PEPTIDE HORMONE ANTIBODIES. CROSS REACTIVITY OF VASOPRESSIN ANALOGUES WITH RABBIT ANTIBODIES AGAINST [8-LYSINE]VASOPRESSIN

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The antibodies against [8-lysine]vasopressin were obtained by immunisation of rabbits with conjugates of the hormone with different carriers — bovine serumalbumin, porcine immunoglobulin, poly-L-lysine. The antibodies prepared were used for the study of immunochemical specificity using a set of vasopressin analogues. The changes of the basic amino acid in position 8 (L-arginine, D-arginine, L- and D-homoarginine and L-ornithine) were with the exception of bulky substituents (L-tosylarginine, L-benzyloxycarbonylornithine) without considerable effect on the affinity to the antibodies. Aminoacylation of the primary aminogroup of cystein of [8-lysine]vasopressin by a mono-, di-, or triglycyl substituent slightly changed the affinity to the antibodies in accordance with the length of the substituent's peptidic chain. Oxytocin and its analogues generally had a lower affinity by three orders of ten.

Antibodies to neurohypophysial hormones are valuable tools for studying these hormones. As is reviewed in papers[1,2], the antibodies to [8-lysine]vasopressin and to [8-arginine]vasopressin with different affinities and specificities were obtained by the immunisation of different animal species (mostly rabbits) by various antigens. In our preceding papers[3,4] we presented the results of the immunisation of pigs and an immunochemical study of antibodies to [8-arginine]vasopressin. This paper describes the immunisation of a group of 12 rabbits by an antigenic form of [8-lysine]-vasopressin and presents the results of an immunochemical study of the rabbit serum with the highest titer.

EXPERIMENTAL

Material. [8-Lysine]vasopressin with a pressoric potency of 240—260 I.U./mg was obtained by the purification of a commercial product purchased from Léčiva according to the procedure published in paper[5]. [8-Lysine]de-9-glycineamide vasopressin was prepared by the tryptic digestion of [8-lysine]vasopressin[6]. All the other analogues were synthesized at the Department of Organic Synthesis of this Institute and are summarized in Table I. Porcine immunoglobulin was isolated according to Franěk and Lankaš[7], poly-L-glutamic acid was prepared by the poly-

merisation of γ-benzyl-N-carboxyl glutamate anhydride, which was synthesized from γ-benzyl glutamate[8,9]. Bovine serum albumin was purchased from the Institute of Serum and Vaccines, Prague, poly-L-lysine was obtained from Koch-Light Labs, Colnbrook, England.

Preparation of antigens. The following conjugates were used as antigens: conjugate of [8-lysine]-vasopressin with bovine serum albumin (antigen A), conjugate of [8-lysine]vasopressin and porcine immunoglobulin (antigen B), conjugate of [8-lysine]de-9-glycineamide vasopressin with poly-L-lysine (antigen C). All the antigens were prepared by means of the carbodiimide reaction, as described elsewhere[3,4]. The conjugate of [8-lysine]vasopressin with poly-L-glutamic acid (antigen D) was prepared by the carbodiimide reaction in dimethylformamide (DMFA). Hormone (2·9 mg) dissolved in 1 ml of DMFA was mixed with 10 mg of poly-L-glutamic acid dissolved in 1 ml of DMFA and 5 mg of dicyclohexyl carbodiimide were then added. After 4 h of stirring at 4°C, the mixture was dialysed 24 h at 4°C against a 0·9% solution of NaCl.

Immunisation. Rabbits were immunised by a mixture of the individual conjugates with Al-Span-Oil adjuvant[10]. The injection was applied *s.c.* in the vicinity of the lymphatic nodes, *i.e.* into the paws and back. One immunisation dose (containing 2—4 mg of antigen) was applied in 3—4 places, and the immunisation was run for 1·5—7 months; an example of the immunisation procedure is given in Table II. Blood samples were taken from the ear vein 10 days after immunisation.

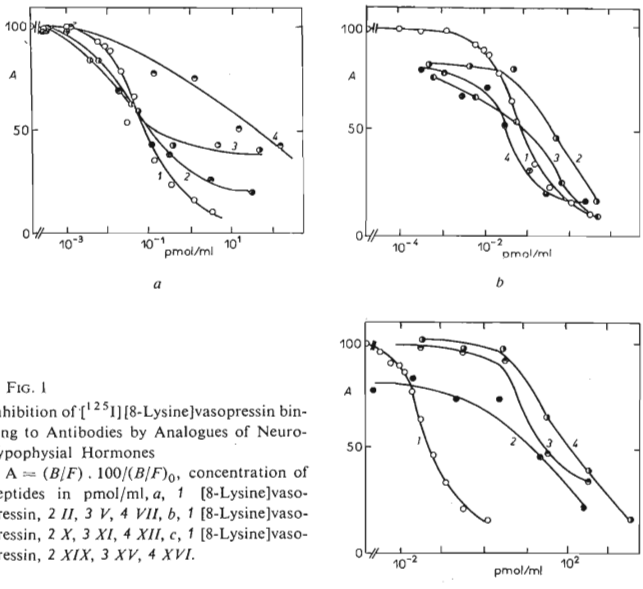

FIG. 1

Inhibition of [^{125}I][8-Lysine]vasopressin binding to Antibodies by Analogues of Neurohypophysial Hormones

$A = (B/F) \cdot 100/(B/F)_0$, concentration of peptides in pmol/ml, *a*, *1* [8-Lysine]vasopressin, *2 II*, *3 V*, *4 VII*, *b*, *1* [8-Lysine]vasopressin, *2 X*, *3 XI*, *4 XII*, *c*, *1* [8-Lysine]vasopressin, *2 XIX*, *3 XV*, *4 XVI*.

TABLE I
Survey of the Analogues Studied

No	Compound	Structure of N-terminal part	Amino acids in position 3 and 4	Structure of C-terminal tripeptide	References
I	[8-L-lysine]vasopressin	Cys-Tyr └	-Phe-Gln-	-Pro-Lys-GlyNH$_2$	—
II	[8-L-arginine]vasopressin	Cys-Tyr- └	-Phe-Gln	Pro-Arg-GlyNH$_2$	—
III	[8-L-arginine]deaminovasopressin	CH$_2$—CH$_2$—CO-Tyr S———S—	-Phe-Gln	-Pro-Arg-GlyNH$_2$	11
IV	[8-D-arginine]deaminovasopressin	CH$_2$—CH$_2$—CO-Tyr- S———S—	-Phe-Gln-	-Pro-[D-Arg]-GlyNH$_2$	12
V	[1,6-α-deaminocystathionine, 8-L-arginine]vasopressin	CH$_2$—CH$_2$—CO-Tyr- S———CH$_2$—	-Phe-Gln	-Pro-Arg-GlyNH$_2$	13
VI	[8-L-homoarginine]deaminovasopressin	CH$_2$—CH$_2$—CO-Tyr- S———S—	-Phe-Gln	-Pro-Har-GlyNH$_2$	14
VII	[1,6-α-deaminocystathionine, 8-L-tosylarginine]vasopressin	CH$_2$—CH$_2$—CO-Tyr S———CH$_2$—	-Phe-Gln	-Pro-Arg(Tos)-GlyNH$_2$	13
VIII	[1,6-α-deaminocystathionine, 8-L-ornithine]vasopressin	CH$_2$—CH$_2$—CO-Tyr- S———CH$_2$—	-Phe-Gln-	-Pro-Orn-GlyNH$_2$	13
IX	[1,6-α-deaminocystathionine, 8-L-benzyloxycarbonylornithine]vasopressin	CH$_2$—CH$_2$—CO-Tyr- S———CH$_2$—	-Phe-Gln	Pro-Orn(Cbz)-GlyNH$_2$	13
X	N^a-glycyl-[8-L-lysine]vasopressin	Gly-Cys-Tyr- └	-Phe-Gln-	Pro-Lys-GlyNH$_2$	15
XI	N^a-glycylglycyl-[8-L-lysine]vasopressin	Gly-Gly-Cys-Tyr └	-Phe-Gln-	Pro-Lys-GlyNH$_2$	16
XII	N^a-glycylglycylglycyl-[8-L-lysine]vasopressin	Gly-Gly-Gly-Cys-Tyr- └	-Phe-Gln	Pro-Lys-GlyNH$_2$	16

XIII	[8-L-lysine]de-9-glycineamide-vasopressin	Cys-Tyr-	-Phe-Gln	Pro-Lys	16
XIV	[2-L-phenylalanine, 8-L-lysine]vasopressin	Cys-Phe-	-Phe-Gln	Pro-Lys-$GlyNH_2$	17
XV	[3-L-isoleucine, 8-L-leucine]vasopressin	Cys-Tyr-	-Ile-Gln	Pro-Leu-$GlyNH_2$	—
XVI	[3-L-isoleucine, 8-L-leucine]-deamino-vasopressin	CH_2—CH_2—CO-Tyr- S——S—	-Ile-Gln-	Pro-Leu-$GlyNH_2$	18
XVII	[1,6-α-deaminocystathionine, 3-L-isoleucine, 6-L-leucine]vasopressin	CH_2—CH_2—CO-Tyr- S——CH_2—	-Ile-Gln	Pro-Leu-$GlyNH_2$	19
XVIII	[1,6-β-deaminocystathionine 3-L-isoleucine, 8-L-leucine]vasopressin	CH_2—CH_2—CO-Tyr CH_2——S—	-Ile-Gln-	Pro-Leu-$GlyNH_2$	20
XIX	[3-L-isoleucine]vasopressin (1,6)hexapeptide amide	Cys-Tyr-	-Ile-Gln	—	21
XX	[3-L-isoleucine, 4-L-leucine, 8-D-arginine]vasopressin	Cys-Tyr	-Ile-Leu-	Pro-[D-Arg]-$GlyNH_2$	22
XXI	[3-L-isoleucine, 4-L-leucine, 8-D-arginine]deaminovasopressin	CH_2—CH_2—CO-Tyr- S——S—	-Ile-Leu-	Pro-[D-Arg]-$GlyNH_2$	23

TABLE II

The Development of Antibody Titer in the Serum of Two Rabbits Immunised by Different Antigens for a Period of 32 Weeks

Immunisation	I	II	III	IV	V	VI	VII
			Rabbit 11				
Type of antigen applied	B	B	B	B	C	A	A
Antibody titer	—	—	1 : 50	1 : 20	1 : 25	1 : 600	1 : 1 000
			Rabbit 12				
Type of antigen applied	A	A	A	B	C	A	A
Antibody titer	—	—	1 : 5	1 : 50	1 : 60	1 : 2 000	1 : 2 500

Ten days after the rabbits received the last injection of antigen, blood was collected by a cardiac puncture. The serum was separated, diluted 2—10 times by saline, divided into small amounts (0·2—1·0 ml), frozen and stored at —20°C.

Detection of antibodies. The ability of the serum to bind radioactively labelled [8-lysine]vasopressin or [8-arginine]vasopressin was estimated. The labelling of hormones by [^{125}I] and the radioimmunological assay were described in one of our previous papers[3].

RESULTS

The immunisation of rabbits by only one antigen, regardless of the type selected (in total, 5 immunisation doses applied during 15 weeks), produced antibodies with a titer lower than 1 : 200. By combining the antigens, increasing the number of applied doses and prolonging the immunisation period to 32 weeks, antibodies with a titer of 1 : 1 000—2 500 were obtained (the titer of the antibodies gives a dilution of the serum at which 50% of the labelled hormone is bound to antibodies in the reaction). The antibodies with the highest titer obtained were used for the cross-reaction study of [8-lysine]vasopressin and a series of analogues of neurohypophysial hormones. The ability of the individual analogues to compete with iodinated [8-lysine]vasopressin in the binding to antibodies was estimated. The curves characterizing the dependence of $(B/F) \cdot 100/(B/F)_0$ on the log of analogue concentration are presented in Figs 1*a—c*. The concentrations of analogues resulting in a 50% decrease of the binding of the labelled hormone to antibodies are presented in Table III.

As can be seen in Table III, the rabbit antibodies are almost insensitive to changes in position 8 of the hormone chain (if the basic character of the amino acid in position

8 is preserved). The replacement of L-lysine by L-arginine or D-arginine or L-ornithine resulted only in a small decrease of affinity of the analogue to the antibodies. A remarkable decrease of affinity was observed when a bulky substituent in position 8 was present (a tosylated or benzyloxycarboxylated basic amino acid). The deamination of the hormone in position 1 and replacement of the sulphur atom in the bridge by a methylene group had no considerable effect on the affinity. All the amino acylated analogues studied had the ability to compete with the hormone in binding to antibodies, the highest ability was found for N^{a}-glycyl-glycyl-glycyl[8-lysine]vasopressin. Only when present in an excess concentration (1400 and higher), were oxytocin and its analogues able to inhibit the binding of [8-lysine]vasopressin to antibodies.

DISCUSSION

We immunised three groups of rabbits, each group consisting of four animals, for different periods of time by conjugates of [8-lysine]vasopressin with different carriers- some of protein nature, the others were non-antigenic homopolymers of amino acids. Antibodies with a higher titer were obtained from animals that were subjected to longer immunisation periods. The radioimmunoassay for [8-lysine]vasopressin was developed using serum with a titer of 1 : 2500 (sensitivity 7 – 12 fmol of [8-lysine]-vasopressin/ml). The sensitivity of the assay performed with other sera was lower by 1 – 2 orders of ten.

TABLE III

Immunochemical Reactivity of [8-Lysine]vasopressin Analogues with *anti*-[8-Lysine]vasopressin Antibodies

The values presented give the ratios of analogues and [8-lysine]vasopressin causing 50% inhibition of binding of the labelled hormone to antibodies.

Compound	Immunochemical reactivity	Compound	Immunochemical reactivity	Compound	Immunochemical reactivity
I	1[a]	*VIII*	2·5	*XV*	1 400
II	1·4	*IX*	3 000	*XVI*	[b]
III	2·2	*X*	2	*XVII*	2 500
IV	1·7	*XI*	1	*XVIII*	[b]
V	1	*XII*	0·7	*XIX*	380
VI	2	*XIII*	6	*XX*	100 000
VII	340	*XIV*	100	*XXI*	34 000

[a] The concentration of [8-lysine]vasopressin causing 50% inhibition of binding of labelled vasopressin to antibodies is equal to 0·047 pmol/ml. [b] 50% inhibition of binding of the labelled hormone to antibodies was not observed.

In comparison with porcine antibodies to [8-arginine]vasopressin[3], rabbit anti[8-lysine]vasopressin antibodies had higher cross-reactivity with the individual analogues and inhibitory curves for the analogues were not parallel. It was not possible to achieve total replacement of the labelled hormone by some analogues. Nor was it possible due to this fact to characterize the antigenic determinants for [8-lysine]vasopressin in the same way as in the case of [8-arginine]vasopressin[3]. [8-Lysine]vasopressin can be bound to the carriers *via* two primary amino groups. Two different antigenic determinants are formed, which is reflected by the heterogeneity of antibodies. The heterogeneity is manifested by a lower specificity for the analogues investigated, by the non-parallel inhibitory curves of the individual analogues and the inability of certain analogues to replace [8-lysine]vasopressin totally in its binding to antibodies. Similar heterogeneity was observed by Czernichow and coworkers[23]. In spite of this drawback, some structural features of analogues affecting the binding affinity to antibodies were found. The insensitivity of antibodies to changes in position 8 of the peptide chain may reflect the fact that the synthesis of a certain antibody population was stimulated by an antigen in which the hormone was bound *via* its ε-amino group of L-lysine. The differences in affinity were observed only in the series with bulky substituents in position 8. The deamination of vasopressin analogues or the substitution of the sulphur atom by a methylene group had a negligible effect on the decrease of affinity. However the substitution of the primary amino group of cystein by an amino acid residue brought about a pronounced change of affinity of the analogue to antibodies. With the prolongation of this substituent (diglycyl or triglycyl) the affinity was considerably enhanced. A similar relation was observed in this series of analogues and *anti* [8-arginine]-vasopressin antibodies[3] where the change of affinity of the compounds tested is attributed to changes of molecular conformation which are more pronounced after the introduction of one glycyl (the interaction of NH_2 of glycine with the ring part of molecule) than after increasing the distance of the amino group from the ring. The fact that triglycyl-[8-lysine]vasopressin is more effective in replacing the labelled hormone than [8-lysine]vasopressin can be explained by the character of the antigens containing hormones bound *via* the primary amino group of cystein. Such antigens stimulate the synthesis of antibodies with a higher affinity to deamino analogues of the hormone or to an analogue with a NH_2-group sufficiently remote from the rest of molecule (triglycyl-[8-lysine]vasopressin).

While oxytocin had a 1000 times lower affinity for both types of antibodies (*anti*-[8-lysine]- and [8-arginine]vasopressin) significant differences were observed in the case of its deamino- and deamino-carba analogues. In comparison with oxytocin these analogues had half the affinity to [8-lysine]vasopressin antibodies and a 30 times lower affinity to [8-arginine]vasopressin.

The cumulation of several structural changes (*e.g.* the replacement of glutamine in position 4 by leucine and of phenylalanine in position 4 by isoleucine in [8-D-argi-

nine]deamino-vasopressin) led to a complete loss of affinity of the resulting analogue to antibodies.

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