THE AMINO AND DEAMINO ANALOGUES OF 8-D-HOMOARGININE-VASOPRESSIN WITH *p*-SUBSTITUTED PHENYLALANINE IN POSITION 2 IN ACTIVATION OF FACTOR VIII AND TISSUE PLASMINOGEN ACTIVATOR IN PRIMATES

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The replacement of L-Tyr in dDAVP by L-Tyr(Me), L- or D-Phe(pMe), L- or D-Phe(pEt) in combination with substitution of D-arginine by D-homoarginine resulted in dissociation of Factor VIII and tPA activating properties or in abilities to decrease the levels of circulating Factor VIII and tPA levels in blood of squirrel monkeys.

In our recent communications on Factor VIII (F VIII) activation by vasopressin analogues, in particular by [D-Arg[8]]deaminovasopressin* (dDAVP) in humans and some animal species, we documented several limitations in our attempts to generalize the results obtained[1–3]. We found different time-courses of F VIII elevation after administration of dDAVP in man and dog and differences in the maximal responses obtained in various species studied. Finally identical structure–activity relationships were not always found when a vasopressin analogue was tested in the species studied[4].

The dDAVP (ref.[5]) is the only vasopressin analogue successfully used in patients with mild or moderate hemophilia A or von Willebrand's disease during either bleeding episodes or prophylactically[6,7]. This compound is the drug of choice for the treatment of diabetes insipidus[8–10] and related problems connected with genetically of surgically conditioned inability to biosynthesize vasopressin. The strong and protracted anti-

* All chiral amino acids belong to the L-series, unless otherwise stated. The nomenclature and symbols of the amino acids, their derivatives and peptides obey the published IUPAC recommendations (Eur. J. Biochem. *138*, 9 (1984)). Har denotes the homoarginine moiety, Phe(pEt) the *p*-ethylphenylalanine, Phe(pMe) the *p*-methylphenylalanine moiety and pNA denotes *p*-nitroanilide.

diuretic effect is not the only side effect accompanying the use of dDAVP for the treatment of hemophilia A. Tachycardia, facial flush, a feeling of pressure in the head and the heat sensations have all been reported in association with dDAVP infusion[11,12].

By evaluating the structure–activity relationship for vasopressin analogues in dogs we have determined the basic structural features which preserve the F VIII activating properties of vasopressin analogues in dogs[3]; namely they are the deamination of the peptide molecule and the presence of a strongly positive charge on the amino acid in position 8 of the vasopressin molecule as the intact length of the amino acid chain. Our demonstration of the ability of dDAVP to enhance F VIII level in primates[2] led us to continue in structure–activity studies in squirrel monkeys. Based on our knowledge of the antidiuretic potencies of L- and D-arginine homologues of dDAVP where the antidiuretic potency is closely related to the position of a guanidine group in the side chain of the amino acid at position 8 (both L- and D-stereoisomers)[13] we selected [D-Har^8]deaminovasopressin[14] as a parent peptide for further modifications. This choice also complies with the experimentally confirmed structural requirements, mentioned above for F VIII activating properties in dogs[15]. In order to change the selectivity of the endocrine properties of dDAVP and [D-Har^8]deaminovasopressin we designed and synthesized analogues of the latter compound with modifications in position two, which normally lead to a change in these peptides[16–19] from agonistic properties to antagonistic ones. L-Tyrosine, in both the vasopressin and deaminovasopressin series, was replaced by stereoisomers of *p*-methyl- and *p*-ethylphenylalanines of *O*-methyltyrosine. Evaluation of the biological properties of these analogues revealed a significant decrease of antidiuretic properties in comparison to [D-Har^8]deaminovasopressin, as well as the appearance of antagonistic properties (to vasopressin in the blood pressure assay and to oxytocin in the rat uterus assay).

In this study we present the results on the F VIII and tPA releasing properties of these analogues when tested in squirrel monkeys.

EXPERIMENTAL

Materials

Analogues of dDAVP were synthesized, purified and biologically evaluated as described in previous communications[16–19]. The dDAVP (ref.[5]), [D-Arg^8]vasopressin[20] and [D-Orn^8]deaminovasopressin[21] were supplied by Ferring Pharmaceuticals, Malmö, Sweden. Examined peptides together with their abbreviations used in this paper are presented in Table I.

Factor VIII Estimation

The Kabi Diagnostic Method (Coatest, Stockholm, Sweden), employing a chromogenic substrate Bz-Ile-Glu-(OR)-Gly-Arg-pNA, was used as previously described[1] with a slight modification. Monkey blood plasma (10 μl) was diluted with 1 200 μl of buffer working solution, and the level of Factor VIII was estimated according to the instructions given by the producer.

Tissue Plasminogen Activator Estimation

This estimation was performed according to the instructions of the producer (Kabi, ref.[22]) with the modification that only 25 μl of plasma was used for the isolation of the euglobulin fraction.

Animals

Squirrel monkeys of both sexes (300 – 400 g), caged in pairs, were used for the experiments. They were anesthetized with Ketalar (0.6 ml) i.m. and the individual peptides were given i.m. in the front of the thigh. Blood (200 μl) was obtained before and 30 min after the injection of peptides through the puncture of a popliteal vein, and collected in Microvettes (Sarstedt, Nümbrecht–Rommelsdorf, Germany) coated with EDTA. The samples were placed on ice and immediately centrifuged for 5 min at 4 °C. Blood plasma was kept at –80 °C until assayed

RESULTS

It has already been documented[1,3] that some vasopressin analogues may, depending on their primary structure, evoke a transient decrease in the level of F VIII. In addition, the responsiveness of the animals to the peptides varies not only among animals, but also

TABLE I
A review of vasopressin analogues used in this study: Z-X-Phe-Gln-Asn-Cys-Pro-Y-Gly-NH_2

Analogue	Abbreviation	Z	X	Y
[8-D-Arginine]vasopressin	[D-Arg^8]VP	Cys	L-Tyr	D-Arg
[8-D-Homoarginine]vasopressin	[D-Har^8]VP	Cys	L-Tyr	D-Har
[2-L-*p*-Methylphenylalanine, 8-D-homoarginine]vasopressin	[L-$Phe(pMe)^2$,D-Har^8]VP	Cys	L-Phe(pMe)	D-Har
[2-D-*p*-Methylphenylalanine, 8-D-homoarginine]vasopressin	[D-$Phe(pMe)^2$,D-Arg^8]VP	Cys	D-Phe(pMe)	D-Har
[2-L-*O*-Methyltyrosine, 8-D-homoarginine]vasopressin	[L-$Tyr(Me)^2$,D-Har^8]VP	Cys	L-Tyr(Me)	D-Har
[8-D-Arginine]deaminovasopressin	dDAVP	Mpa	L-Tyr	D-Arg
[8-D-Ornithine]deaminovasopressin	[D-Orn^8]dVP	Mpa	L-Tyr	D-Orn
[8-D-Homoarginine]deaminovasopressin	[D-Har^8]dVP	Mpa	L-Tyr	D-Har
[2-L-*p*-Methylphenylalanine, 8-D-homoarginine]deaminovasopressin	[L-$Phe(pMe)^2$,D-Har^8]dVP	Mpa	L-Phe(pMe)	D-Har
[2-D-*p*-Methylphenylalanine, 8-D-homoarginine]deaminovasopressin	[D-$Phe(pMe)^2$,D-Har^8]dVP	Mpa	D-Phe(pMe)	D-Har
[2-L-*p*-Ethylphenylalanine, 8-D-homoarginine]deaminovasopressin	[L-$Phe(pEt)^2$,D-Har^8]dVP	Mpa	L-Phe(pEt)	D-Har
[2-L-*O*-Methyltyrosine, 8-D-homoarginine]deaminovasopressin	[L-$Tyr(Me)^2$,D-Har^8]dVP	Mpa	L-Tyr(Me)	D-Har

in that the basal levels of F VIII and tPA change over time, as seen during two years of observation[4]. These variations contribute to a higher scatter of experimental values. Tables II and III give the mean values of F VIII and tPA increments following administration of a number of vasopressin analogues.

TABLE II
Biological activities of deaminovasopressin analogues with modification in positions 2 and 8

Compound	AD[a]	BP[a]	tPA	F VIII	*N*[b]
dDAVP	12 000[c] 100%[d]	0	293 ± 51	323 + 83	4
[D-Orn^8]dVP	79 ± 10[c] 1%[d]	8.58 ± 1.38	144 ± 15	103 + 6	4
[D-Har^8]dVP	5%[d]	0.28	88 ± 5	127 ± 13	5
[L-Phe(pMe)^2,D-Har^8]dVP	0.02%[d]	pA_2 = 6.2[e]	67 ± 6	155 ± 36	3
[D-Phe(pMe)^2,D-Har^8]dVP	0.02%[d]	0.0	82 ± 15	112 ± 13	8
[L-Phe(pEt)^2,D-Har^8]dVP	0.02%[d]	pA_2 = 6.2	79.4	11.5	3
[D-Phe(pET)^2,D-Har^8]dVP	0.02%[d]	pA_2 = 6.35	102	96.5	2
[L-Tyr(Me)^2,D-Har^8]dVP	n.d.[f]	0.0	116.5	98.5	2

[a] Activity IU/mg, AD denotes the antidiuretic activity, BP the blood pressor activity, tPA the fibrinolytic activity and F VIII the Factor VIII activation. [b] Number of experiments related to tPA and F VIII determination. [c] Antidiuretic activity determined according to ref.[25]. [d] Antidiuretic activity (in brackets) of dDAVP estimated according to ref.[13] was laid equal to 100%. [e] pA_2 parameter characterizing the intensity of inhibition, see refs[26,27]. [f] Not determined.

TABLE III
Biological activities of vasopressin analogues with modification in positions 2 and 8

Compound	AD[a]	BP[a]	tPA	F VIII	*N*[b]
[D-Arg^8]VP	629 ± 65[c] 1.7%[d]	1.46	157 ± 45	124.5 ± 9.2	6
[D-Har^8]VP	43.5	0.204	61 ± 4	85.6 ± 3.8	6
[L-Phe(pMe)^2,D-Har^8]VP	0.1%[d]	0.04	77	85	3
[D-Phe(pMe)^2,D-Har^8]VP	0.1%[d]	0.04	65	55	3
[L-Tyr(Me)^2,D-Har^8]VP	n.d.[e]	0.04	126.5	114	3

[a,b,c,d] See Table II. [e] Not determined.

Some peptides which have been recently tested in dogs[3] confirm, that molecules F VIII activation properties depend on a strongly positively charged group in position 8 of the peptide chain of the amino acid (compare the F VIII properties of dDAVP and [D-Arg^8]VP (Table II) with those of [D-Orn^8]dVP). Deamination in position 1 seems to potentiate the F VIII activating properties of the peptide (compare [D-Arg^8]VP and dDAVP). A shift of the guanidine group by one methylene group further from the peptide backbone significantly decreases the F VIII releasing activity of vasopressin derivatives (compare [D-Arg^8]VP with [D-Har^8]VP), however the decrease in the deamino series (dDAVP and [D-Har^8]dVP) observed was even higher (see Table II). In the vasopressin series no change occurred when Tyr in position 2 was substituted by stereoisomers of *p*-methylphenylalanine (see Table III). Surprisingly, replacement of *p*-methyl with a methoxy group causes a slight enhancement of F VIII releasing properties. In the deamino series of D-homoarginine vasopressin modifications in position 2 are reflected by an increase of F VIII properties in one case, i.e. with L-*p*-methylphenylalanine derivative. The other substitutions decreased the F VIII activating properties as compared to [D-Har^8]dVP (Table II).

After analyzing the structural demands for potentiation of tPA activities of the vasopressin analogues studied, it is evident that the loss of primary amino group from the cysteine in position 1 results in an increase of tPA activity (see Table II). The insertion of D-Har in the place of D-Arg sharply decreases tPA releasing potencies ([D-Arg^8]VP and [D-Har^8]VP, Table III). In the [D-Har^8]vasopressin series the modifications performed in position 2 (Table III) diminish the impact of shifting the positively charged group further from the peptide backbone. In the deamino series modifications such as insertion of L-*p*-methylphenylalanine or L-*p*-ethylphenylalanine potentiates the effect of D-Har in position 8. Only in two types of substitutions that of tyrosine with either *O*-methyltyrosine or D-*p*-ethylphenylalanine could be observed a partial compensation of the decrease evoked by the changing position 8. Moreover, comparing the effects following deamination in positions 2 and 1 it appears that these modifications attenuate the impact of changes in position 8.

To summarize substitutions either in three positions of AVP or in two positions of dDAVP one can observe that the performed changes generally affected the tPA activities more strongly than the F VIII activating properties of the peptides. We discovered compounds which had a pronounced inhibitory effect on both activities (e.g. [D-Phe(pMe)^2,D-Har^8]VP). At the same time we found a compound with dissociated properties (i.e. [L-Phe(pMe)^2,D-Har^8]deaminovasopressin) with decreased tPA activity and stimulated F VIII activation.

DISCUSSION

The ability of dDAVP to activate F VIII and tPA is well documented[23–25]. The dDAVP, which was originally introduced as a potent antidiuretic agent for the treatment of pa-

tients suffering from diabetes insipidus[8], serves to enhance F VIII and fibrinolytic activity (tPA), if given in doses which by one order of magnitude exceed those recommended for establishing water balance in patients with diabetes insipidus. The occasional side effects which accompany F VIII elevation create a therapeutical demand for a drug with selective F VIII releasing properties.

Results obtained in dogs experiment allowed us to draw some conclusions concerning the basic structural requirements of the vasopressin molecule to sustain the F VIII activating properties[3]. All these structural requirements were also found to be of importance for preserving the antidiuretic potency of the analogues tested. We subsequently tried to reduce the antidiuretic activity by synthesizing a series of dDAVP homologues, compounds in which the D-arginine in position 8 was replaced by lower or higher homologues of this amino acid. The antidiuretic potency was found to be rather sensitive to these changes, and one such homologue of dDAVP [D-Har[8]]deaminovasopressin, retained only 5% of the antidiuretic potency of dDAVP, while 50% of the weak pressoric activity of dDAVP remained intact. Our preliminary experiments in dogs with [D-Har[8]]dVP indicated a partial preservation of F VIII activating properties compared to dDAVP (ref.[15]). On the basis of this finding and keeping in mind that the cardiovascular effects could be significant (as in the case of dDAVP when larger doses of the peptide are used), we designed and synthesized a series of 8-D-homoarginine analogues of vasopressin and deaminovasopressin modified in position 2 of the molecule, one of the critical sites for the selectivity of the biological effects of vasopressin and oxytocin. In both series of peptides the alterations in position 2 practically eliminated the antidiuretic potency of dDAVP. In addition, the D-Har homologues lost more than 90% of their pressoric potency through this modification of the parent molecules. In some cases the analogues proved to be inhibitory to vasopressin in the blood pressure assay, having pA_2 values between 6.2 and 6.5 (refs[16–19]). All the analogues had pronounced inhibitory effects to oxytocin in the rat uterus in vitro assay. The deamino derivatives with the voluminous substituent in the *para*-position of phenylalanine proved to be the strongest inhibitors described so far.

It should be noted that all but one of the deaminopeptides modified in position 2 exhibited F VIII releasing activity lower than that of the parent peptide [D-Har[8]]dVP. The sole exception was the derivative with L-*p*-methylphenylalanine in position 2 and because its tPA activity was substantionally reduced by the structural modifications, it has become a compound of potential interest. The tPA activities of all the peptides had already been strongly influenced by the insertion of D-Har instead of D-Arg. In the amino series, the various modifications introduced at position 2 partly restored the tPA releasing properties, while in deamino series only derivatives with D-*p*-ethylphenylalanine or L-*O*-methyltyrosine partly enhanced tPA activating properties of the peptides. The remaining three substituents contributed to a further decrease of tPA activity. It is worth noticing the peptides with a pronounced inhibitory effect on both the coagu-

lating and fibrinolytic systems of the blood. These are the parent molecule [D-Har[8]]vasopressin and analogues with methyl substituents in *para*-position of L- and D-phenylalanine. They differ from other derivatives in bringing about small agonistic effects in the blood pressure assay, while the remaining peptides in this series are inhibitory to vasopressin in this assay. Concerning the tPA activity the existence of a guanidine group in the side chain of the basic amino acid in position 8 seems to be a prerequisite for further design and synthesis of potentially useful vasopressin analogues in the selective stimulating on the fibrinolytic system.

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REFERENCES

1. Vilhardt H., Barth T., Falck J., Nilsson I. M.: Thrombosis Res. *47*, 585 (1987).
2. Vilhardt H., Barth T.: *Proc. IVth Conf. Neurohypophysis, Copenhagen, July 1989*, p. 165. Oxford University Press, Oxford 1989.
3. Vilhardt H., Barth T.: Receptor Res. *11*, 233 (991).
4. Vilhardt H., Barth T.: Exp. Clin. Endocrinol. (Life Sci. ed.) *11*, 183 (1992).
5. Zaoral M., Kolc J., Sorm F.: Collect. Czech. Chem. Commun. *32*, 1250 (1967).
6. Warrier A. I., Lusher J. M.: J. Pediatrics *102*, 228 (1983).
7. Nilsson I. M., Felding P., Timberg L., Jonsson G., Harris A. S. in: *Factor VIII (von Willebrand Factor), Biological and Clinical Advances* (N. L. Ciavarella, Z. M. Ruggerri and T. S. Zimmerman, Eds), p. 63. Wichling Editore, Milano 1986.
8. Vavra I., Machova A., Holecek V., Cort J. H., Zaoral M., Sorm F.: Lancet *I*, 948 (1968).
9. Becker D. J., Foley T. P.: J. Pediatrics *92*, 1011 (1978).
10. Monson J. P., Richard P.: Br. Med. J. *1*, 24 (1978).
11. Kostering H., Ehrlich U., Wieding J., Wigger W. in: *Minirin* (A. H. Sutor, Ed.), p. 171. Schattauer Verlag, Stuttgart 1980.
12. Blatter W.: Ref.[11], p. 173.
13. Skopkova J., Hrbas P., Slaninova J., Barth T.: Collect. Czech. Chem. Commun. *46*, 1850 (1981).
14. Zaoral M., Brtnik F.: Collect. Czech. Chem. Commun. *40*, 905 (1975).
15. Vilhardt H.: Unpublished results.
16. Prochazka Z., Zertova M., Barth T., Slaninova J., Maletinska L., Blaha I., Velek J. in: *Peptides 1990 Proc. 21st EPS, Platja d'Aro 1990* (E. Giralt and D. Andreu, Eds), p. 663. Escom, Leiden 1991.
17. Zertova M., Prochazka Z., Blaha I., Barth T., Slaninova J., Maletinska L., Lebl M.: Collect. Czech. Chem. Commun. *55*, 3000 (1990).
18. Zertova M., Prochazka Z., Barth T., Slaninova J., Skopkova J., Blaha I., Lebl M.: Collect. Czech. Chem. Commun. *56*, 1761 (1991).
19. Zertova M., Prochazka Z., Slaninova J., Skopkova J., Barth T., Lebl M.: Collect. Czech. Chem. Commun. *57*, 604 (1992).
20. Zaoral M., Kolc J., Sorm F.: Collect. Czech. Chem. Commun. *31*, 382 (1966).
21. Zaoral M., Brtnik F., Barth T., Machova A.: Collect. Czech. Chem. Commun. *41*, 2088 (1976).
22. Vilhardt H., Barth T.: J. Receptor Res. *11*, 239 (1991).
23. Cash J. D., Gader A. M. A., Da Costa J.: Br. J. Haematol. (Abstr.) *27*, 263 (1974).

24. Mannucci P. M., Aberg, M., Nilsson I. M., Robertson B.: Br. J. Haematol. *30*, 81 (1975).
25. Vilhardt H., Nilsson I. M. in: *Recent Progress in Pituitary Hormones* (S. Yoshida and L. Share, Eds), p. 177. Elsevier Science Publishers, New York 1988.
26. Shild H. O.: Br. J. Pharmacol. Chemotherap. *4*, 277 (1949).
27. Larson L. E., Lindenberg G., Melin P., Pliska V.: J. Med. Chem. *21*, 352 (1978).