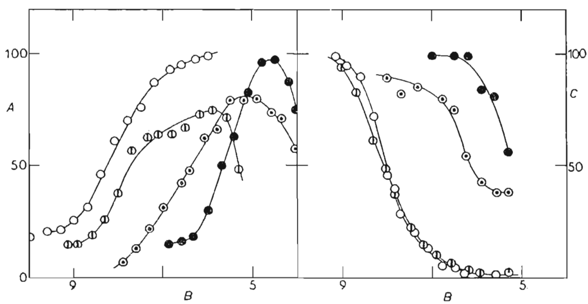
Activation of adenylate cyclase and binding to the rat kidney system. Oxytocin analogues

Peptide	Adenylate cyclase		A_{max} / $A_{max LVP}$%	Binding pK_D
	pK_a	n, Hill		
Oxytocin	7·21	0·83	86	7·47
Deamino-oxytocin	7·44	0·71	67	6·99
[2-*p*-Ethylphenylalanine]oxytocin	6·20	1·04	82	6·12
Deamino-1-carba-oxytocin	7·20	0·71	94	6·29
Deamino-6-carba-oxytocin	7·43	0·73	99	7·49
Deamino-dicarba-oxytocin	6·47	1·09	98	6·04
[2-Phenylalanine]deamino-6-carba-oxytocin	6·72	1·03	89	6·28
[2-*p*-Methylphenylalanine]deamino-6-carba-oxytocin	7·54	0·62	87	7·80
[2-*p*-Ethylphenylalanine]deamino-6-carba-oxytocin	7·48	0·72	83	7·60
[2-*p*-Aminophenylalanine]deamino-6-carba-oxytocin	6·72	0·79	70	6·62
[2-*p*-Nitrophenylalanine]deamino-6-carba-oxytocin	5·96	0·94	69	6·54
[2-Methionine]deamino-6-carba-oxytocin	5·00	0·77	65	5·24
[2-O-Ethyltyrosine]deamino-6-carba-oxytocin	5·70	1·15	34	6·39
[2-O-Methyltyrosine]deamino-1-carba-oxytocin	7·39	0·74	77	6·74
[4-Isoleucine]deamino-1-carba-oxytocin	5·59	0·99	78	6·73
[4-Glutamic acid]deamino-1-carba-oxytocin	4·28	0·87	76	4·31

late cyclase (Fig. 1), whereas the replacement of the disulfide bond by the 1-mono-carba bridge had no effect on the binding and only slightly decreased the affinity for the enzyme system as compared with [8-arginine]deamino-vasopressin. The elimination of the carboxy terminal glycinamide pronouncedly decreased the binding and activating properties of the analogues studied. Introduction of one or two iodine atoms in the *ortho* position of tyrosine sharply decreased the affinity to both the systems on the average by three orders of ten (Fig. 2). In the series of deamino vasopressin analogues negative cooperativity was observed.

In the oxytocin series, the absence of the primary amino group was without effect on the binding, while the character of carba bridge played a role in the magnitude of binding (Fig. 3). The 6-carba analogue had the same affinity as oxytocin, the other two, 1-carba and di-carba, had less than 10% of the affinity of deamino-oxytocin for binding. The di-carba substitution finds the same expression in activation of adenylate cyclase. Both the mono-carba analogues of oxytocin had similar activation properties as oxytocin.

Several analogues of deamino-6-carba-oxytocin with modifications in position 2 were studied (Fig. 4). The elimination of the hydroxy group (2-phenylalanine derivative) decreased the activation by one order of ten; no change was found when methyl



a *b*

FIG. 1

Activation of adenylate cyclase (*a*) and binding (*b*) to the kidney membrane preparation. *A* Adenylate cyclase activation (normalized to [8-lysine]vasopressin activation), *B* log concentration of added unlabelled peptide (molarity), *C* binding of ^{3}H-LVP to membrane, competition with unlabelled peptides. For details see Methods. ○ [8-lysine]vasopressin, ● N^{α}-glycyl-glycyl-glycyl-[8-lysine]vasopressin, ⊙ [8-lysine, 9-desglycinamide]vasopressin, ⦶ [8-lysine]deamino-vasopressin or ethyl group was introduced instead of hydroxyl. Substitution of the hydroxy by ethoxy group decreased the affinity for binding by one order of ten and the activating properties by two orders. A very similar modification, the replacement of the hydroxy by methoxy group in deamino-1-carba-oxytocin did not affect the binding and activating properties. Finally, the introduction of amino or nitro groups had small effect on the binding and activating properties when compared with [2-phenylalanine]deamino-6-carba-oxytocin.

Two replacements of the amino acid in position 4 were studied: replacement of glutamine by lipophilic isoleucine or by glutamic acid. The binding properties of the 4-isoleucine derivative were diminished by one order of ten whereas the activating properties were lowered by two orders of ten. The appearance of the free carboxyl in position 4 significantly depressed both the binding and activating properties.

DISCUSSION

Ample pharmacological data concerning the antidiuretic potencies of analogues of neurohypophysial hormones have already been obtained in experiments with rats. The rat kidney medullary membrane system has been proved to be well suited for investigating the structure-activity relationship of vasopressin analogues at the molecular level.

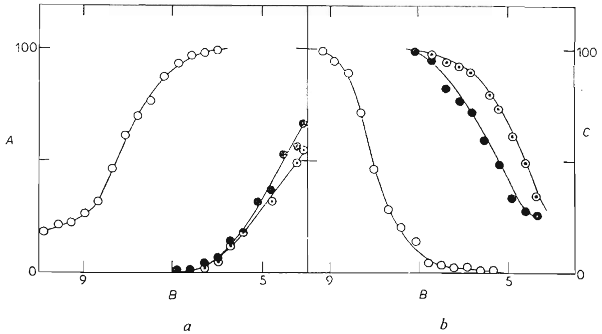


FIG. 2

Activation of adenylate cyclase (*a*) and binding (*b*) to the kidney membrane preparation. *A*, *B*, *C*, the same meaning as in figure 1. ○ [8-Lysine]vasopressin, ● [2-(3-iodotyrosine), 8-lysine]-vasopressin, ⊙ [2-(3,5-diodotyrosine), 8-lysine]vasopressin

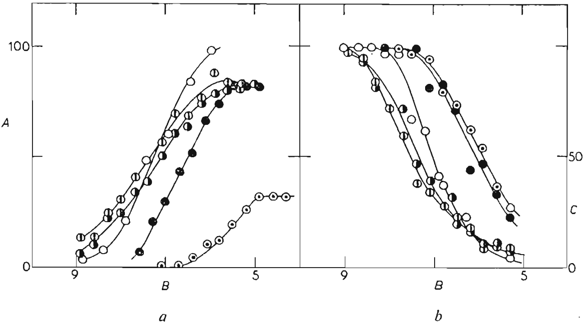


FIG. 3

Activation of adenylate cyclase (*a*) and binding (*b*) to the kidney membrane preparation. *A*, *B*, *C* the same meaning as in Fig. 1. ◑ Oxytocin, ○ deamino-oxytocin, ● deamino-1-carba-oxytocin, ⦶ deamino-6-carba-oxytocin, ⊙ deamino-dicarba-oxytocin

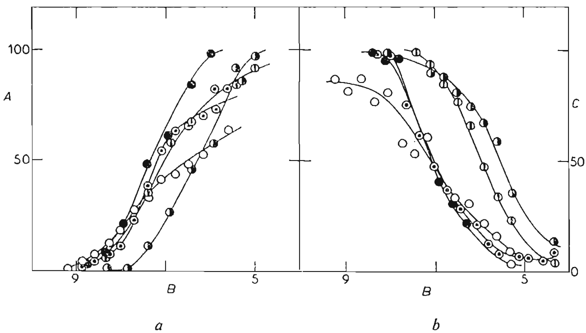


FIG. 4

Activation of adenylate cyclase (*a*) and binding (*b*) to the kidney membrane preparation. *A*, *B*, *C* the same meaning as in Fig. 1. ⊙ Deamino-6-carba-oxytocin, ◑ [2-phenylalanine]deamino-6-carba-oxytocin, ⦶ [2-O-ethyltyrosine]deamino-6-carba-oxytocin ● [2-*p*-methylphenylalanine]-deamino-6-carba-oxytocin, ○ [2-*p*-ethylphenylalanine]deamino-6-carba-oxytocin

Note added in proof: In the left part of Fig. 4 the symbols ○ and ⦶ should be mutually substituted.

This study was undertaken in order to obtain more detailed information on the structure–activity relationship, to gain knowledge of the consequences of the modification of molecular structure and finally to characterize several groups of analogues which have an altered specificity of biological action.

So far, two important features of the structure and resulting biological activity have been examined. For the analogues derived from [8-L-arginine]deamino-vasopressin (carba substitution in position 6 and alteration of amino acid in position 8), known as very potent antidiuretic agents, no higher affinity of V_{max} of activation was proved. Their prolonged antidiuretic action is probably due to changed distribution and metabolic stability as has already been proposed[4,34–37].

Several analogues of oxytocin and vasopressin which may be structurally characterized by alterations performed in the amino-terminal part – modification of position 1 and 2 – were found to inhibit the adenylate cyclase activation by [8-lysine]-vasopressin (ref.[4,38]). All had low, antidiuretic activity[4,37,39,40]. When they were applied in subthreshold doses to anesthetized rats[39], no clear inhibitory effect on the antidiuretic action of oxytocin or vasopressin was observed. Some of the analogues studied are characterized by the absence of primary amino group. [8-Arginine]deamino-vasopressin and [8-lysine]deamino-vasopressin had lower affinity and activating properties than the natural hormones. Deamino-oxytocin was a weaker activator of adenylate cyclase than oxytocin. The carba substitution in the cyclic part of the molecule is one of the favorable structural modifications which promotes greater metabolic stability of the molecule and provides some molecules even with a higher intrinsic activity.

[8-Arginine]deamino-6-carba-vasopressin was already studied in the rat kidney medullary membrane system[3]. Here we present the results obtained with 1-carba derivative. A certain preference of the receptor for 6-carba modification can be seen comparing the data on activation. Antidiuretically superactive vasopressin analogues were obtained by deamination in position 1 and stereoplacement in position 8 (ref.[4]). Each of the modifications by itself decreased the binding and activating properties. Nevertheless the combination of the two modifications provided [8-D-arginine]deamino-vasopressin, an analogue that had a much higher antidiuretic potency than [8-arginine]vasopressin. Contrary to our expectation, when the 6-carba modification known to produce analogues with prolonged antidiuretic action[31] was introduced into the molecule of [8-D-arginine]deamino-vasopressin, the activating properties of the resultant analogue decreased[4]. The presence of carboxy terminal glycinamide in vasopressin analogue is known to be necessary for their biological activity (with the exception of the activity on the CNS). It was therefore not surprising that its absence was accompanied by a substantial loss of affinity and activating properties: the desglycinamide derivative of N^{α}-glycyl-glycyl-glycyl[8-lysine]vasopressin is practically devoid of binding and activating properties.

Two analogues of triglycyl[8-lysine]vasopressin with modifications in position 8, 7 and 9 were studied. The data obtained with carba analogues[3] indicated that the activating properties decreased when arginine was replaced by ornithine. These results indicate that the recognition system of the receptor might distinguish the position of amino group in the side chain of the basic amino acid. A decrease of affinity and activating properties was observed when this group was shifted nearer to the backbone of the peptide molecule. The ability of triglycyl[7-glycine, 8-ornithine]vasopressin, which is not included in the Tables to compete with ^{3}H-LVP for binding did not differ significantly from the binding properties of two other hormonogens.

In the oxytocin series both the deamination and 1-carba and 6-carba substitution enhanced the antidiuretic potency of the analogues[40] as compared with oxytocin. The higher antidiuretic potency, especially that of deamino-6-carba-oxytocin, could not be attributed only to stronger binding and activation properties in the kidney receptor system. No prolonged antidiuretic activity was observed in the case of the monocarba derivatives of oxytocin[31]. Deamino-dicarba-oxytocin also had the same antidiuretic potency as oxytocin, but ten times lower binding and activating properties and no indication of prolonged antidiuretic activity.

Our studies concerning processes occurring at the molecular level in the kidney did not provide an explanation of the unexpectedly high antidiuretic response of carba analogues of oxytocin.

A series of deamino-6-carba-oxytocin derivatives that had higher natriuretic potency[20] was studied. The replacement of the tyrosine hydroxyl by methyl or ethyl group did not influence the natriuretic properties, while its elimination or substitution by ethoxy, amino or nitro group significantly lowered the natriuretic activity. The same holds for the binding and activating properties in the kidney receptor system. Both the analogues with the alkyl in place of the hydroxy group have the same or better ability than oxytocin or deamino-6-carba-oxytocin to interact with the receptor.

The methylation of the tyrosine hydroxyl ([2-O-methyltyrosine]deamino-1-carba-oxytocin) led to a decrease of the A_{max} (the maximum rate of cAMP production induced by [8-lysine]vasopressin) to 77%. The introduction of the ethoxy group caused a drop to 33% of A_{max}. The previously reported data[4] also give evidence of the importance of this kind of modification; nevertheless, the character and bulkiness of the groups used for modifying the amino acid in position 1 must also be taken into consideration.

While the substitution of the hydroxyl in position 2 of deamino-6-carba-oxytocin by either ethyl or methyl group had no effect on the binding and activating properties, the replacement of the tyrosine hydroxyl of oxytocin by ethyl group reduced binding and activation ten times. The presence of a free carboxyl in side chain of the amino acid residue in position 4 practically eliminated the interaction of the compound with the kidney receptor. Our results showed that carba substitution affected the binding and activating properties of resulting oxytocin and vasopressin analogues in the same way. This was not so in the case of other modifications (substitutions in positions 2 and 4) when the binding and activating properties were dependent on the already performed substitutions in the primary structure of the peptide.

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