

OXYTOCIN AND VASOPRESSIN ANALOGUES CAPABLE OF COMPETITION WITH VASOPRESSIN BINDING TO RAT LIVER MEMBRANES

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A number of vasopressin and oxytocin analogues modified in positions 1, 2, 4, 6 and 9 of the peptide chain were tested regarding their affinity to the rat liver membrane receptor. The affinities were estimated from the ability of the analogues to compete with the binding of tritiated vasopressin to rat liver membranes. In the series of vasopressin agonists, the degree of competition was in good agreement with the corresponding pressor activities. In the case of inhibitors of vasopressin pressor action, binding to the membrane system was also observed.

The discovery of separate vasopressin receptors in liver, the interaction of which with the hormone resulted in the activation of glycogen phosphorylase^{1,2}, opened the way for research in this field. Latest data show that there is a good correlation between the activation of liver glycogen phosphorylase and liver membrane binding. The basic parameters of the interaction between vasopressin and the receptor in liver membranes were already recognized³ and the method was introduced and standardized for the evaluation of structure-activity relationship of vasopressin analogues.

We studied several groups of analogues of vasopressin in order to supplement the available data on biological activities. The analogues had the following structural modifications: absence of the primary amino group of the amino acid in position 1, carba substitution, modifications in the C-terminal part of the linear chain, amino acid substitutions in positions 2 and 4, and, finally, combinations of the above-mentioned alterations. All these structural modifications are known to influence the biological effects.

EXPERIMENTAL

Materials

[2-³H]Tyrosine, 8-lysine]vasopressin (³H-LVP) was prepared and purified as previously described^{4,5}, its specific radioactivity was 8.5 Ci (3.14 · 10¹¹ Bcq) per mmol and its biological properties

were the same as those published in our previous paper³. The following peptides, oxytocin, vasopressin and their analogues were used in the study: [8-lysine]deamino-vasopressin^{6,7}, [8-arginine]deamino-vasopressin⁸, [8-arginine]deamino-1-carba-vasopressin⁹, [8-lysine, 9-desglycinamide]vasopressin¹⁰, [8-arginine, 9-desglycinamide]deamino-vasopressin^{11,12}, N^α-glycyl-glycyl-glycyl[8-ornithine]vasopressin¹³, N^α-glycyl-glycyl-glycyl[8-lysine]vasopressin^{14,15}, N^α-glycyl-glycyl-glycyl[7-glycine, 8-ornithine]vasopressin¹⁶, N^α-glycyl-glycyl-glycyl[8-lysine, 9-desglycinamide]vasopressin¹⁵, deamino-oxytocin¹⁷, deamino-1-carba-oxytocin¹⁸, deamino-6-carba-oxytocin¹⁹, deamino-dicarba-oxytocin¹⁹, [2-*p*-ethylphenylalanine]oxytocin²⁰, [2-*p*-ethylphenylalanine]deamino-6-carba-oxytocin²¹, [2-*p*-methylphenylalanine]deamino-6-carba-oxytocin²¹, [2-phenylalanine]deamino-6-carba-oxytocin²¹, [2-*O*-ethyltyrosine]deamino-6-carba-oxytocin²¹, [2-*p*-aminophenylalanine]deamino-6-carba-oxytocin²¹, [2-*p*-nitrophenylalanine]deamino-6-carba-oxytocin²¹, [2-methionine]deamino-6-carba-oxytocin²², [2-*O*-methyltyrosine]oxytocin²³, [2-*O*-methyltyrosine]deamino-1-carba-oxytocin²⁴, [4-isoleucine]deamino-1-carba-oxytocin²⁵, [4-glutamic acid]deamino-1-carba-oxytocin²⁶. Oxytocin and [8-lysine]vasopressin were prepared in the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences. Our thanks are due to Dr M. Flegel for a sample of [8-arginine]deamino-vasopressin. Monoicdo- and diiodo-substituted [8-lysine]vasopressin²⁷ were prepared in the Paris laboratories.

Creatine phosphate disodium salt and creatine kinase were purchased from Boehringer-Mannheim (GFR), bacitracin, bovine albumin (fraction V) were provided by Sigma, Mo (USA) together with Trisma (tris-hydroxymethylaminomethan).

Methods

Purified plasma membranes were prepared from rat liver according to the procedure of Neville and coworkers²⁸ up to step 11. Wistar rats weighing 200–250 g were used. Protein concentration was determined according to Lowry and coworkers²⁹.

Binding assays were performed in 100 μ l of incubation medium, containing TRIS-HCl buffer pH 7.4 in final concentration 50 mmol l⁻¹, MgCl₂ (10 mmol), bovine albumin (1 mg/ml), ³H-LVP in a concentration corresponding approximately to its K_D for binding (2–5 · 10⁻⁹ mol l⁻¹) and various concentrations of peptide analogues.

The reaction was initiated by the addition of membranes (50–100 μ g of protein per tube). After 15 min of incubation at 30°C, the reaction was stopped by the addition of 2 ml of cold solution of 10 mol l⁻¹ Tris-HCl buffer pH 7.0 and 1 mmol l⁻¹ MgCl₂ and the whole volume was filtered through Gelman filters (Metricel^R membrane filter), GA-3, 1.2 μ m. The filters were washed three times with a total volume of 7 ml of the same buffer as that used for the dilution of the sample. The radioactivity was counted by liquid scintillation spectrometry (5 ml of Aquasure, High Performance LSC Cocktail, New England Nuclear). Nonspecific binding of ³H-LVP was determined in the presence of unlabelled vasopressin (5 · 10⁻⁵ mol l⁻¹).

The data on the displacement of ³H-LVP by nonlabelled analogues were treated by the method published in our previous paper³. The values of specific binding of ³H-LVP measured in the presence of unlabelled peptide (*B*) were expressed as a fraction of the specific binding measured in the absence of the competing analogue (*B*).

RESULTS

The results will be dealt with considering the type of structural modification of the parent molecules of vasopressin and oxytocin. The absence of the primary amino group in the vasopressin molecule resulted in a decrease of affinity to the receptors

in liver membranes. This conclusion was drawn from the comparison of the affinities of [8-lysine]vasopressin and its deamino-derivative, oxytocin and deamino-oxytocin (Table I and II) (Fig. 1) and also from the comparison of AVP³ and [8-arginine]deamino-vasopressin. The extension of the molecule at the amino end by a short peptide chain thus placing the free amino group far from the skeleton of the parent hormone, caused a dramatic fall in the value of affinity. The elimination of the carboxy terminal glycinamide also decreased the affinity significantly. Nevertheless, if we compare the difference between [8-lysine]vasopressin and its desglycinamide derivative on the one hand, and [8-arginine]deamino-vasopressin and its desglycinamide derivative on the other, we can see that the drop of affinity in the latter case is not so pronounced. The desglycinamide derivative of the triglycyl[8-lysine]vasopressin was almost unable to compete for binding with ³H-LVP. The replacement of one sulfur atom by a methylene group was another rather important modification of the cyclic moiety of the peptide molecule. As can be seen in the Fig. 1b when [8-arginine]deamino-vasopressin was modified in this way, the affinity to the receptor increased significantly. A slight enhancement of the affinity was observed also in the

TABLE I

Dissociation constants for the binding of vasopressin structural analogues to purified liver membranes and pressoric potencies

Compound	pK _d dm mol l ⁻¹	Pressoric potency	Ref. ^a
[8-Lysine]vasopressin	8.33	285	7
[8-Lysine]deamino-vasopressin	8.29	120	7
[8-Arginine]deamino-vasopressin	7.92	395	8
[8-Lysine, 9-desglycineamide]vasopressin	6.36	5	10
[8-Arginine, 9-desglycineamide]deamino-vasopressin	7.47	^b	11
[8-Arginine]deamino-1-carba-vasopressin	8.72	550	9
N ^α -Glycyl-glycyl-glycyl[8-lysine]vasopressin (Glypressin)	5.90	0.5	15
N ^α -Glycyl-glycyl-glycyl[8-lysine, 9-desglycinamide]- vasopressin	5.35	0.1	15
N ^α -Glycyl-glycyl-glycyl[8-ornithine]vasopressin	5.92	1.5	13
N ^α -Glycyl-glycyl-glycyl[7-glycine, 8-ornithine]- vasopressin	6.40	<i>anti</i>	16
[2-(3'-Iodo)tyrosine, 8-lysine]vasopressin	6.22	^b	27
[2-(3',5'-Diiodotyrosine), 8-lysine]vasopressin	5.32	^b	27

^a Ref. for the biological activity; ^b not determined.

case of monocarba derivatives of deamino-oxytocin. Fig. 1c illustrates the differences among the affinities of carba analogues in relation to the position of the methylene group in the bridge (1-carba and 6-carba substitution) and the effect of the replacement of the disulfide bridge by an ethylene group (dicarba analogue).

In the oxytocin and carba oxytocin series, we studied mainly analogues with modifications in position 2. Some of the substances had an inhibitory effect on the pressoric action of vasopressin: this was most pronounced in the case of the compound with the methylated hydroxyl. While the methylation of the hydroxyl group of tyrosine in oxytocin decreased the affinity by more than one order of magnitude, only a fourfold decrease was observed when the modification was introduced into the molecule of deamino-1-carba-oxytocin. On comparing this finding with the effect produced by the replacement of the tyrosine hydroxyl by an ethyl group, we observed that this change influenced the affinity only in 6-carba series and was without effect in the oxytocin series.

TABLE II

Dissociation constants for the binding of oxytocin structural analogues to purified liver membranes

Compound	pK_{dm} mol^{-1}	Pressoric potency I.U./mg	Ref. ^a
Oxytocin	7.11	3	7
Deamino-oxytocin	6.48	1.43	17
Deamino-1-carba-oxytocin	7.21	17.5	31
Deamino-6-carba-oxytocin	6.71	1.5	31
Deamino-dicarba-oxytocin	5.44	0.25	31
[2-Phenylalanine]deamino-6-carba-oxytocin	5.87	0.9	32
[2- <i>p</i> -Methylphenylalanine]deamino-6-carba-oxytocin	6.48	1.0	32
[2- <i>p</i> -Ethylphenylalanine]deamino-6-carba-oxytocin	6.25	anti	32
[2- <i>p</i> -Ethylphenylalanine]oxytocin	7.09	^b	
[2-O-Ethyltyrosine]deamino-6-carba-oxytocin	5.86	<0.2	32
[2- <i>p</i> -Aminophenylalanine]deamino-6-carba-oxytocin	5.36	<0.2	32
[2- <i>p</i> -Nitrophenylalanine]deamino-6-carba-oxytocin	5.86	<0.2	32
[2-Methionine]deamino-6-carba-oxytocin	4.53	^b	
[2-O-Methyltyrosine]oxytocin	5.89	anti	7
[2-O-Methyltyrosine]deamino-1-carba-oxytocin	6.59	anti	24
[4-Isoleucine]deamino-1-carba-oxytocin	5.3	2.8	25
[4-Glutamic acid]deamino-1-carba-oxytocin	3.75	<0.2	^c

^a Ref. for biological activity, ^b not determined; ^c not published.

Several analogues of deamino-6-carba-oxytocin were included in this study. If we arrange the analogues with modifications in the *para*-position of the tyrosine ring according to the decrease of affinity we can see that the replacement of the hydroxyl group by methyl or by ethyl groups did not decrease the affinity significantly. However, its elimination or replacement by ethoxy, nitro or amino groups lowered the affinity by one order of magnitude. From the vasopressin series we can deduce (Table I) that the introduction of bulky atoms of iodine is of even greater consequence,

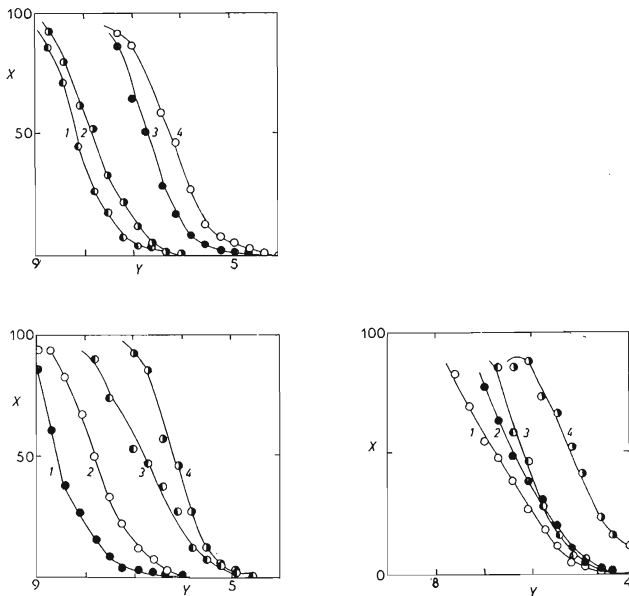


FIG. 1

Binding of vasopressin and oxytocin analogues to liver membranes

For details see Experimental. $X B/B_0 \times 100$, $Y [-\log C]$, mol l^{-1} a 1 [8-lysine]vasopressin, 2 [8-lysine]deamino-vasopressin, 3 oxytocin, 4 deamino-oxytocin; b 1 [8-arginine]deamino-1-carba-vasopressin, 2 [8-arginine]deamino-vasopressin, 3 deamino-1-carba-oxytocin, 4 deamino-oxytocin; c 1 deamino-1-carba-oxytocin, 2 deamino-6-carba-oxytocin, 3 deamino-oxytocin, 4 deamino-dicarba-oxytocin

the introduction of one or two atoms in tyrosine decreases the affinity by two or three orders of ten, resp.

From the results obtained with analogues of deamino-1-carba-oxytocin having a modification in position 4, we can see that the replacement of glutamine by glutamic acid resulted in a sharp decrease of affinity by more than three orders of magnitude, and the introduction of isoleucine led to 82 fold lowering of affinity.

DISCUSSION

Studies of structure-activity relationships of suitable selected analogues of natural hormones on the molecular level can not only provide information on the topography of the corresponding receptor but can promote the synthesis of structural analogues with desired biological properties. This approach can prove useful for the evaluation of analogues the biological properties of which are already known because it may help in the interpretation of the observed character of the biological response.

Several conclusions concerning the effect of structural changes in analogues on the binding affinity can already be made. The elimination of the primary amino group from the first amino acyl residue caused a decrease of binding affinity. This effect was counteracted by the further alteration of the analogue molecule — by carba substitution; both 1-carba and 6-carba analogues³ have higher affinity than [8-arginine]deamino-vasopressin.

The low affinity of desglycinamide analogues clearly demonstrates that the integrity of the carboxy-terminal sequence is necessary for the binding to liver membranes.

In the group of hormonogens, where a pronounced effect of prolongation of the peptide chain on the affinity was demonstrated, N²-glycyl-glycyl-glycyl[7-glycine, 8-ornithine]vasopressin was of special interest. It had an inhibitory effect on the pressoric action of exogenous [8-lysine]vasopressin in the despinalized rat and it was capable of decreasing the basal blood pressure. In experiments *in vitro*, the compound inhibited the effect of [8-lysine]vasopressin on the activation of glycogen phosphorylase in liver hepatocytes³⁰. The replacement of proline by glycine enhanced the affinity by 50% as compared with triglycyl[8-ornithine]vasopressin. Like in the case of the two other desglycinamide analogues mentioned earlier, the elimination of the carboxy-terminal glycineamide from triglycyl[8-lysine]vasopressin nearly abolished the affinity to the receptor in the liver.

Considering the carba substitution in the cyclic part of molecule, we could study the effect of this modification in both the series of analogues. In the vasopressin series, in spite of the already mentioned decreasing effect caused by the absence of the α -amino group, both the 1-carba and 6-carba modifications of the bridge enhanced the affinity as compared with [8-arginine]vasopressin³. The same holds for the oxytocin series, where deamino-1-carba-oxytocin and deamino-6-carba-oxy-

tocin bind to the receptor more firmly than deamino-oxytocin. The introduction of the ethylene bridge instead of mono carba or disulfide bridges caused a decrease of affinity by one order of magnitude as compared with deamino-oxytocin.

In the carba series of oxytocin, several structural changes have been cumulated in the molecule. Together with deamination and 1-carba or 6-carba substitution, the replacement of the amino acid in position 4 or 2 was realized. The consequences of altering the amino acid in position 2 by replacing the tyrosine hydroxyl by methoxy or ethyl groups differed in the oxytocin and carba series. While these modifications had a slight effect in the carba series (compare deamino-1-carba-oxytocin with [2-O-methyltyrosine]deamino-1-carba-oxytocin and deamino-6-carba-oxytocin with [2-*p*-ethylphenylalanine]deamino-6-carba-oxytocin), the affinity of the O-methylated analogue of oxytocin was lower by more than one order of ten than that of oxytocin and [2-*p*-ethylphenylalanine]oxytocin.

The influence of the character of the substituent in *para* position of phenylalanine was studied in a series of seven analogues. The substituents can be roughly divided in two groups according to their effect on the affinity. The first group includes methyl, ethyl and hydroxyl groups; the analogues containing them have the same affinities or higher than those of deamino-oxytocin. The second group is formed by substituents which have the same effect as the absence of any group in this position.

From the results obtained with analogues of deamino-1-carba-oxytocin having modifications in position 4, we can confirm previous data on the importance of this position for the expression of biological properties. The deamidation of glutamine resulted in a decrease of affinity equal to three orders of ten, replacement of glutamine by isoleucine by 1.5 order of ten. Similarly Cantau and coworkers³ found for the analogues of [8-arginine]vasopressin, [8-D-arginine]vasopressin and [8-D-arginine]-deamino-vasopressin, in which glutamine was replaced by valine, a decrease approximately by an half order of ten, whereas no change was found when glutamine was replaced by threonine in [8-D-arginine]deamino-vasopressin. It can be seen that modifications of this position substantially affect the affinity of the peptide to the liver receptor. Our results show that in the vasopressin series, the character of the structure-activity (or affinity) relationships was similar in both the tests performed, *i.e.* in the pressoric assay as well as in studies of the binding to liver membranes. This holds for the effect of the absence of the amino group, carba substitution and the removal of the C-terminal glycinamide from the molecule of hormone or analogue.

In the oxytocin series the above mentioned conclusion is valid so far as the absence of the group and carba substitution are concerned. The alteration of the amino acid in the position 2 seems to be critical when the shift from agonism to antagonism is taken into consideration. Some analogues are known to have an inhibitory effect on the pressoric activity of exogenous vasopressin, others have been found to be without intrinsic pressoric activity even when high doses are applied^{20,21,23-24}.

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