CROSS-REACTIVITY STUDY OF OXYTOCIN ANTIBODIES WITH OXYTOCIN AND ITS ANALOGUES MODIFIED IN THE CYCLIC PART OF THE MOLECULE

Pavel HRBAS[a], Jiřina SLANINOVÁ[a], Tomislav BARTH[a] and František FRANĚK[b]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 and*
[b] *Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 160 00 Prague 6*

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The specificity of oxytocin antibodies prepared by immunizing a pig with oxytocin that had been bound to thyreoglobulin by means of carbodiimide, was investigated using oxytocin analogues modified in positions 1, 2, 4 and 6. All the modifications (deamination, substitution of sulfur in the disulfide bridge by a methylene group, methylation of the OH group of tyrosine or the substitution of the amino acid in this position and in position 4) resulted in analogues with a lower affinity to the antibodies than that of oxytocin.

Specific antibodies against oxytocin have been obtained by administering a high-molecular-weight immunogenic form of the hormone[1-5], oxytocin in combination with carbodiimide[6,7], oxytocin adsorbed on carbon microparticles[8], or oxytocin alone[9,10]. The preparation of specific antibodies against analogues of neurohypophysial hormones, should be based on some knowledge of the properties of the binding site of the antibodies for the parent hormones. Vasopressin antibodies have been studied with the aim of understanding the topology of the binding site of the antibody or the antigen determinant by means of evaluating the cross-reactivity of a number of analogues[11-16]. There is much less information available concerning antioxytocin antibodies[4,8,17]. In the present paper, we describe a detailed study of the specificity of pig antiserum prepared by immunization with a conjugate of oxytocin with bovine thyreoglobulin[18] *via* carbodiimide. The specificity was determined in experiments with ^{125}I-oxytocin and a number of analogues of oxytocin.

EXPERIMENTAL

Material: Oxytocin, [8-arginine]vasopressin, [8-lysine]vasopressin and [8-arginine]vasotocin were synthetized at the Department of Organic Synthesis of this Institute and by the firm Léčiva, Prague. The analytical values and biological activities were the same as those described earlier[19]. The following analogues of oxytocin were also used: 1-deaminooxytocin[20], deamino-1-carba-oxytocin[21], carba-1-oxytocin[22], deamino-6-carba-oxytocin[23], deamino-dicarba-oxytocin[23], deamino-1-carba-oxytocin sulfoxide[24], [4-isoleucine]deamino-1-carba-oxytocin[25], [4-leucine]-deamino-1-carba-oxytocin[25], [4-glutamic acid]deamino-1-carba-oxytocin[26], [2-isoleucine]de-

amino-6-carba-oxytocin[27], [2-methionine]deamino-6-carba-oxytocin[27], [2-O-methyltyrosine]deamino-6-carba-oxytocin[27], [2-O-methyltyrosine]oxytocin[28], [2-O-methyltyrosine]deamino-1--carba-oxytocin[29] and N^{α}-acetyl-[2-O-methyltyrosine]oxytocin[30]. Na ^{125}I was supplied by Isotop (Hungary), Al-Span-Oil adjuvant was purchased from Velaz (Prague). 1-Ethyl-3/3-dimethylaminopropylcarbodiimide was a product of Fluka (Switzerland). Bovine thyreoglobulin was isolated according to Tarutani and Shulman[31]. The final product was checked by gel electrophoresis and elemental analysis (2·33% I).

Preparation of the immunogenic form of oxytocin and immunization: Synthetic oxytocin (10 mg) dissolved in 0·02M Na-phosphate buffer (1 ml), pH 7·4, was conjugated by the carbodiimide reaction to 50 mg of bovine thyreoglobulin (2 ml). The reaction mixture was stirred for 4 h at room temperature, then dialyzed against water and the conjugate was emulsified with Al-Span-Oil adjuvant. The immunization of pigs was performed as described earlier[32].

Iodination of oxytocin: Synthetic oxytocin was labelled according to the method of Greenwood and coworkers[33], as modified by Vaněčková and coworkers[15,16]. The labelled hormone was purified on a column of Sephadex G-25 and its binding capacity was determined in every peak fraction of the eluate. The purity of the iodinated preparation was estimated by paper electrophoresis.

Determination of the titre and the cross-reactivity of the analogues: Incubation was performed in 0·1M-Tris-HCl buffer, pH 7·8, containing 0·2% bovine serum albumin. To 0·1 ml of suitably diluted unlabelled hormone or analogue and 0·1 ml of labelled oxytocin (5000—10000 cpm), 0·4 ml of diluted serum was added. The mixture was incubated for 24 h at 4°C. The free hormone was separated from its bound form by means of dextrane-coated charcoal. All the experiments were performed in pairs; the serum of unimmunized animals was used as a blank for the determination of nonspecific binding. Cross-reactivity experiments were performed at the dilution which produced 50% binding of the total counts. The concentration of the analogue at which 50% of inhibition binding was achieved was estimated from the inhibition curves.

RESULTS

Under the given experimental conditions, the porcine serum had a titre of 1 : 5000. This concentration was used for studying the cross-reactivity of the analogues. The

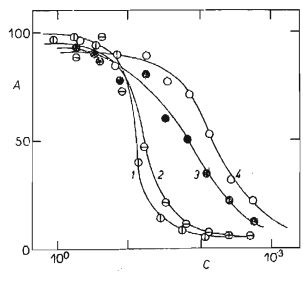

FIG. 1

Inhibition of the Binding of ^{125}I-Oxytocin to Antibodies by Oxytocin and Some of its Analogues

$A = B/B_0 \cdot 100$, c concentration of peptide in ng/ml. *1* Oxytocin, *2* deamino-oxytocin, *3* carba-1-oxytocin, *4* deamino--1-carba-oxytocin.

affinities of the naturally occurring hormones [8-arginine]vasopressin, [8-lysine]--vasopressin and [8-arginine]vasotocin were lower by several orders of ten than that of oxytocin (Table I). Next, we investigated oxytocin analogues modified in positions

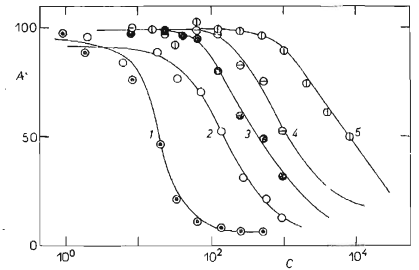

FIG. 2

Inhibition of the Binding of ^{125}I-Oxytocin to Antibodies by Some Analogues of Oxytocin Modified in the N-Terminal Part of the Molecule

A and *c* are explained in Fig. 1. *1* Deaminooxytocin, *2* deamino-1-carba-oxytocin, *3* deamino-6-carba-oxytocin, *4* deamino-dicarba-oxytocin, *5* deamino-1-carba-oxytocin sulfoxide.

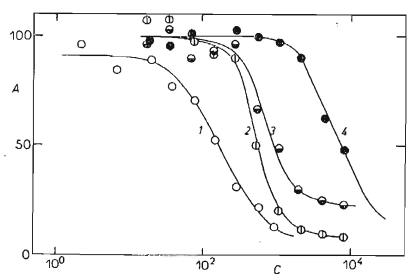

FIG. 3

Inhibition of the Binding of ^{125}I-Oxytocin to Antibodies by Analogues of Deamino-1-carba--oxytocin with Modifications in Position 4

A and *c* are explained in Fig. 1. *1* Deamino-1-carba-oxytocin, *2* [4-isoleucine]deamino-1--carba-oxytocin, *3* [4-glutamic acid]deamino-1-carba-oxytocin, *4* [4-leucine]deamino-1-carba--oxytocin.

1 and 6. Deaminooxytocin had only slightly decreased affinity to oxytocin antibodies (Fig. 1). The substitution of the sulfur atom in the disulfide bridge by a methylene group (carba-1-oxytocin in Fig. 1) resulted in a more pronounced decrease. The combination of the two modifications led to a further decrease in affinity. It is apparent from Fig. 2 that the affinity of the analogue depends on the position in which the substitution is performed. The substitution of the sulfur atom of cysteine in position 6 decreased the affinity to a greater extent (Table I). When the electronegativity of the bridge was increased by the oxidation of sulfur in position 6 to form a sulfoxide, the affinity of the analogue to the antibodies decreased markedly. We also investigated the effect of the substitution of glutamine in position 4 of the peptide chain and tyrosine in position 2. The presence of a free carboxyl group in position 4 decreased the affinity of the analogue four times (compare deamino-1-carba-oxytocin

TABLE I

Specificity of Oxytocin Antibodies

The values express the affinity to the individual analogues in percentages of the affinity to oxytocin.

Compound	Immunochemical reactivity
Oxytocin	100
[8-Arginine]vasopressin	0·060
[8-Lysine]vasopressin	0·0045
[8-Arginine]vasotocin	0·025
Deaminooxytocin	50
Deamino-1-carba-oxytocin	6·25
Deamino-6-carba-oxytocin	1·56
Deamino-dicarba-oxytocin	0·75
Carba-1-oxytocin	9·1
[4-Isoleucine]deamino-1-carba-oxytocin	3·1
[4-Glutamic acid]deamino-1-carba-oxytocin	1·58
[4-Leucine]deamino-1-carba-oxytocin	0·042
Deamino-1-carba-oxytocin sulfoxide	0·1
[2-O-Methyltyrosine]oxytocin	20
[2-O-Methyltyrosine]deamino-1-carba-oxytocin	0·33
[2-O-Methyltyrosine]deamino-6-carba-oxytocin	0·126
[2-Methionine]deamino-6-carba-oxytocin	0·11
[2-Isoleucine]deamino-6-carba-oxytocin	0·042
N^{α}-Acetyl-[2-O-methyltyrosine]oxytocin	0·055

and [4-glutamic acid]deamino-1-carba-oxytocin). The fact that the affinities of the three analogues modified in position 4 differed greatly (Fig. 3 and Table I) indicated that the antibodies were very sensitive to changes in this part of the hormone molecule.

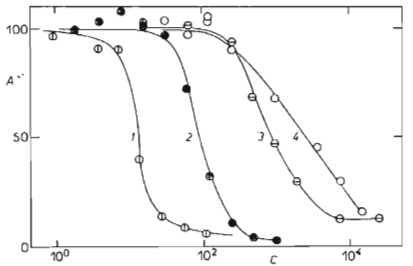

FIG. 4

Inhibition of the Binding of ^{125}I-Oxytocin to Antibodies by Oxytocin Analogues with Methyltyrosine in Position 2

A and *c* are explained in Fig. 1. *1* Oxytocin, *2* [2-O-methyltyrosine]oxytocin, *3* [2-O-methyltyrosine]deamino-1-carba-oxytocin, *4* [2-O-methyltyrosine]deamino-6-carba-oxytocin).

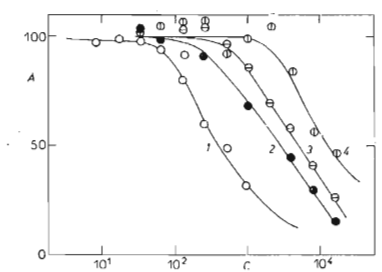

FIG. 5

Inhibition of the Binding of ^{125}I-Oxytocin to Antibodies by Analogues of Deamino-6-carba-oxytocin with Modifications in Position 2 of the Peptide Chain

A and *c* are explained in Fig. 1. *1* Deamino-6-carba-oxytocin, *2* [2-O-methyltyrosine]deamino--6-carba-oxytocin, *3* [2-methionine]deamino-6-carba-oxytocin, *4* [2-isoleucine]deamino-6-carba--oxytocin.

Analogues with methylated tyrosine (Fig. 4) had 5–20 times lower affinity than nonmethylated analogues. A similar effect on the affinity as that produced by the methylation of tyrosine in position 2 was observed when tyrosine was substituted by methionine or isoleucine (Fig. 5). The substitution of the primary amino group of [2-O-methyltyrosine]oxytocin by an acetyl group led to a further 500fold decrease of the affinity to antibodies.

DISCUSSION

The possibility of preparing defined immunogenic forms[34] of biologically active peptides enables the preparation of antibodies with increased specificity for a chosen part of the molecule. This approach was used in the preparation of antibodies against [8-D-arginine]deaminovasopressin; in this case, [8-D-arginine]vasopressin, bound by the primary amino group of cysteine to a high-molecular-weight carrier, was used as a hapten[35,36]. When antibodies against oxytocin bound by its primary amino group of cysteine in position 1 were prepared, it was expected that they would have a high affinity to deaminooxytocin. The data published on antibodies against [8--arginine]vasopressin and [8-D-arginine]vasopressin[15,16,35,36] state that they had a slightly higher affinity to deamino derivatives. By contrast, antibodies against oxytocin had half the affinity to the deaminoderivative, as compared with oxytocin. Data obtained in different laboratories concerning the affinity of oxytocin analogues to antibodies can be compared only when the immunogenic form of the hormone used is the same. In the case of the natural analogues of oxytocin, *i.e.* vasotocin and vasopressins, the cross-reactivity of which has been tested by many authors and found to equal 1–0·1%, we obtained lower values (less than 0·1%). The decreased ability of deaminooxytocin to react with antibodies we observed was also published by Czernichov and coworkers[4]. As far as the carba analogues are concerned, we found only one value[4] for deamino-dicarba-oxytocin; it had 25 times lower affinity than oxytocin. In our experiments, the value was even lower (130 times). The comparison of the affinities of monocarba analogues shows that the character of the bridge responsible for the cyclic structure of the peptide also influences the binding to antibodies. The two sulfur atoms are not equivalent in this respect. For the binding to antibodies, it is more important that the electronegative atom be in position 6 than in position 1 (Table I). To our knowledge, data concerning the other analogues we studied have not been published. The following conclusions can be drawn from Table I and Figs 3–5: The substitution of glutamine in position 4 by glutamic acid or isoleucine slightly decreases the affinity, whereas the substitution by leucine decreases the affinity 100 times. It would be necessary to study a greater number of analogues with this type of modification in order to elucidate this question. The last group of analogues we studied were substituted in position 2. [2-O-Methyltyrosine]oxytocin had 5 times lower affinity than oxytocin. The affinity of deamino-carba analogues of [2-O-methyl-

tyrosine]oxytocin was still lower (10–20 times); the deamination and carba substitution resulted in a greater decrease of affinities when it concerned [2-O-methyltyrosine]-oxytocin than oxytocin. The methylation of the OH group of tyrosine influenced the interaction with antibodies to the same extent as the substitution of tyrosine by methionine or isoleucine. A similar decrease of affinity was observed in the case of N^{α}-acetyl[2-O-methyltyrosine]oxytocin. Our results show that the antibodies against oxytocin are more sensitive to modifications in position 1 (deamination and carba modification) of the molecule than the antibodies against vasopressin prepared in the same way.

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