SYNTHESIS, BIOLOGICAL ACTIVITY AND RECEPTOR BINDING AFFINITY OF TWO [8-ARGININE]VASOPRESSIN ANALOGUES WITH INHIBITORY PROPERTIES

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Dedicated to the memory of Dr Karel Bláha.

Two analogues of arginine-vasopressin: [1-(β-mercapto-β-β-cyclopentamethylenepropionic acid), 2-D-phenylalanine, 7-sarcosine, 8-arginine]vasopressin and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-phenylalanine, 7-N-methylalanine, 8-arginine]vasopressin were synthesized by solid-phase peptide synthesis method. Both peptides exhibit antioxytocic, antivasopressor and antiglycogenolytic activities, and on the other hand they are weak antidiuretic agonists. The binding affinities of both analogues to oxytocic receptor (guinea pig myometrium membranes) and to hepatic V_1 receptor (rat liver membranes) are practically the same as for the parent hormones, whereas the binding affinities to renal V_2 receptor (bovine kidney membranes) are 60—90 times lower than for vasopressin.

Potent antagonists of neurohypophyseal hormones are valuable tools to study the role of oxytocin and vasopressin in health and disease, to elucidate mechanisms of their actions, and to characterize receptor subtypes for both hormones in different tissues. In recent years a strong impetus can be seen in the design, synthesis and use of oxytocin and vasopressin antagonists[1-3]. We have synthesized many vasopressin antagonists[4-6] which retained high binding affinities to vasopressin receptors, and some of them were used in the identification and characterization of receptor subunits[5]. All of these peptides had in position 7 N-methylalanine (MeAla) or sarcosine (Sar) residues*.

* Unless stated otherwise, all chiral amino acids are of the L-series. Abbreviations used are: AVP, [8-arginine]vasopressin; Cpp, β-mercapto-β,β-cyclopentamethylenepropionic acid; Sar, sarcosine; MeAla, N-methylalanine. All other symbols follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: Eur. J. Biochem., *138*, 9 (1984).

Now, we would like to present the synthesis, biological activity and receptor binding affinity of two new vasopressin analogues: [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-phenylalanine,7-sarcosine,8-arginine]-vasopressin, ([Cpp^1,D--Phe^2,Sar^7]AVP, *IIa*) and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-phenylalanine,7-N-methylalanine,8-arginine]vasopressin, ([Cpp^1, D-Phe^2, MeAla^7]AVP, *IIb*).

It is known that the introduction of β-mercapto-β,β-cyclopentamethylenepropionic acid residue (Cpp) in position 1 of vasopressin brings about a sharp decrease of agonistic potencies and leads to compounds with inhibitory properties[7]. The combination of Cpp substitution at position 1 and the D-phenylalanine substitution at position 2 gave an analogue with high antivasopressor activity (pA_2 8·35), and with distinct antiantidiuretic activity (pA_2 7·21) as well[7]. We have previously found that the substitution of proline residue at position 7 for sarcosine or N-methylalanine residues in arginine-vasopressin have strikingly different effects on the interaction of such analogues with physiologically important V_1 and V_2 receptors[8].

Both AVP analogues, [Cpp^1, D-Phe^2, Sar^7]AVP and [Cpp^1, D-Phe^2, MeAla^7]-AVP, were obtained by solid-phase peptide synthesis[9-11] as described previously[12]. In general, the tert-butyloxycarbonyl group was used for the protection of α-amino groups, and was removed by treatment with 1·3M-HCl/AcOH. The side chain functional groups were protected as S-benzyl (Cpp and Cys residues), or as N-tosyl (Arg residue) derivatives. Coupling was accomplished by dicyclohexylcarbodiimide with the addition of N-hydroxybenztriazole, or by *p*-nitrophenyl esters for the asparaginyl and glutaminyl residues. The protected peptides were split from the resin by ammonolysis. They were deblocked with sodium in liquid ammonia and the resulting disulfhydryl derivatives were subjected to oxidative cyclization with $K_3Fe(CN)_6$. The vasopressin analogues were purified by gel filtration on Sephadex G-15.

The antiuretonic, antigalactogogic, antivasopressor, antiglycogenolytic and antidiuretic potencies of [Cpp^1, D-Phe^2, Sar^7]AVP and [Cpp^1, D-Phe^2, MeAla^7]AVP are presented in Table I. Both analogues appear to be very potent as antiglycogenolytic agents, in fact the MeAla substituted peptide is the most potent glycogenolytic antagonist reported to date. They retain high antivasopressor activity as compared to [Cpp^1, D-Phe^2]AVP[7]. However, there is a qualitative difference between the two compounds. *IIa* has a protracted inhibitory effect, with the highest intensity of inhibitory potency 10–20 min after administration. The pA_2 value of analogue *IIa* estimated from the doses of agonist administered 10–20 min after the doses of inhibitor increases from 7·80 to 8·90. The inhibitory effect persists several hours. The Sar and MeAla substituted AVP analogues are also strong antagonists of oxytocin in the uterotonic tests in vitro and in vivo.

Because the [Cpp^1, D-Phe^2]AVP was found[7] to be antidiuretic antagonist (pA_2 7·21) we have expected for the synthesized analogues *IIa* and *IIb* the inhibitory

TABLE I

Biological activities of [Cpp[1], D-Phe[2], Sar[7]]AVP and [Cpp[1], D-Phe[2], MeAla[7]]AVP

Peptide	Antiuterotonic pA_2		Antigalactogogic pA_2	Antivasopressor pA_2	Antiglycogenolytic pA_2	Antidiuretic IU/mg
	in vitro	in vivo				
[Cpp[1], D-Phe[2]]AVP[a]	8·59	—	—	8·35	—	$pA_2 = 7·21$
[Cpp[1], D-Phe[2], Sar[7]]AVP (*IIa*)	8·10	7·60	7·10	7·80[b]	9·13	$0·08 \pm 0·02$[c]
[Cpp[1], D-Phe[2], MeAla[7]]AVP (*IIb*)	8·55	7·50	—	7·72	9·37	$0·43 \pm 0·06$[c]

[a] Data taken from ref.[7]. [b] The determined pA_2 value from the doses of agonist administered 10 min after the doses of inhibitor was 8·90. [c] Estimated by Melin P., Ferring Research lab., Malmö.

TABLE II

Binding affinities of vasopressin analogues to oxytocin receptor in the guinea pig myometrium membranes and to vasopressin receptors in the rat liver membranes (V_1 receptor) and in the bovine kidney membranes (V_2 receptor)

Peptide	Oxytocin receptor		Hepatic V_1 receptor		Renal V_2 receptor	
	K_D mol l^{-1}	$\frac{K_D(\text{analogue})}{K_D(\text{OXT})}$	K_D mol l^{-1}	$\frac{K_D(\text{analogue})}{K_D(\text{AVP})}$	K_D mol l^{-1}	$\frac{K_D(\text{analogue})}{K_D(\text{AVP})}$
Oxytocin (OXT)	$(6·9 \pm 0·5) \cdot 10^{-9}$					
[8-Arginine]vasopressin (AVP)			$(6·5 \pm 0·3) \cdot 10^{-10}$		$(1·1 \pm 0·1) \cdot 10^{-9}$	
[Cpp[1], D-Phe[2], Sar[7]]AVP (*IIa*)	$(6·6 \pm 0·9) \cdot 10^{-9}$	0·96	$(9·6 \pm 1·4) \cdot 10^{-10}$	1·49	$(9·8 \pm 1·2) \cdot 10^{-8}$	89
[Cpp[1], D-Phe[2], MeAla[7]]AVP (*IIb*)	$(8·7 \pm 1·2) \cdot 10^{-9}$	1·3	$(3·1 \pm 0·6) \cdot 10^{-10}$	0·46	$(6·4 \pm 0·7) \cdot 10^{-8}$	58

potency as well. However, they were found to be very weak antidiuretic agonists.

Table II shows the binding affinities of *IIa* and *IIb* to oxytocic receptor in guinea pig myometrium membranes and to vasopressin receptors in the rat liver membranes (V_1 receptor) and in the bovine kidney membranes (V_2 receptor).

Both analogues retain a high binding affinity to uterotonic receptor and to hepatic V_1 receptor. However, there is a distinct difference between both analogues. Sarcosine substituted analogue showed higher affinity to myometric membranes than the N-methylalanine substituted analogue. On the other hand, the latter peptide had much higher affinity to hepatic V_1 receptor than the sarcosine analogue. The K_D value for [Cpp[1], D-Phe[2], MeAla[7]]AVP (*IIb*) is even lower than the K_D value of the parent hormone.

As can be seen from the data presented in Table II both analogues are characterized by the decreased binding affinities to renal V_2 receptor in comparison to AVP. The antidiuretic effect is mediated by the formation of cyclic AMP (refs.[13,14]). Therefore, it was of interest to study if both synthesized compounds have completely lost or retained some activity for stimulating the renal vasopressin sensitive adenylate cyclase. The data presented in Table III show that both *IIa* and *IIb* have completely lost the agonistic property on the kidney plasma membranes.

The discrepancy between: (i) the loss in activity for stimulating the renal vasopressin sensitive adenylate cyclase, (ii) the lack of antiantidiuretic activity, and (iii) to some extent retained binding affinity to V_2 receptors, can result from different species used in antidiuretic assay (rat) and in recetor binding assay, as well as in activation of vasopressin sensitive adenylate cyclase (bovine kidney)[15]. The different concentrations of ligands used in these three assays can also influence the experimental data presented here.

TABLE III

Activation of adenylate cyclase by [8-arginine]vasopressin and its analogues[a]

Peptide	Concentration mol . l^{-1}	V_{max}[b]
—	—	114 ± 11[c]
[8-Arginine]vasopressin	1 . 10^{-7}	604 ± 70
[Cpp[1], D-Phe[2], Sar[7]]AVP (*IIa*)	1 . 10^{-5}	111 ± 21
[Cpp[1], D-Phe[2], MeAla[7]]AVP (*IIb*)	6 . 10^{-6}	118 ± 21

[a] The maximal activation of adenylate cyclase in plasma membranes from bovine kidney was measured as described previously[8]; the concentration of peptides was about 100-fold higher than their K_D value for binding to vasopressin receptors in the same membranes. The results are the mean ± S.E. (n = 3). [b] Amount of cAMP (in pmol) per mg of protein in 10 min; [c] basal activity.

EXPERIMENTAL

The procedure of solid-phase peptide synthesis conformed to the published[12]. Chloromethylated resin (Bio-Rad, Bio-Beads SX-1) was esterified[16] by Boc-Gly to an incorporation of 0·76 mmol/g. Triethylamine was distilled from ninhydrin; dimethylformamide was distilled under reduced pressure. Other solvents and reagents were of analytical grade. Thin-layer chromatography was carried out on silica gel plates (Merck), and the products were detected by ninhydrin or iodine vapor. The following solvent systems were used: BAW, 1-butanol–acetic acid–water (4 : 1 : 5, v/v, upper phase); BAWP, 1-butanol–acetic acid–water–pyridine (15 : 3 : 3 : 10, v/v). Loads of 10—80 μg were applied, and chromatograms were of minimum length 10 cm. Melting points are uncorrected. Optical rotations were determined with a Hilger–Watts polarimeter with an accuracy of 0·01°. Samples for analytical purposes were dried over P_2O_5 in vacuo for 24 h. Analytical results were determined on a Carlo Erba Model 1 106 analyzer. For amino acid analysis, peptides (c. 0·5 mg) were hydrolyzed with constant boiling hydrochloric acid (400 μl) containing phenol (20 μl) in evacuated and sealed ampules for 18 h at 110°C. The analyses were performed on a AAA 881 Mikrotechna analyzer. Sarcosine and N-methylalanine ratios were not calculated, considering the difficulties in detection of N-methylamino acids[17]. The presence of Sar and MeAla in the structure of the synthesized peptides was determined by TLC of hydrolysates on silica gel. Two-dimensional solvent systems: (1) 1-butanol–acetic acid–water (4 : 1 : 5, v/v, upper phase); (2) phenol–water (3 : 1, v/v). Molecular ions of pure peptides were determined by mass spectrometry field desorption technique with a Varian MAT 711 instrument.

S-Benzyl-β-mercapto-β,β-cyclopentamethylenepropionyl-D-phenylalanyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteinyl-sarcosyl-N^{δ}-tosylarginyl-glycine Amide (*Ia*)

Boc-Gly-resin (1·31 g, 1 mmol) was converted in eight cycles of deprotection, neutralization and coupling[12] to 9-peptide-resin: yield 2·41 g (91%). The resin was ammonolyzed in methanol for 48 h. Following evaporation of the solvent the protected peptide was extracted into hot DMF, precipitated with boiling water and left overnight at room temperature. The product was collected by filtration, washed with water and dried over P_2O_5. Peptide was further purified by dissolving in DMF and reprecipitating with boiling EtOH: yield 601 mg (42% based on Gly); m.p. 172—174°C; $[\alpha]_D^{20}$ −12·1° (*c* 1, DMF); TLC: R_F 0·48 (BAW), 0·64 (BAWP). For $C_{70}H_{90}N_{14}O_{13}S_3$ (1 431) calculated: 58·72% C, 6·34% H, 13·70% N; found: 58·50% C, 6·51% H, 13·54% N. Mass spectrum *m/z*: 1 431.

S-Benzyl-β-mercapto-β,β-cyclopentamethylenepropionyl-D-phenylalanyl-phenylalanyl--glutaminyl-asparaginyl-S-benzylcysteinyl-N-methylalanyl-N^{δ}-tosylarginyl-glycine Amide (*Ib*)

The protected nonapeptide was synthesized in the same manner as the peptide *Ia*. Yield: 650 mg (45% based on Gly); m.p. 112—115°C; $[\alpha]_D^{20}$ −16·5° (*c* 0·66, DMF); TLC: R_F 0·54 (BAW), 0·68 (BAWP). For $C_{71}H_{92}N_{14}O_{13}S_3$ (1 445) calculated: 58·98% C, 6·41% H, 13·57% N; found: 58·63% C, 6·60% H, 13·33% N. Mass spectrum *m/z*: 1 445.

[1-β-Mercapto-β,β-cyclopentamethylenepropionic acid, 2-D-phenylalanine, 7-sarcosine,8-arginine]vasopressin (*IIa*)

The protected nonapeptide *Ia* (198 mg, 0·138 mmol) was dissolved in 400 ml of ammonia freshly distilled from sodium and treated at the boiling point, with stirring, with sodium from a stick of the metal contained in a small bore glass tube, until a light blue color persisted in the solution for 20 s. The color was discharged by addition of NH_4Cl, and the clear solution was evaporated.

The residue was dissolved in N_2-flushed 0·2% aqueous AcOH, and aqueous ammonia (2M) was added gradually to give a solution of pH 7. The solution was treated with 0·02M-$K_3Fe(CN)_6$ until a permanent yellow color was observed and stirred for next 20 min. Anion-exchange resin (Amberlite IR 45, acetate form) was added to remove the ferri- and ferrocyanide ions. The mixture was filtered through a bed of the resin, and the filtrate was lyophilized. The residue was desalted on Sephadex G-15 column (100 × 2·5 cm), eluting with 50% AcOH with a flow rate of 4 ml/h. The eluate was monitored at 280 nm and fractionated. The major peak was pooled and lyophilized, and the residue (38 mg) was further subjected to gel filtration on Sephadex G-15 column (120 × 1·2 cm), eluting with 0·2M-AcOH with a flow rate of 2·5 ml/h. The [8-arginine]vasopressin analogue (29·8 mg, 19·7%) was isolated from the fractions comprising the single symmetrical peak by lyophilization. $[\alpha]_D^{20}$ −86·0° (*c* 0·5, 1M-AcOH); TLC: R_F 0·26 (BAW), 0·31 (BAWP). Mass spectrum *m/z*: 1 095. Amino acid analysis: Phe 2·05, Glu 0·98, Asp 1·02, Arg 1·04, Gly 1·00.

[1-β-Mercapto-β,β-cyclopentamethylenepropionic acid,2-D-phenylalanine, 7-N-methylalanine,8-arginine]vasopressin (*IIb*)

The peptide derivative *Ib* (180 mg, 0·124 mmol) was reduced by sodium in liquid ammonia, oxidized to the disulfide, and purified by gel filtration on Sephadex G-15 as detailed above in the preparation of compound *IIa*: yield 22 mg (16%); $[\alpha]_D^{20}$ −92·0° (*c* 0·5, 1M-AcOH); TLC: R_F 0·25 (BAW), 0·34 (BAWP). Mass spectrum *m/z*: 1 109. Amino acid analysis: Phe 1·98, Glu 1·04, Asp 0·97, Arg 0·93, Gly 1·00.

Pharmacological Methods

Uterotonic activity was determined on an isolated strip of rat uterus[18,19], the activity in vivo was estimated according to Pliška[20]. Galactogogic activity was determined on ethanol anesthetized rats[21,22], pressor activity on despinalized rats[23] and antidiuretic potency on anesthetized rats[24,25]. Inhibitory properties were expressed by the pA_2 value[26] from the determined effective doses. The antiglycogenolytic potency of both analogues on isolated hepatocytes was determined from their capacity to shift the dose-response curve of phosphorylase activation by AVP to higher hormone concentration[27].

Receptor Binding Assays: Plasma membranes from rat liver containing 2—3 pmol V_1 receptor/mg protein were prepared by modification[8] of the method of Lesko et al.[28] using two-phase polymer system. Plasma membranes from bovine kidney inner medulla were prepared by differential centrifugation followed Percoll density gradient centrifugation as described previously[29,30]. Membrane preparations obtained by this procedure had a specific binding capacity of 4—6 pmol [^{3}H]AVP/mg protein. Plasma membranes derived from the myometrium of guinea pig in late pregnancy were prepared following the procedure described for rat uterine and mammary membranes[31].

The K_D values of AVP analogues and of oxytocin and vasopressin were obtained from competition binding experiments. Plasma membranes from rat liver or bovine kidney (50 μg) were equilibrated at 30°C for 30 min with 10 nmol [^{3}H] AVP and varying concentrations of the non-labelled peptides. The binding assay with rat liver membranes contained bacitracin (1 mg/ml). The binding assay with guinea pig myometrium membranes consisted of 50 mmol Hepes, pH 7·6 containing 10 mmol $MnCl_2$. The tests for binding of [^{3}H]oxytocin (20 Ci/mmol, Ammersham) and for competition experiments with 10 nmol [^{3}H]oxytocin and unlabelled analogues were performed as described for the binding of AVP to renal and hepatic vasopressin receptors[8].

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REFERENCES

1. Lebl M. in: *Handbook of Neurohypophyseal Hormone Analogs* (K. Jošt, M. Lebl and F. Brtník, Eds), Vol. II, Part 1, p. 17. CRC Press, Boca Raton 1987, and references cited therein.
2. Manning M., Sawyer W. H. in: *Vasopressin* (R. W. Schrier, Ed.), p. 131. Raven Press, New York 1985.
3. Sawyer W. H., Manning M. in: *Oxytocin: Clinical and Laboratory Studies* (J. A. Amico and A. G. Robinson, Eds), p. 423. Elsevier, Amsterdam 1985.
4. Gazis D., Schwartz I. L., Lammek B., Grzonka Z.: Int. J. Pept. Protein Res. *23*, 78 (1984).
5. Fahrenholz F., Kojro E., Müller M., Boer, R. Löhr R., Grzonka Z.: Eur. J. Biochem. *161*, 321 (1986).
6. Kruszyński M., Lammek B., Manning M., Seto J., Haldar J., Sawyer W. H.: J. Med. Chem. *28*, 364 (1980).
7. Manning M., Olma A., Kliś W. A., Seto J., Sawyer W. H.: J. Med. Chem. *28*, 1607 (1983).
8. Fahrenholz F., Boer R., Crause P., Fritsch G., Grzonka Z.: Eur. J. Pharmacol. *100*, 47 (1984).
9. Merrifield R. B.: J. Am. Chem. Soc. *85*, 2149 (1963).
10. Merrifield R. B.: Biochemistry *3*, 1385 (1964).
11. Manning M.: J. Am. Chem. Soc. *90*, 1348 (1968).
12. Grzonka Z., Lammek B., Kasprzykowski F., Gazis D., Schwartz I. L.: J. Med. Chem. *26*, 555 (1983).
13. Chase L. R., Aurbach G. D.: Science *159*, 545 (1968).
14. Guillon G., Butlen D., Cantau B., Barth T., Jard S.: Eur. J. Pharmacol. *85*, 291 (1982).
15. Stassen F. L., Bryan W., Gross M., Kavanagh B., Shue D., Sulat L., Wiebelhaus V. D., Yim N., Kinter L. B. in: *The Neurohypophysis: Structure, Function and Control*, Progress in Brain Research, (B. A. Cross and G. Leng, Eds), Vol. 60, p. 395. Elsevier, Amsterdam 1983.
15. Gisin B. F.: Helv. Chim. Acta *56*, 1476 (1973).
17. Yamashiro D., Li C. H., Tseng L. F., Loh H. H.: Int. J. Pept. Protein Res. *11*, 251 (1978).
18. Holton P.: Brit. J. Pharmacol. *3*, 328 (1948).
19. Munsick R. A.: Endocrinology *66*, 451 (1960).
20. Pliška V.: Eur. J. Pharmacol. *5*, 253 (1969).
21. Bisset G. W., Clark B. J., Haldar J., Harris M., Lewis G. P., Roche e Silva M.: Brit. J. Pharmacol. Chemotherap. *31*, 537 (1967).
22. Barth T., Jošt K., Rychlík I., Endocrinol. Exper. *9*, 35 (1975).
23. Krejčí I., Kupková B., Vávra I.: Brit. J. Pharmacol. Chemotherap. *30*, 497 (1967).
24. Jeffers W. A., Livezey M. M., Austin J. H.: Proc. Soc. Exp. Biol. Med. *50*, 184 (1942).
25. Pliška V., Rychlík I.: Acta Endocrinol. *54*, 129 (1967).
26. Slaninová J. in: Handbook of Neurohypophyseal Hormone Analogs (K. Jošt, M. Lebl and F. Brtník, Eds), Vol II, Part 1, p. 83. CRC Press, Boca Raton 1987.
27. Keppens S., De Wulf H.: Biochim. Biophys. Acta *588*, 63 (1979).
28. Lesko L., Donlon M., Marinetti G. V., Hare J. D.: Biochim. Biophys. Acta *311*, 173 (1973).
29. Crause P., Fahrenholz F.: Mol. Cell. Endocrinol. *28*, 528 (1982).
30. Fahrenholz F., Crause P.: Biochem. Biophys. Res. Commun. *122*, 974 (1984).
31. Fuchs A. R., Periyasamy S., Alexandrova M., Soloff M. S.: Endocrinology *113*, 742 (1983).