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PHARMACOLOGICAL CHARACTERIZATION OF LINEAR ANALOGUES OF VASOPRESSIN GENERATED BY THE SYSTEMATIC SUBSTITUTION OF POSITIONS 1 AND 6 BY L-AMINO ACIDS

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Abstract—Eighteen linear analogues of [Arg^8]vasopressin (AVP) were synthesized by systematically substituting the cysteine residues at positions 1 and 6 with a range of L-amino acids. Screening by competition ligand binding revealed that the combinations of amino acid residues tolerated at these positions was very restricted with respect to retention of vasopressin receptor (VPR) binding. Consequently, only three of the eighteen analogues investigated, [Pro^1,Met^6]AVP, [Gly^1,Met^6]AVP and [Phe^1,Lys^6]AVP, bound to the V_{1a} receptor. Furthermore, these three peptides were all selective for the V_{1a} receptor rather than the V_{1b}, V_2 and vasotocin receptors. In addition, although very homologous to the natural agonist, these analogues were in fact antagonists at V_{1a} receptors. These data provide insights into the biophysical requirements at positions 1 and 6 of linear ligands for binding to V_{1a} receptors and furthermore, supply clues to the nature of the receptor:ligand interaction.

Key words: ligand; receptor; subtypes; peptide; vasotocin; oxytocin

The peptide hormone [Arg^8]vasopressin has a disulphide bond between Cys^1 and Cys^6. However, Manning *et al.* [1] demonstrated that this cyclic conformation is not a pre-requisite for ligands to bind to VPRs‖. Subsequently, many linear ligands have been synthesized, some of which are subtype-selective. Such ligands have been used as selective radioiodinated labels [2, 3] and for the development of heterofunctional ligands [4–6]. Although a range of structurally diverse, non-amino acids, has been employed successfully at position 1 [7–10], it has been reported that amino acids *per se* are not tolerated at this position [9].

In this study, positions 1 and 6 of AVP have been systematically substituted with L-amino acids to generate eighteen related analogues possessing a wide diversity of side-chain characteristics at these two positions. Pharmacological characterization of these peptides revealed that only very restricted combinations of substitution are compatible with binding to VPRs but not with retention of agonism. Furthermore, the data provide clues to future ligand design and to the nature of VPR:ligand interaction.

MATERIALS AND METHODS

Peptide synthesis. Eighteen related peptides (Fig. 1) were synthesized on a 5 μmol scale using *N*-α-Fmoc-protected amino acid pentafluorophenyl esters on 4-(2′,4′-Dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin (Novabiochem, Nottingham, U.K.). Peptides were deprotected and cleaved from the

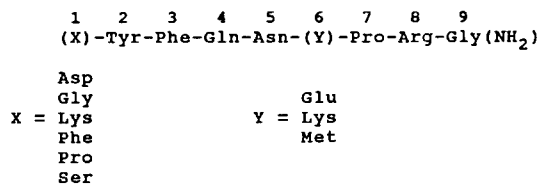

Fig. 1. Structural formulae of synthetic analogues of AVP. Linear peptides were produced substituting positions 1 (X) and 6 (Y) with the amino acids shown using all possible 18 combinations. The natural agonist AVP has Cys at these positions. In contrast to AVP, these linear peptides lack the conformational restraint of a disulphide bond between these two Cys residues at positions 1 and 6.

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‖ Abbreviations: AVP, [8-arginine]vasopressin; [d(CH$_2$)$_5$Tyr(Me)^2] AVP, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine, 8-arginine]-vasopressin; VT, vasotocin; VPR, vasopressin receptor; K_d, dissociation constant; pK_d, $-\log[K_d(M)]$; TFA, trifluoroacetic acid; EDT, ethanedithiol. Linear vasopressin analogues: [Gly^1,Met^6]AVP, [1-glycine, 6-methionine, 8-arginine]-vasopressin; [Phe^1,Lys^6]AVP, [1-phenylalanine, 6-lysine, 8-arginine]vasopressin; [Pro^1,Met^6]AVP, [1-proline, 6-methionine, 8-arginine]vasopressin.

resin with 95% (v/v) TFA/5% (v/v) EDT (12 hr) and lyophilized to yield the appropriate peptide amide. These peptides were dissolved in 0.05% (v/v) acetic acid at a concentration of 1 mM and stored frozen at −20°. Larger scale synthesis of [Phe^1,Lys^6]AVP, [Gly^1,Met^6]AVP and [Pro^1,Met^6]AVP used similar methodology but on a 0.2–0.5 mmol scale and the amino acid content was then confirmed by analysis. Peptides were purified and analysed by reverse-phase HPLC as previously described [5, 11].

Ligand binding assays. Membrane preparations of rat liver (expressing V_{1a}VPR) and kidney medulla (expressing V_2 VPR) were as described previously [4, 5, 11]. Ovine anterior pituitary membranes were prepared according to the method of Shen *et al.* [12]. An enriched membrane fraction of the kidney of adult Pekin ducks was prepared by a minor modification of the method of Schütz *et al.* [13]. Briefly, fresh avian kidneys were washed, minced and the crude homogenate washed through nylon sieves with mesh apertures of 200, 150 and 105 μm using cold buffer (30 mM Tris/HCl, 100 mM NaCl, 20 mM sucrose, 0.1 mM phenylmethylsulphonylfluoride; pH 7.4). At mesh apertures of 150 and 105 μm, the superficially adherent nephrons were homogenized and centrifuged (45,000 *g*, 20 min, 4°). The upper layer of the resulting pellet was homogenized in cold buffer to a final concentration of 0.6 mg/mL.

Competition binding assays were optimized for the receptor type and the tissue source. Binding assays on rat liver membranes [11, 14, 15] used both [^{3}H]AVP and the labelled V_{1a}-selective antagonist [^{3}H][d(CH$_2$)$_5$Tyr(Me)^2]AVP ([1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine]arginine vasopressin [16]) at concentrations of 0.3–1.4 nM and 0.4–0.9 nM respectively. Assays with rat kidney medulla membranes [4, 5, 11] used [^{3}H]AVP at 0.6–1.9 nM. Binding analysis of synthetic peptides to ovine pituitary membranes was performed according to the method of Shen *et al.* [12] using [^{3}H]AVP as a tracer ligand at a final concentration of 1 nM. Similar studies on the duck kidney vasotocin receptor were performed at 22° for 90 min using [^{3}H]AVP at a concentration of 2 nM according to the previously described receptor binding assay protocol [13].

Binding data were analysed using non-linear regression (Fig. 2 'Fig.P' software, Biosoft) and dissociation binding constants (K_d) were calculated from IC_{50} values [17] using experimentally determined dissociation constants for the affinity of [^{3}H][d(CH$_2$)$_5$Tyr(Me)^2]AVP, K_d V_{1a} = 0.28 nM [4, 5, 11]; [^{3}H]AVP K_d V_{1b} = 1.9 nM [12].

Measurement of inositol phosphate production in WRK-1 cells. The rat mammary tumour cell line WRK-1, which expresses a well characterized V_{1a} VPR [18], was cultured and labelled with myo-[2-^{3}H]inositol (10 μCi/mL; 108.4 Ci/mmol, NEN Du Pont Ltd, Herts, U.K.) for 3 days as previously described [4, 19]. Cells, in 35 mm culture dishes, were washed with, and transferred into, a medium containing 0.44 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 138 mM NaCl, 8.1 mM Na_2PO_4 and 10 mM $LiCl_2$ [4, 18]. Vasopressin antagonists were added 20 min after washing at the concentrations indicated and AVP (1 nM) was added 10 min later. Incubations were terminated with $HClO_4$ (5% w/v final concentration) after a further 20 min. A mixed inositol phosphate fraction containing inositol mono-, bis- and tris-phosphates was resolved by elution from a 0.8 mL Bio-Rad AG1-X8 column with 10 mL 0.8 M NH_4COOH/0.1 M HCOOH having first eluted inositol and glycerophosphoinositol with 10 mL water and 60 mM NH_4COOH respectively.

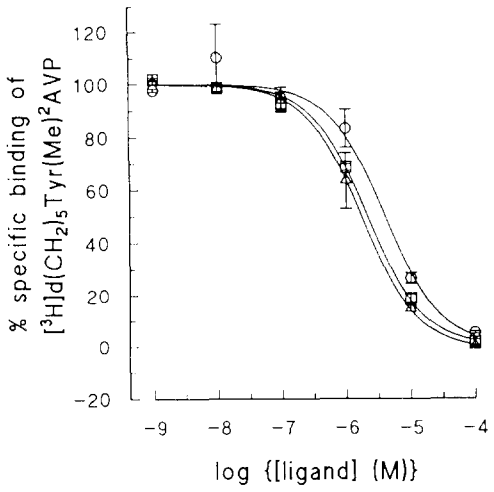

Fig. 2. Competition of [^{3}H][d(CH$_2$)$_5$Tyr(Me)^2]AVP binding to the V_{1a} vasopressin receptor by linear vasopressin analogues. Rat liver membranes were incubated with [^{3}H][d(CH$_2$)$_5$Tyr(Me)^2]AVP (0.4–0.9 nM) and various concentrations of unlabelled peptides: △, [Pro^1,Met^6]AVP; □, [Gly^1,Met^6]AVP; ○, [Phe^1,Lys^6]AVP. Points are mean values (±SEM) from a single typical experiment performed in triplicate. Values for the specific binding of [^{3}H]-[d(CH$_2$)$_5$Tyr(Me)^2]AVP in the presence of unlabelled peptide are expressed as a percentage of the specific binding in the absence of competing ligand. Curves represent the best fit of a simple Langmuir isotherm to the experimental data ('Fig.P.', Biosoft).

RESULTS

Pharmacological characterization using ligand binding studies

Linear analogues of AVP were synthesized with either an acidic (Glu), basic (Lys) or hydrophobic (Met) residue at position 6 instead of the naturally occurring Cys. For each of these substitutions at position 6, six analogues were synthesized with different amino acid residues at position 1 as detailed in Fig. 1. The position 1 substitutions were selected so as to provide a diverse range of side-chain characteristics at the N-terminus. This generated a series of eighteen homologous linear AVP analogues with systematic substitutions at positions 1 and 6.

All of the peptides in the series were screened for their ability to bind to VPRs. This was done by competition ligand binding studies as described in

Table 1. Comparative binding affinities of linear vasopressin analogues for neurohypophysial hormone receptor subtypes

Ligand	Receptor	pK_d value			
		V_{1a}	V_2	V_{1b}	VT
AVP		9.2	8.9	8.7	8.7
$[d(CH_2)_5Tyr(Me)^2]AVP$		9.6	7.2	5.4	5.3
$[Pro^1,Met^6]AVP$		6.6	<4	4.4	<4
$[Gly^1,Met^6]AVP$		6.2	<4	5.1	<4
$[Phe^1,Lys^6]AVP$		5.9	<4	5.3	<4

pK_d values are calculated from experimentally determined mean values of the dissociation binding constant (K_d) at rat V_{1a}, rat V_2, ovine V_{1b} and duck vasotocin (VT) receptor subtypes. A value of <4 indicates that a concentration of peptide $\leq 10\,\mu M$ failed to displace the appropriate [^{3}H]tracer ligand from its receptor. Shown for comparison are data for the agonist AVP and the cyclic V_{1a}-selective antagonist $[d(CH_2)_5Tyr(Me)^2]AVP$.

Materials and Methods. A final linear analogue concentration of 1–10 μM was employed with rat liver and kidney medulla membranes which express V_{1a} and V_2 receptors respectively. These initial studies revealed that three of the peptides competed with both [^{3}H]AVP and [^{3}H][$d(CH_2)_5Tyr(Me)^2$]AVP binding to the rat liver V_{1a}VPR *viz.* $[Pro^1,Met^6]AVP$, $[Gly^1,Met^6]AVP$ and $[Phe^1,Lys^6]AVP$. The remaining fifteen analogues did not bind to the V_{1a} receptor. At the concentrations tested, no synthetic peptide competed for [^{3}H]AVP binding to the rat kidney medulla V_2 VPR. The three peptides cited above were then synthesized on a larger scale to facilitate a more detailed pharmacological characterization.

Full competition binding curves for the three selected linear ligands are presented in Fig. 2. The affinity (K_d) of the analogues for the V_{1a} receptor was calculated as $2.75 \pm 0.50 \times 10^{-7}$ M, $6.88 \pm 0.85 \times 10^{-7}$ M and $1.32 \pm 0.08 \times 10^{-6}$ M (mean ± SEM, N = 3) for $[Pro^1,Met^6]AVP$, $[Gly^1,Met^6]AVP$ and $[Phe^1,Lys^6]AVP$, respectively. When binding studies were performed with pituitary membranes, all of these peptides competed for [^{3}H]AVP binding, however the affinity that they exhibited for the ovine V_{1b} receptor was consistently lower than that for the V_{1a} receptor (Table 1). In addition, the rank order of potency of the synthetic analogues for the V_{1b} receptor also differed from that observed with the V_{1a} receptor (Table 1). When tested at rat V_2 receptors and avian vasotocin receptors, none of the three analogues competed with [^{3}H]AVP binding, even when present at a concentration of 10 μM, thereby indicating a very low affinity at these receptor subtypes (Table 1).

Effect of selected linear peptides on AVP-stimulated inositol phosphate accumulation in WRK-1 cells

It can be seen from Table 2 that AVP (1 nM) produced a characteristic increase in the accumulation of [^{3}H] inositol phosphates in WRK-1 cells. All three synthetic analogues of AVP were devoid of agonist activity as, even at a saturating concentration of 10–50 μM, application of these peptides alone had no effect upon basal inositol phosphate levels (Table 2). However, when these concentrations of the synthetic peptides $[Pro^1,Met^6]AVP$, $[Gly^1,Met^6]AVP$ and $[Phe^1,Lys^6]AVP$ were employed in conjunction with AVP, they completely blocked the AVP-stimulated inositol phosphate accumulation. In contrast, $[Asp^1,Glu^6]AVP$ present at either 1 μM or 50 μM had no effect on AVP-stimulated inositol phosphate accumulation (Table 3). $[Asp^1,Glu^6]AVP$ did not compete for [^{3}H]AVP in the binding studies described above, so it would not be expected to prevent receptor-mediated effects of AVP. The results presented in Table 3 demonstrate that the antagonism of AVP-induced inositol phosphate production by $[Pro^1,Met^6]AVP$, $[Gly^1,Met^6]AVP$ and $[Phe^1,Lys^6]AVP$ was not due to a non-specific

Table 2. Effect of synthetic peptides on inositol phosphate accumulation in WRK-1 cells

Treatment	Accumulation of mono-, bis-, and tris-phosphates (10^{-3} × dpm/plate)
Control (no addition)	4.32 ± 0.48
AVP	12.09 ± 0.36
$[d(CH_2)_5Tyr(Me)^2]AVP$	4.83 ± 0.73
AVP + $[d(CH_2)_5Tyr(Me)^2]AVP$	4.75 ± 0.22
$[Pro^1,Met^6]AVP$	5.14 ± 1.17
AVP + $[Pro^1,Met^6]AVP$	4.56 ± 0.24
$[Gly^1,Met^6]AVP$	3.76 ± 0.38
AVP + $[Gly^1,Met^6]AVP$	4.37 ± 0.05
$[Phe^1,Lys^6]AVP$	4.67 ± 0.44
AVP + $[Phe^1,Lys^6]AVP$	5.34 ± 0.93

AVP (1 nm) stimulated a significant accumulation of labelled inositol phosphates. The effect of AVP was completely antagonized by the cyclic V_{1a}-selective antagonist $[d(CH_2)_5Tyr(Me)^2]AVP$ (1 μM) and the linear analogues $[Pro^1,Met^6]AVP$ (10 μM), $[Gly^1,Met^6]AVP$ (50 μM) and $[Phe^1,Lys^6]AVP$ (50 μM). Values are means (±SEM) from a single representative experiment performed in triplicate.

Table 3. Lack of effect of the peptide $[Asp^1,Glu^6]AVP$ on inositol phosphate accumulation in WRK-1 cells

Treatment	Accumulation of mono-, bis-, and tris-phosphates (10^{-3} × dpm/plate)
Control (no addition)	2.04 ± 0.07
AVP	6.65 ± 0.44
AVP + $[Asp^1,Glu^6]AVP$ (1 μM)	6.96 ± 0.92
AVP + $[Asp^1,Glu^6]AVP$ (50 μM)	7.43 ± 0.31

AVP-stimulated inositol phosphate accumulation was assayed in the presence and absence of $[Asp^1,Glu^6]AVP$ as described in Materials and Methods. Values are means (±SEM) from a single representative experiment performed in triplicate. The AVP concentration was 1 nM in all cases.

cytotoxic effect of synthetic AVP analogues on WRK-1 cells. Taken together these data demonstrate that [Pro^1,Met^6]AVP, [Gly^1,Met^6]AVP and [Phe^1,Lys^6]AVP were all antagonists at the V_{1a} receptor.

DISCUSSION

Our data show that linear analogues of AVP synthesized entirely from L-amino acids can bind to V_1 receptors. However, the combinations of residues at positions 1 and 6, which retain the peptides' ability to recognize VPRs, are very restricted. The hydrophobic/uncharged residues Phe, Pro and Gly were tolerated at position 1 but the polar, basic and acidic residues (Ser, Lys and Asp respectively) were not. This correlates well with the non-amino acid substituents previously employed at position 1 which have included adamantaneacetyl, phenylacetyl and tert-butylacetyl [7, 8]. Although varying considerably in bulk, these moieties all share an hydrophobic nature. G-protein-coupled receptors which have small amines as their agonists bind their ligands deep in the hydrophobic regions of the receptor [20, 21]. Given the architectural similarities of the V_{1a} receptor to these biogenic amine receptors [22], the hydrophobic/uncharged requirement of the N-terminus could be due to this part of the ligand binding to the receptor within the hydrophobic transmembrane domains. An acidic residue at position 6 seems incompatible with VPR binding in contrast to Lys^6 or Met^6 substitutions. However, the requirements for binding are not merely a hydrophobic/uncharged group at position 1 and Lys^6/Met^6, as out of six such peptides only three recognized VPRs. Hence it can be concluded that the nature of the moiety tolerated at position 1 is critically dependent on the residue at position 6 and *vice versa* (Table 1).

The three analogues which recognized V_{1a} receptors were antagonists (Table 2) and had an affinity 2–3 orders of magnitude lower than AVP ($K_d = 0.68$ nM [11]). However, all high affinity V_{1a} receptor linear antagonists reported to date have D-Tyr^2 or D-Tyr(Et/Me)^2 residues [2, 7–10]. Consequently, it would be predicted that substitution of L-Tyr^2 with D-Tyr(Et)^2 would increase our peptides' affinity. The rationale for employing L-Tyr^2 rather than D-Tyr(Et)^2 in this study was to ensure that the possibility of synthesizing V_{1a} receptor linear agonists was not precluded. Although linear V_2 receptor agonists have been reported with D-Tyr^2 [3] these are V_{1a} receptor antagonists. Despite this precaution, none of the analogues synthesized were agonists (Table 2). A much earlier observation [23] that the peptide [Ala^1,Ala^6]AVP has only extremely weak pressor activity (*ca.* 20,000-fold less than AVP) is consistent with these data.

From the data presented in Table 1, it can be seen that V_{1a} receptors have the least stringent structural requirements for binding, as V_2 and VT receptors did not bind any of the linear analogues. Linear peptides did however bind to V_{1b} receptors but with lower affinity and different rank order than V_{1a} receptors.

In conclusion, systematic substitution of the residues at positions 1 and 6 in AVP has provided clues to the complex structural requirements for linear peptides to bind to VPRs.

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