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Tyr115 is the key residue for determining agonist selectivity in the V1a vasopressin receptor

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Using a three-dimensional model of G protein-coupled receptors (GPCR), we have previously succeeded in docking the neurohypophysial hormone arginine-vasopressin (AVP) into the V1a receptor. According to this model, the hormone is completely embedded in the transmembrane part of the receptor. Only the side chain of the Arg residue at position 8 projects outside the transmembrane core of the receptor and possibly interacts with a Tyr residue located in the first extracellular loop at position 115. Residue 8 varies in the two natural neurohypophysial hormones, AVP and oxytocin (OT); similarly, different residues are present at position 115 in the different members of the AVP/OT receptor family. Here we show that Arg8 is crucial for high affinity binding of AVP to the rat V1a receptor. Moreover, when Tyr115 is replaced by an Asp and a Phe, the amino acids naturally occurring in the V2 and in the OT receptor subtypes, the agonist selectivity of the V1a receptor switches accordingly. Our results indicate that the interaction between peptide residue 8 and the receptor residue at position 115 is not only crucial for agonist high affinity binding but also for receptor selectivity.

Key words: agonist selectivity/dDAVP/molecular modelling/receptor mutagenesis/vasopressin receptor

Introduction

G protein-coupled receptors (GPCRs) belong to a family of structurally related proteins activated by a broad spectrum of molecules that includes biogenic amines, nucleotides, prostaglandins, small peptides and large glycoproteins. Due to a huge variation in the chemical nature of the ligands, different mechanisms probably account for specific receptor–ligand interactions (Strosberg, 1991; Ostrowski *et al.*, 1992; Savarese and Fraser, 1992; Schwartz, 1994).

The arginine-vasopressin/oxytocin (AVP/OT) receptor family is particularly suitable to investigate structure/function relationships among GPCRs and small peptide ligands. First of all, different receptor subtypes of this receptor family have been described in vertebrates and invertebrates. Recently, several of these receptors have been cloned in mammals: the OT receptor (Kimura *et al.*, 1992; Gorbulev *et al.*, 1993), the V1a (Morel *et al.*, 1992; Wheatley *et al.*, 1993; Thibonnier *et al.*, 1994), V1b (Sugimoto *et al.*, 1994) and V2 vasopressin receptors (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Gorbulev *et al.*, 1993). A related Arg8-vasotocin (AVT) receptor has also been cloned from a teleost fish (Mahlmann *et al.*, 1994). From sequence comparisons and three-dimensional (3-D) modelling, clues to regions crucial for agonist binding and selectivity might now be deduced. Finally, extensive pharmacological characterization of these receptors exists. In general, they are activated by closely related nonapeptides characterized by a cyclic backbone (Berde and Boissonnas, 1968; Hruby and Smith, 1987; Acher, 1993). In human and rat, two hypothalamic hormones, AVP and OT, bind to and activate their respective receptors in a selective way. The two hormones differ at position 3, in the cyclic part of the peptide, and at position 8, in the C-terminal tripeptide. A large variety of peptide analogues has been synthesized and analysed to assess the contribution of single residues of these peptides to their functional properties (Hruby and Smith, 1987; Hruby and Chow, 1990; Manning and Sawyer, 1993). However, these analogues have usually been assayed for their biological activity on intact tissues and only limited data is available on binding properties and efficacy on specific receptor subtypes.

Here, we have evaluated the contribution of peptide positions 3 and 8 to high affinity binding and receptor selectivity by studying the pharmacological profile of several peptide analogues on the cloned rat V1a (Morel *et al.*, 1992), human V2 (Birnbaumer *et al.*, 1992) and human OT receptors (Kimura *et al.*, 1992). Our results indicate that the two residues exert different effects in mediating the high affinity binding of AVP and OT to these three receptor subtypes. Interestingly, only residue 8 appears to play a crucial role in high affinity binding of AVP to the V1a receptor.

To understand the molecular basis of ligand binding and selectivity, we have analysed the possible chemical interactions between hormone and receptor residues. We took advantage of a general model of AVP/V1a receptor interaction based on both computer modelling and site-directed mutagenesis. According to this model, which was validated by point mutation of residues located in transmembrane regions 2, 3, 4 and 6, the AVP binding domain is situated in the transmembrane core of the receptor, as described for the cationic neurotransmitter receptors (Mouillac *et al.*, manuscript in preparation).

Interestingly, the exocyclic part of AVP fills the upper part of the binding pocket, and the side chain of residue 8 is in the direct neighbourhood of the first extracellular loop. We postulate here that an interaction might take place between the side chain of Arg8 in AVP and a Tyr residue in position 115 in the first extracellular loop of the V1a receptor. This interaction might contribute significantly to the high affinity binding of AVP to the V1a receptor. Since different amino acids are present at the corresponding position in the different members of the AVP/OT receptor family, we hypothesize that this hormone/receptor interaction might also contribute to agonist selectivity. By substituting Tyr115 in the V1a receptor with a Leu or with the amino acids present at equivalent positions in the V2 and OT receptors, Asp and Phe, we show that this receptor residue indeed plays a major role in hormone high affinity binding and receptor selectivity.

Results

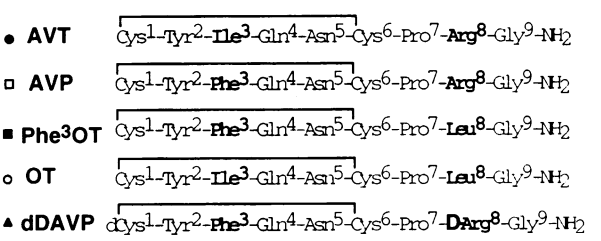
Role of hormone residues in high affinity binding and selectivity

Our first goal was to investigate the relative contributions of single peptide residues to hormone binding and selectivity in the recently cloned rat V1a (Morel *et al.*, 1992), human V2 (Birnbaumer *et al.*, 1992) and human OT receptor subtypes (Kimura *et al.*, 1992).

AVP and OT are two nonapeptides characterized by the presence of a ring formed by a disulphide bridge between Cys1 and Cys6 (Figure 1). The two hormones differ only at two positions: in the cyclic part of the peptide, residue 3 is a Phe in AVP and an Ile in OT; at position 8, in the C-terminal tripeptide, the Arg present in AVP is replaced by a Leu in OT. The role of these two positions was analysed by using AVP, OT and two peptide analogues of AVP in which only one substitution is present at a time: AVT, where Phe3 is replaced by an Ile, and Phe3-OT where Arg8 is substituted by a Leu (for references to original syntheses and pharmacological properties of AVP, OT, AVT and Phe3-OT, see Katsoyannis, 1957; Berde and Boissonnas, 1968).

We first examined the affinities of the peptides for the V1a receptor subtype. Displacement curves of the linear V1a antagonist [^{125}I]OH-phenylaceyl1, D-Tyr(Me)2,Phe3, Gln4,Asn5,Arg6,Pro7,Arg8-NH_2 (OH-LVA; Manning *et al.*, 1992; Barberis *et al.*, 1995) by the different peptides are shown in Figure 1; K_i values are reported in Table I. AVP and AVT, sharing an Arg at position 8, have the same high affinity for the V1a receptor, whereas the affinity of Phe3-OT and OT, characterized by a Leu at position 8, is >100-fold lower. The presence of either a Phe or an Ile residue in position 3 does not seem to have any influence on peptide affinity in the V1a receptor. Finally, very low affinities were found for the selective V2 agonists 1-deamino, D-Arg8-vasopressin (dDAVP; Zaoral *et al.*, 1967b) and D-Arg8-vasopressin (DAVP; Zaoral *et al.*, 1967a), in which an Arg in the D configuration substitutes for the naturally occurring L-Arg at position 8.

An homologous study was performed on the human V2 and OT receptors. For these two receptors, a more complex situation was found. In the V2 receptor, AVP shows a high affinity and OT a low affinity, whereas AVT and



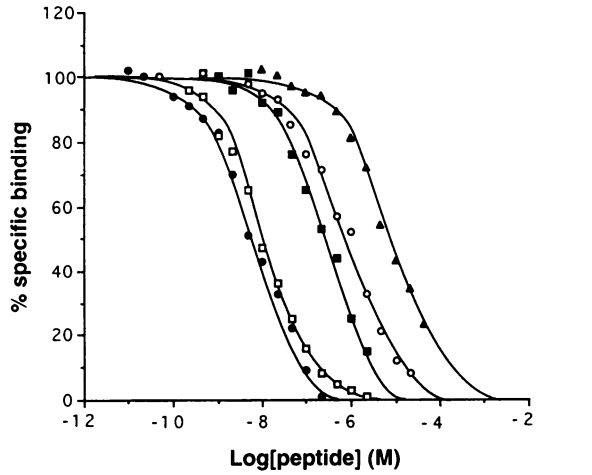


Fig. 1. Binding properties of nonapeptide analogues to the rat V1a receptor. Competition experiments were performed using membrane preparations of COS7 cells transiently transfected with the wild-type rat V1a receptor. The specific linear V1a receptor antagonist [^{125}I]OH-LVA was used at a concentration of 100–200 pM; non-specific binding was determined in the presence of 100 nM OH-LVA. Data are expressed as percent of bound [^{125}I]OH-LVA in the absence of cold competitor. Curves are representative of three independent assays performed in triplicate on 2–3 different batches of transfected cells.

Phe3-OT have an intermediate affinity with respect to AVP and OT (Table I). An Arg residue in position 8 is not sufficient to confer high affinity for the peptides and the presence of a Phe in position 3 is apparently necessary to give to the AVP its high affinity for the V2 receptor. We also tested two analogues known for their selective activity on the rat and human V2 receptor subtypes: DAVP and dDAVP. As expected, these two peptides bound with the best affinity to the V2 receptor subtype. Since no difference was found in affinity for dDAVP and DAVP, we concluded that it is the presence of the D-Arg in position 8 that determines the binding selectivity of these analogues to the V2 receptor.

In the OT receptor, a similar affinity for the four peptides was found (Table I). Both positions 3 and 8 seem to be involved in interactions leading to high peptide affinity for the OT receptor. A slightly higher affinity was however observed for OT and AVT. An Ile in position 3 seems to contribute specifically to the binding of these peptides to the OT receptor.

Role of receptor residues in high affinity binding and selectivity

Since residue 8 but not residue 3 of AVP seems to contribute to high affinity peptide binding in the V1a receptor, this receptor represents the most favourable subtype for studying the contribution of residue 8 to

Table I. Inhibition constants (K_i in nM) for wild-type and mutant receptors

Analogue	V1a wild-type	V2 wild-type	OTR wild-type	V1a Y115L	V1a Y115D	V1a Y115F
AVP	4.2 ± 1.4	1.06 ± 0.35[a]	1.65 ± 0.49	219 ± 61	2.2 ± 0.99[a]	1.04 ± 0.25
AVT	3.2 ± 0.68	25 ± 4.7	0.36 ± 0.10	209 ± 63	6.7 ± 1.81	3.23 ± 0.73
Phe3-OT	548 ± 126	88 ± 21	1.54 ± 0.53	1296 ± 761	391 ± 52	28 ± 9.7
OT	845 ± 99	1544 ± 263	0.79 ± 0.22	2067 ± 670	839 ± 28	45 ± 13
dDAVP	2466 ± 402	31 ± 11	203 ± 32	4006 ± 554	38 ± 7.8	2212 ± 612
DAVP	871 ± 74	25 ± 3.4	ND	ND	16 ± 4.5	ND
[^{125}I]OH-LVA	0.063 ± 0.017[b]	ND	ND	0.048 ± 0.002[b]	0.100 ± 0.002[b]	0.054 ± 0.005[b]
[^{125}I]OTA	ND	ND	0.095 ± 0.033[c]	ND	ND	ND

For the wild-type rat V1a receptor and the two V1a receptor mutants Y115L and Y115F, K_i values were obtained by displacement of [^{125}I]OH-LVA, after determination of antagonist K_d values (indicated by [b]) by Scatchard analysis. Similarly, K_i values for the human V2 receptor and for the V1a receptor mutant Y115D were obtained by displacement of [^{3}H]AVP (K_d values indicated by [a]). Finally, the specific OT antagonist [^{125}I]OTA (K_d value indicated by [c]) was used to determine the K_i values for the human OTR. In competition experiments, the labelled ligands were used at a concentration which approximately equals the K_d determined in the saturation experiments (100–200 pM for [^{125}I]OH-LVA and [^{125}I]OTA; 2–4 nM for [^{3}H]AVP). All values are the means ± SEM of three different experiments performed in triplicate. ND: not determined.

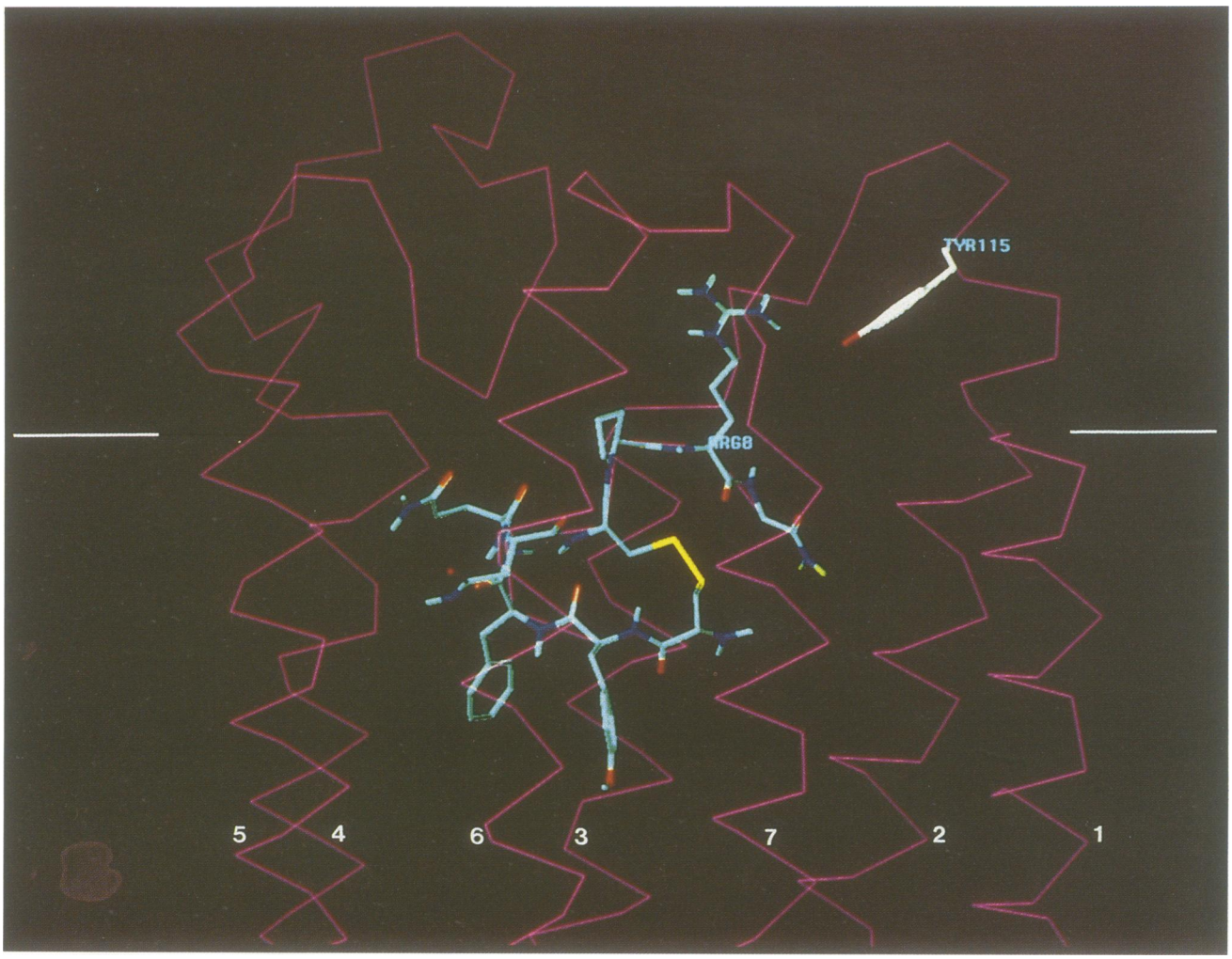


Fig. 2. 3-D model of AVP docked in the V1a receptor. Side view of the upper third of the transmembrane regions of the receptor and of its three extracellular loops from a direction parallel to the cell membrane surface. The boundary between the extracellular space and the lipid bilayer is indicated by white bars. Transmembrane regions 1–7 are arranged anticlockwise. The C_α-chain backbone of the receptor is displayed in magenta. The AVP C_α-chain trace is displayed in green. The carbon skeleton of the side chains of Tyr115 in the first extracellular loop is displayed in white. Oxygen atoms are in red, nitrogen atoms are in dark blue, sulphur atoms are in yellow.

peptide binding in the AVP/OT receptor family. We looked for V1a receptor residues that might interact with the side chain of the Arg in position 8 by using a 3-D model of the AVP/V1a receptor (Figure 2). According to this model, the lateral chain of Arg8 lies in proximity to the first extracellular loop of the receptor and might interact with the polar Tyr residue at position 115 (Tyr115). Moreover, sequence comparisons of the different receptor subtypes

RECEPTOR	1st extracellular loop										
	•			•		•	•		•	•	•
Teleost fish AVT	W	E	I	T	**F**	R	F	Y	G	P	D
Pig OT	W	D	I	T	**F**	R	F	Y	G	P	D
Human OT	W	D	I	T	**F**	R	F	Y	G	P	D
Human V1b	W	D	I	T	**Y**	R	F	Q	G	P	D
Rat V1a	W	D	I	T	**Y**	R	F	R	G	P	D
Human V1a	W	D	I	T	**Y**	R	F	R	G	P	D
Pig V2	W	D	A	T	**Y**	R	F	R	G	P	D
Rat V2	W	D	A	T	**D**	R	F	H	G	P	D
Bovine V2	W	D	A	T	**D**	R	F	R	G	P	D
Human V2	W	K	A	T	**D**	R	F	R	G	P	D

Fig. 3. Comparison of amino acid sequences of the first extracellular loop of teleost fish AVT receptor (Mahlmann *et al.*, 1994), pig and human OT receptors (Kimura *et al.*, 1992; Gorbulev *et al.*, 1993), rat and human V1a vasopressin receptors (Morel *et al.*, 1992; Wheatley *et al.*, 1993; Thibonnier *et al.*, 1994), pig, rat, bovine and human V2 vasopressin receptors (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Gorbulev *et al.*, 1993; Kojro *et al.*, 1993). The putative first extracellular loop, between transmembrane segments II and III, is shown. Residues at position 115 in the V1a receptor and at the equivalent position in the other receptor subtypes are printed in bold and shadowed. Dots indicate conserved residues.

showed that this residue is not conserved in the AVP/OT receptor family (Figure 3). We thus postulated that residue 115 might play a crucial role in agonist binding and receptor selectivity in the AVP/OT receptor family.

To test our hypothesis, site-directed mutagenesis was performed on the V1a receptor subtype and the pharmacological properties of receptor mutants were studied in transiently transfected COS7 cells. The affinity for the specific linear V1a antagonist [^{125}I]OH-LVA was determined by Scatchard analysis on the three following mutants: Y115L (K_d 0.048 ± 0.005 nM), Y115D (K_d 0.100 ± 0.002 nM) and Y115F (K_d 0.054 ± 0.005 nM). In all mutant receptors the K_d values for this analogue were comparable to the wild-type V1a receptor (K_d 0.063 ± 0.017 nM) (Figure 4). Furthermore, no change concerning the B_{max} values in the saturation experiments was noticed with respect to the wild-type V1a receptor (not shown), indicating a comparable level of expression of the different mutants. These data indicate that the general properties of the mutant receptors are unchanged with respect to the wild-type V1a receptor.

When Tyr115 is replaced by a Leu residue, whose lateral chain is not expected to interact with the lateral chain of the position 8 residue in AVP, we observed a 50-fold decrease in the affinities for AVP and AVT, the two peptides with an Arg in position 8 (Table I). On the contrary, affinities of OT and Phe3-OT, sharing a Leu in position 8, were only slightly affected, as was the affinity of the specific V2 agonist dDAVP .

When we replaced Tyr115 by an Asp residue, the amino acid naturally occurring at this position in the human and rat V2 subtypes, we did not observe any significant change in the affinity for AVP, AVT, Phe3-OT and OT (Table I). On the contrary, a gain in affinity for dDAVP and DAVP was observed which makes this V1a mutant comparable to the wild-type V2 subtype with respect to the specific interaction between the D-Arg8 and the receptor (Figure 4).

Finally, we exchanged Tyr115 for a Phe, the residue naturally occurring in the OT receptor. In this case, the

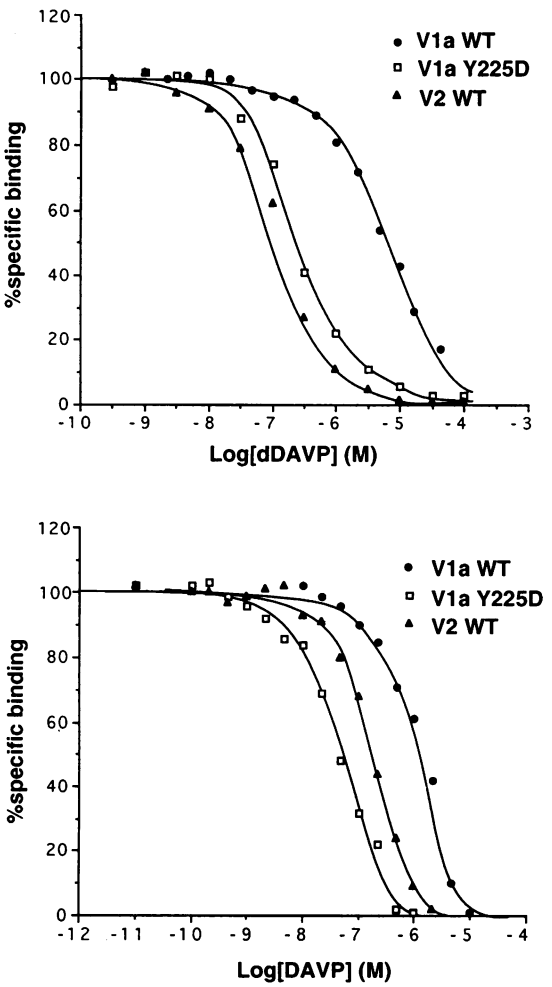


Fig. 4. Comparison of the ligand binding properties of the wild-type rat V1a receptor, the wild-type human V2 receptor and the Y115D V1a receptor mutant. Top panel: competition of [^{3}H]AVP (2–4 nM) by dDAVP; bottom panel: competition of [^{3}H]AVP (2–4 nM) by DAVP. Non-specific binding was determined in the presence of 0.5 μM AVP. Data are expressed as percent of [^{3}H]AVP bound to each receptor in the absence of cold competitor. Curves are representative of three independent assays performed in triplicate on 2–3 different batches of membrane preparations of transiently transfected COS7 cells.

V1a receptor was shifted towards the pharmacological profile of the OT receptor (Figure 5). A 17-fold increase in the affinity for OT and a 19-fold increase in the affinity for Phe3-OT was observed, while the affinity for AVP, AVT and dDAVP remained unchanged (Table I).

Coupling properties of mutant receptors

The receptor–effector coupling properties of the three mutants, Y115L, Y115D and Y115F, were examined in COS7 cells transiently expressing the receptors. Inositol phosphate (InsP) accumulation induced by AVP was measured; dose–response curves of total InsPs production (see Materials and methods for details) are reported in Figure 6. These experiments demonstrated that the three mutants were able to activate phospholipase C (PLC) after stimulation by AVP. The Y115F mutant and the wild-type V1a receptor showed comparable K_{act} values (K_{act} 0.44 ± 0.06 nM and 0.67 ± 0.16 nM respectively). In the two other mutants, Y115L and Y115D, a 100-fold increase in

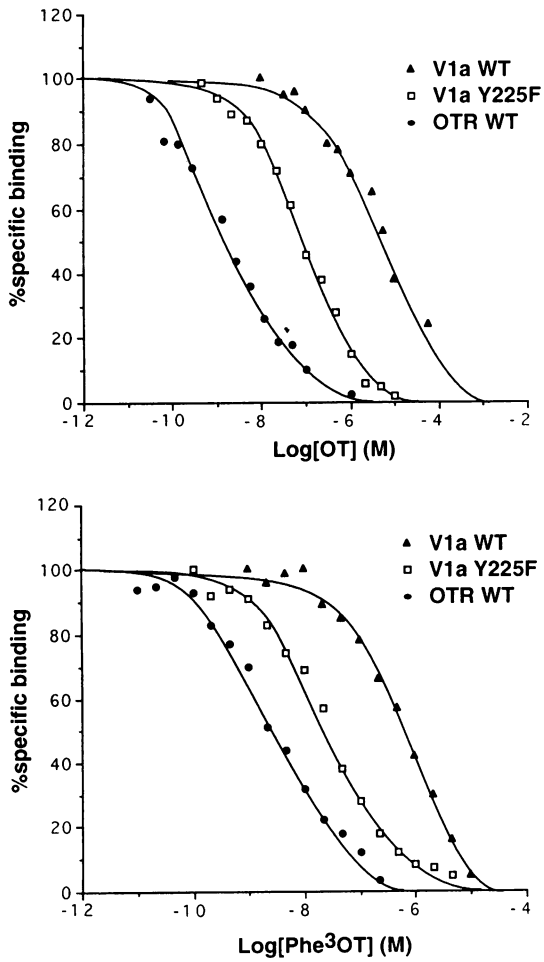


Fig. 5. Comparison of the ligand binding properties of the wild-type rat V1a receptor, the wild-type human OT receptor (OTR) and the Y115F V1a receptor mutant. For the wild-type V1a and the mutant Y115F V1a receptor, competition binding of [^{125}I]OH-LVA (100–200 pM) by OT (top panel) and Phe3-OT (bottom panel) was determined; non-specific binding was determined in the presence of 100 nM OH-LVA. For the wild-type OT receptor, competition binding of [^{125}I]OTA (100–200 pM) by OT (top panel) and Phe3-OT (bottom panel) was performed; non-specific binding was determined in the presence of 100 nM OTA. Data are expressed as percent of labelled antagonist bound to each receptor in the absence of cold competitor. Curves are representative of three independent assays performed in triplicate on 2–3 different batches of membrane preparations of transiently transfected COS7 cells.

K_{act} values was observed (K_{act} 46.5 ± 7.5 nM and 46.5 ± 13.1 nM, respectively). In the case of the Y115L, this increase in K_{act} correlates with the 100-fold decrease in the affinity of AVP. However, in the mutant Y115D, we found that the increase in the K_{act} value does not correlate with a variation in the affinity of the agonist.

Discussion

In peptide receptor families, agonist selectivity might be due to ligand interactions with non-conserved receptor residues located at equivalent positions in the different receptor subtypes. Moreover, residues located at different positions in different receptors may also contribute to determine agonist selectivity. For these reasons, difficulties have been encountered in identifying single residues

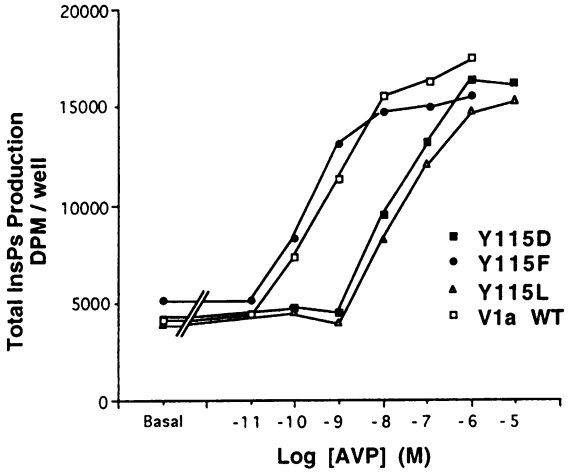


Fig. 6. Analysis of second messenger responses of wild-type and mutant V1a receptors. Inositol phosphate production was measured after stimulation of transiently transfected COS7 cells with increasing concentrations of AVP. Each point represents the total amount of inositol phosphates (total InsPs) and is expressed as d.p.m./well. Curves are representative of three independent assays performed in triplicate.

responsible for agonist selectivity in peptide receptors. Agonist selectivity of the neurokinin receptors is not affected by any single residue substitution analysed so far (Strader *et al.*, 1994). In the endothelin receptors, the residue responsible for agonist selectivity in the hET_A receptor subtype is not apparently involved in hET_B receptor selectivity (Krystek *et al.*, 1994; Lee *et al.*, 1994). Determinant factors for agonist selectivity may be located in the transmembrane region of the receptors, as in the endothelin receptors (Krystek *et al.*, 1994; Lee *et al.*, 1994), or in their extracellular part, as in the thrombin receptors (Gerszten *et al.*, 1994). Nevertheless, general models of interaction among ligands and receptors have been proposed which may help in elucidating the most important ligand–receptor interactions in each receptor family (for recent reviews, see Hoflack *et al.*, 1993; Schwartz, 1994; Strader *et al.*, 1994). We have developed such a model in order to analyse ligand–receptor interactions in the AVP/OT receptor family (Mouillac *et al.*, manuscript in preparation).

In our 3-D model of the V1a receptor, the side chain of Arg8 points towards the outside of the transmembrane core of the receptor and might interact with receptor residues in the first extracellular loop. In particular, it might form a hydrogen bond with a Tyr residue at position 115. Our model is in complete agreement with the data obtained by Fahrenholz and co-workers using a peptide analogue of AVP with a photoactivatable group on the lateral chain of residue 8 (Kojro *et al.*, 1993). This analogue was demonstrated to react with two amino acids located in the first extracellular region of the bovine V2 receptor. The authors suggested that an interaction might occur between the side chain of residue 8 in the natural hormone and one of the three Asp residues present in the first extracellular loop of the V2 receptor. Notably, one of these Asp residues is located at the same position as Tyr115 in the V1a receptor.

Our present data indicate that the electrostatic interaction between the lateral chain of the residue in position 8 and

the hydroxyl group of Tyr115 is crucial to stabilize the peptide into the binding pocket. Other findings in the literature support our hypothesis. Peptides with positively charged residues in position 8, such as Lys or Orn, are reported to have a high affinity for the V1a receptor, whereas the biological activity of analogues with uncharged residues at position 8 is in general low (Berde and Boissonnas, 1968; Cantau *et al.*, 1980; Hruby and Smith, 1987). Similarly, if the amino group of the side chain of residue 8 is masked by a formyl group, as in [Ne-For-Lys8]AVP, the activity on the V1a receptor is greatly reduced (Berde and Boissonnas, 1968).

Tyr115 also plays a crucial role in receptor selectivity. A selective change in the pharmacological profile of the V1a receptor is in fact observed when this residue is substituted with the residue present at the equivalent position in the V2 or OT receptor.

In the human V2 receptor, an Asp residue replaces Tyr115; in this case, an ionic bond between this Asp and Arg8 could account for the high affinity that this receptor displays for DAVP and dDAVP, an analogue of AVP which is currently used in the diagnosis and treatment of some forms of diabetes insipidus (Mannucci *et al.*, 1977; Bichet *et al.*, 1988). When an Asp is introduced in the V1a receptor, as in the Y115D mutant, this ionic bond can be established and the V1a receptor becomes comparable to the V2 receptor as far as dDAVP and DAVP binding is concerned. Interestingly, the mutant Y115D is less efficiently coupled to PLC than the wild-type V1a receptor, even if a maximal response comparable to that of the wild-type receptor is observed at higher doses of AVP. The introduction of an Asp residue in the first extracellular region of the V1a subtype not only influences the binding properties of the receptor, but also affects its activation. The first extracellular loop connects helices 2 and 3 and it is very possible that a slight change in the conformation of this region influences the arrangement of the helices, thereby influencing the activation process.

If residue 115 is the major determinant of the V1a versus V2 selectivity, the substitution of the Asp residue by a Tyr in the V2 receptor should induce a decrease in the affinity of this receptor subtype for dDAVP. We know from sequence comparison that the Asp residue at position 115 is conserved in rat, bovine and human V2 subtypes (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Kojro *et al.*, 1993) but not in the pig V2 receptor (Gorbulev *et al.*, 1993). In the pig V2 receptor, nothing else but a Tyr substitutes for the Asp at position 115. In complete agreement with our predictions, the pig V2 receptor is well known for its low affinity for dDAVP (Roy *et al.*, 1975; Gorbulev *et al.*, 1993) and thus resembles the V1a receptor in the way it interacts with the side chain of D-Arg8 of dDAVP. Notably, this is the only residue in the first extracellular loop that might account for this species-specific pharmacological difference because it is the only residue in this loop that is common to the V1a and pig V2 receptors, but not to the human, bovine and rat V2 receptors.

An aromatic Phe residue is present at a corresponding position in the human OT receptor and in the teleost fish AVT receptor, two receptors characterized by a high affinity or functional activity of OT (Kimura *et al.*, 1992; Mahlmann *et al.*, 1994). Mutation of Tyr115 into a Phe conferred a higher affinity for OT on the V1a receptor. Furthermore, the affinity of the receptor for Phe3-OT, the other analogue that presents a Leu in position 8, also increased. One possible explanation is that the switch from a Tyr to a Phe in this extracellular loop leads to a more lipophilic local environment which is more suitable for a Leu side chain. No loss in the affinity for AVP and AVT was observed in this mutant, probably because the aromatic ring of the Phe residue might contribute to stabilization of the amino group of the side chain of residue 8 (see Burley and Petsko, 1986 for a discussion on interactions between protonated amines and aromatic residues). The same interaction might account for the high affinity shown by AVP and AVT for the human OT receptor.

The Y115F mutation did not completely convert the V1a receptor into an OT receptor. Similarly, the mutant Y115D does not behave like the V2 receptor with respect to binding affinities of AVT and Phe3-OT. This is most likely due to the fact that interactions with peptide position 3 also contribute to high affinity binding in the V2 and OT receptors.

In conclusion, by carefully selecting a few peptide analogues, we have been able to determine the most important interactions responsible for agonist selectivity in several members of the AVP/OT receptor family. These interactions involve the two non-conserved positions of the peptides, position 3 and position 8. Using a 3-D model. we have identified receptor residues likely to interact with the peptide ligand at position 8 and we have validated the prediction experimentally. Our data indicate that the interaction of the side chain of residue 8 with a non-conserved residue in the first extracellular region plays a crucial role in agonist binding and selectivity of AVP/OT receptors.

Materials and methods

Three-dimensional modelling

The construction of the V1a receptor 3-D model has been described in detail elsewhere (Mouillac *et al.*, manuscript in preparation). Briefly, the transmembrane part of the V1a receptor was constructed by using the 3-D model first developed for cationic neurotransmitter receptors (Hibert *et al.*, 1991; Trumpp-Kallmeyer *et al.*, 1992) and refined on the bovine rhodopsin footprint (Schertler *et al.*, 1993). The extracellular regions of the receptor were then built and the disulphide bridge connecting Cys124, at the beginning of helix 3, and Cys205, in the second extracellular loop, was added. The whole receptor structure was energy minimized in order to relax the structure and to remove unfavourable steric constraints. Finally, AVP was manually docked in order to optimize the structural and physicochemical complementarity between the ligand and the receptor.

Cell transfection and mutagenesis

Rat V1a (Morel *et al.*, 1992) receptor cDNA was kindly provided by Dr A.Morel; human OT cDNA (Kimura *et al.*, 1992) receptor was a generous gift of Dr T.Kimura. Human V2 receptor gene was amplified from human genomic DNA using specific primers designed on the 5′- and 3′-untranslated regions of the published sequence (Birnbaumer *et al.*, 1992); the PCR product was subcloned into a suitable vector (pBluescript II, Stratagene) and completely sequenced (in collaboration with Dr D.Bichet, Montreal, Canada). Mutants were constructed by oligonucleotide-directed mutagenesis (Sculptor Kit, Amersham) and identified by dideoxy sequencing (^{T7}sequencing^{TM} kit, Pharmacia). After insertion into an eukaryotic expression vector under the control of the cytomegalovirus promoter, wild-type and mutant receptors were transiently transfected into COS7 cells (American Type Culture Collection) by electroporation. COS7 cells were grown in Dulbecco's modified Eagles medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (Eurobio, France),

100 IU/ml penicillin and streptomycin (Gibco BRL), in 5% CO_2 in air, at 37°C. Electroporation (280 V, 960 μF, Bio-Rad gene pulser electroporator) was performed in a total volume of 300 μl, with 20 μg of carrier DNA (vector without any insert), 75 ng of plasmid DNA (vector with a subcloned insert) and 10^6 cells in electroporation buffer (50 mM K_2HPO_4; 20 mM CH_3COOK, 20 mM KOH). After electroporation, cells were split into 6-well clusters (for determination of InsP accumulation), or in 150 mm Petri dishes (for membrane preparation).

Drugs

AVP, OT, AVT (Berde and Boissonnas, 1968; Hruby and Smith, 1987) and dDAVP (Zaoral *et al.*, 1967b) were from Bachem, Switzerland. DAVP (Zaoral *et al.*, 1967a) was from Ferring AB, Sweden. Phe3-OT (Katsoyannis, 1957), the V1a antagonist OH-LVA (Manning *et al.*, 1992; Barberis *et al.*, 1994) and the OT receptor antagonist d(CH_2)$_5$ [Tyr(Me)2,Thr4,Tyr9-NH_2]-OVT (OTA) (Elands *et al.*, 1987) were synthesized in the laboratory of Dr M.Manning. [^{3}H]AVP (60–80 Ci/mmol) was from Dupont-NEN.

Binding assays

To prepare membranes for the binding assays, transfected COS7 cells were homogenized, washed twice and resuspended in the binding buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4). The linear peptidic V1a antagonist OH-LVA and the specific OT receptor antagonist OTA were iodinated as described (Elands *et al.*, 1987; Manning *et al.*, 1992). [^{125}I]OH-LVA, [^{125}I]OTA and [^{3}H]AVP were used for Scatchard analysis and competition experiments. When [^{125}I]OH-LVA and [^{125}I]OTA were used, 1–5 μg of membrane proteins were incubated for 60 min at 30°C; when [^{3}H]AVP was used, incubation lasted 30 min in the presence of 5–10 μg of membrane proteins. Non-specific binding was determined in the presence of a 250- to 1000-fold excess of unlabelled analogues. Bound and free radioactivity were separated by filtration over Whatman GF/C filters pre-soaked in either 0.5% polyethylenimine ([^{125}I]OH-LVA) or 10 mg/ml BSA ([^{3}H]AVP and [^{125}I]OTA). In competition experiments, the labelled ligand was used at a concentration which approximately equals the K_d determined in the saturation experiments (70–100 pM for [^{125}I]OH-LVA and [^{125}I]OTA, 2–4 nM for [^{3}H]AVP).

The binding data were analysed with non-linear model fitting programs (Kinetic, Ebda, Ligand, Lowry – Release 2; Biosoft)

Inositol phosphate assays

InsP accumulation was determined as previously described (Kirk *et al.*, 1986). Briefly, transfected cells were grown on 6-well dishes for 12 h. Cells were then labeled for 48 h with myo-[2-^{3}H]inositol (10–20 Ci/mmol; Dupont-NEN) at a final concentration of 2 μCi/ml. Before agonist stimulation, cells were incubated in a serum-free, myo-[2-^{3}H]inositol-free medium for 30 min, washed twice with phosphate-buffered saline (PBS), and incubated at 37°C in PBS supplemented with 10 mM LiCl for 10 min. Cells were then stimulated for 15 min with an increasing concentration of AVP (from 10^{-11} to 10^{-5}). After stopping the reaction with perchloric acid, InsPs were extracted and separated on a strong anionic exchange column (Dowex AG1x8, formate form, 200–400 mesh; Bio-Rad). For each point, a fraction containing inositol monophosphates, inositol diphosphates and inositol triphosphates was collected and radioactivity was determined by scintillation counting. This fraction is referred to as total labelled inositol phosphates (total InsPs) and is expressed as d.p.m./well. K_{act} values were obtained from three different dose–reponse curves performed in triplicate and correspond to the concentration of AVP leading to the half-maximal production of total InsPs.

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References

Acher,R. (1993) *Regul. Pept.*, **45**, 1–13.
Barberis,C., Balestre,M., Jard,S., Tribollet,E., Arsenijevic,Y., Dreifuss, J.J., Bankowski,K., Manning,M., Chan,W.Y., Schlosser,S.S., Holsboer,F. and Elands,J. (1995) *Neuroendocrinology*, in press.
Berde,B. and Boissonnas,R.A. (1968) In Berde, B. (ed.), *Handbook of Experimental Pharmacology. Neurohypophysial Hormones and Similar Polypeptides*. Springer-Verlag, Berlin, Heidelberg, New York, Vol. 23, pp. 802–870.
Bichet,D.G., Razi,M., Lonergan,M., Arthus,M.F., Papukna,V., Kortas,C. and Barjon,J.N. (1988) *New Engl. J. Med.*, **318**, 881–887.
Birnbaumer,M., Seibold,A., Gilbert,S., Ishido,M., Barberis,C., Antaramian,A., Brabet,P. and Rosenthal,W. (1992) *Nature*, **357**, 333–335.
Burley,S.K. and Petsko,G.A. (1986) *FEBS Lett.*, **203**,139–143.
Cantau,B., Keppens,S., De Wulf,H. and Jard,S. (1980) *J. Receptor Res.*, **1**, 137–168.
Elands, J., Barberis, C., Jard, S., Tribollet, E., Dreifuss, J., Bankowski, K., Manning,M. and Sawyer,W.H. (1987) *Eur. J. Pharmacol.*, **147**, 197–207.
Gerszten,R.E., Chen,J., Ishii,M., Ishii,K., Wang,L., Nanevicz,T., Turck,C.W., Vu,T.H. and Coughlin,S.R. (1994) *Nature*, **368**, 648–651.
Gorbulev,V., Buchner,H., Akhundova,A. and Fahrenholz,F. (1993) *Eur. J. Biochem.*, **215**, 1–7.
Hibert,M., Trumpp-Kallmeyer,S., Bruinvels,A. and Hoflack,J. (1991) *Mol. Pharmacol.*, **40**, 8–15.
Hoflack,J., Trumpp-Kallmeyer,S. and Hibert,M. (1993) In Kubinyi, H. (ed.), *3D QSAR and Drug Design. Theory, Methods and Applications*. Escom, pp. 355–372.
Hruby,V.J. and Chow,M.S. (1990) *Annu. Rev. Pharmacol. Toxicol.*, **30**, 501–534.
Hruby,V.J. and Smith,C.W. (1987) In Smith.,C.W. (ed.), *The Peptides. Chemistry, Biology and Medicine of Neurohypophysial Hormones and Their Analogs*. Academic Press, Orlando, FL, Vol. 8, pp. 77–207.
Katsoyannis,P.G. (1957) *J. Am. Chem. Soc.*, **79**, 109–111.
Kimura,T., Tanizawa,O., Mori,K., Brownstein,M.J. and Okayama,H. (1992) *Nature*, **356**, 526–529.
Kirk,C.J., Guillon,G., Balestre,M. and Jard,S. (1986) *Biochem. J.*, **240**, 197–204.
Kojro,E., Eich,P., Gimpl,G. and Fahrenholz,F. (1993) *Biochemistry*, **32**, 13537–13544.
Krystek,S.R., Patel,P.S., Rose,P.M., Fisher,S.M., Kienzle,B.K., Lach,D.A., Liu,E.C.K., Lynch,J.S., Novotny,J. and Webb,M.L. (1994) *J. Biol. Chem.*, **269**, 12383–12386.
Lee,J.A., Elliott,J.D., Sutiphong,J.A., Friesen,W.J., Ohlstein,E.H., Stadel,J.M., Gleason,J.G. and Peishoff,C.E. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7164–7168.
Lolait,S.J., O'Carroll,A.M., McBride,O.W., Konig,M., Morel,A. and Brownstein,M.J. (1992) *Nature*, **357**, 336–339.
Mahlmann,S., Meyerhof,W., Hausmann,H., Heierhorst,J., Schonrock,C., Zwiers,H., Lederis,K. and Richter,D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1342–1345.
Manning,M. and Sawyer,W.H. (1993) *J. Receptor Res.*, **13**, 195–214.
Manning,M., Bankowski,K., Barberis,C., Jard,S., Elands,J. and Chan,W.Y. (1992) *Int. J. Peptide Protein Res.*, **40**, 261–267.
Mannucci,P.M., Ruggeri,Z.M., Pareti,F.I. and Capitanio,A. (1977) *Lancet*, **1**, 869–872.
Morel,A., O'Carroll,A., Brownstein,M.J. and Lolait,S.J. (1992) *Nature*, **356**, 523–526.
Ostrowski,J., Kjelsberg,M.A., Caron,M.G. and Lefkowitz,R.J. (1992) *Annu. Rev. Pharmacol. Toxicol.*, **32**, 167–183.
Roy,C., Barth,T. and Jard,S. (1975) *J. Biol. Chem.*, **250**, 3149–3156.
Savarese,T.M. and Fraser,C.M. (1992) *Biochem. J.*, **283**, 1–19.
Schertler,G.F.X., Villa,C. and Henderson,R. (1993) *Nature*, **362**, 770–772.
Schwartz,T.W. (1994) *Curr. Opin. Biotechnol.*, **5**, 434–444.
Strader,C.D., Fong,T.M., Tota,M.R. and Underwood,D. (1994) *Annu. Rev. Biochem.*, **63**, 101–132.
Strosberg,A.D. (1991) *Eur. J. Biochem.*, **196**, 1–10.
Sugimoto,T., Saito,M., Mochizuki,S., Watanabe,Y., Hashimoto,S. and Kawashima,H. (1994) *J. Biol. Chem.*, **269**, 27088–27092.
Thibonnier,M., Auzan,C., Madhun,Z., Wilkins,P., Berti-Mattera,L. and Clauser,E. (1994) *J. Biol. Chem.*, **269**, 3304–3310.
Trumpp-Kallmeyer,S., Hoflack,J., Bruinvels,A. and Hibert,M. (1992) *J. Med. Chem.*, **35**, 3448–3462.
Wheatley,M., Howl,J., Morel,A. and Davies,A.R.L. (1993) *Biochem. J.*, **296**, 519.
Zaoral,M., Kolc,J. and Sorm,F. (1967a) *Collect. Czech. Chem. Commun.*, **32**, 1242–1249.
Zaoral,M., Kolc,J. and Sorm,F. (1967b) *Collect. Czech. Chem. Commun.*, **32**, 1250–1257.

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