Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin

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Abstract We investigated the mechanisms that regulate the efficacy of agonists in the arginine-vasopressin (AVP)/oxytocin (OT) receptor system. In this paper, we present evidence that AVP, a full agonist of the vasopressin receptors, acts as a partial agonist on the oxytocin receptor. We also found that AVP becomes a full agonist when two aromatic residues of the oxytocin receptor are replaced by the residues present at equivalent positions in the vasopressin receptor subtypes. Our results indicate that these two residues modulate the response of the oxytocin receptor to the partial agonist AVP.

Key words: Oxytocin receptor; Vasopressin; G protein coupled receptor; Partial agonism; Mutagenesis

1. Introduction

Evolutionarily related peptides of the oxytocin (OT)/vasopressin (AVP) family are found throughout the animal kingdom [1]. These hormones bind to and activate a number of receptor subtypes: in mammals, one oxytocin receptor (OTR) [2,3] and three vasopressin receptors (V1a, V1b and V2) [4–9] have been recently cloned; closely related receptors have also been identified in bony fish and invertebrates [10–12]. All of the OT and AVP receptors cloned so far belong to the 7 transmembrane spanning domain receptor family and are characterised by a high degree of sequence homology. The neurohypophyseal peptides are also structurally linked: they are all characterised by the presence of a cyclic backbone formed by six amino acids and a short C-terminus tripeptide.

In a previous study, we showed that the high affinity binding of AVP and related peptides is mainly determined by interactions of the hormones with the residues located in the transmembrane part of the V1a receptor [13]. These residues are conserved in all of the OT/AVP receptors cloned so far, suggesting that a common binding pocket is shared by all of these receptor subtypes. On the other hand, it is very likely that the selective actions of the different peptides on the different receptor subtypes are due to interactions involving non-conserved peptide and/or receptor residues. OT and AVP, the two nonapeptides present in humans, differ in terms of two residues: one is located at position 3 in the cyclic part of the peptide, the other at position 8 in the linear part (Table 1). We have previously shown that part of the binding selectivity in neurohypophyseal receptors is due to the interaction between the lateral chain of peptide residue 8 and a receptor residue located in the first extracellular loop [14] (see also [15]). In the present study, we investigated in detail the binding and coupling properties of the cloned human OTR and the role played by the interactions of peptide position 3 in determining the pharmacological properties of the agonist response.

2. Materials and methods

2.1. Drugs

Arg^8-vasopressin (AVP), arginine^8-vasotocin (AVT), Phe^2Orn^8-vasotocin (Phe^2Orn^8-VT) and oxytocin (OT) came from Bachem, France. Thr^4Gly^7-oxytocin (Thr^4Gly^7OT), Phe^3-oxytocin (Phe^3OT) and the OT receptor antagonist $d(CH_2)_5[Tyr(Me)^2,Thr^4,Tyr^9\text{-}NH_2]$OVT (OTA) were synthesised in the laboratory of Dr. M. Manning; the OTA was iodinated as previously described [16]. [^{3}H]Arg^8-vasopressin ([^{3}H]AVP) and [^{3}H]oxytocin ([^{3}H]OT) (60–80 Ci/mmol) came from Dupont-NEN.

2.2. Mutagenesis and transfection

The human OT cDNA [2] receptor was a generous gift from Dr. T. Kimura. The mutants were constructed by means of oligonucleotide-directed mutagenesis (Sculptor Kit, Amersham). COS7 cells, mouse L fibroblasts (LTK^- cells) and CHO cells were from the American Type Culture Collection. For transfection, the wild type and mutant OTR cDNAs were inserted in an expression vector under the control of the CMV promoter. Transient transfections in COS7 cells were performed by means of electroporation as described [14]. The CHO and LTK^- cells were transfected with DOTAP (Boehringer Mannheim) according to the manufacturer's instructions; stable clones were selected on the basis of their resistance to neomycin sulphate (used at a concentration of 200 μg/ml for LTK^- cells and 400 μg/ml for CHO cells).

2.3. Binding assays and inositol phosphates measurements

[^{125}I]OTA, [^{3}H]AVP and [^{3}H]OT were used for Scatchard analysis and competition experiments on cell homogenates [13,14]. The binding data were analysed by means of Ligand [17]. Inositol phosphates (InsPs) accumulation was determined as described [14,18]. The data were analysed by means of non-linear regression using a sigmoidal dose-response equation (Graph Pad Prism).

2.4. Computer modelling

The modelling of the oxytocin receptor was based on the same procedures already used for modelling the rat V1a receptor, and extensively described in a previous paper [13].

3. Results and discussion

3.1. Agonist selectivity of the human OTR

In order to better characterize the agonist selectivity of the cloned human OTR, we studied the binding properties of this receptor and its activation by OT and AVP in transiently

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Table 1
Inhibition constants (K_i in nM) for the wild-type and mutant receptors

Analogue		OTR wild-type	OTR Y509 F	OTR F609 Y
OT	Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6-Pro^7-Leu^8-Gly^9-NH$_2$	0.79 ± 0.22	0.31 ± 0.07	0.86 ± 0.12
AVP	Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Gly^9-NH$_2$	1.65 ± 0.49	1.41 ± 0.16◊	3.6 ± 0.7
Phe^{3}OT	Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Leu^8-Gly^9-NH$_2$	1.54 ± 0.53	0.53 ± 0.03	11.3 ± 3.4
AVT	Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-GLy^9-NH$_2$	0.36 ± 0.10	0.25 ± 0.11	1.86 ± 0.9
Phe^{2}Orn^{8}VT	Cys^1-Phe^2-Ile^3-Gln^4-Asn^5-Cys^6-Pro^7-Orn^8-GLy^9-NH$_2$	21.9 ± 3.30	7.23 ± 0.50	92.00 ± 17.4
Thr^{4}Gly^{7}OT	Cys^1-Tyr^2-Ile^3-Thr^4-Asn^5-Cys^6-Gly^7-Leu^8-Gly^9-NH$_2$	6.62 ± 1.22	0.99 ± 0.35	17.65 ± 4.2
[^{125}I]OTA		0.09 ± 0.03°	3.62 ± 1.27•	0.19 ± 0.02°

For the wild-type OTR and the OTR mutant F609Y, the K_i values were obtained by means of the displacement of [^{125}I]OTA, after determination of the antagonist K_d values (indicated by ○) using Scatchard analysis. The K_i values for the OTR mutant F509Y were obtained by means of displacement of [^{3}H]AVP (K_d value indicated by ◊); • indicates the K_i value obtained for the unlabelled iodinated I-OTA. 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]OTA; 2–4 nM for [^{3}H]AVP). All of the values are the means ± S.E.M. of at least three different experiments each performed in triplicate. The amino acid sequence of the peptide analogues is reported in the three-letter code.

transfected COS7 cells and in stably transfected CHO and LTK$^-$ cells.

As shown in Table 1, the cloned human OTR expressed in COS7 cells binds OT, AVP, AVT, Phe^3-OT and the selective OTR agonist Thr^{4}Gly^7-OT [19] with similar affinity; it also binds the selective V1a agonist Phe^{2}Orn^8-VT [20], although with a lower affinity. In LTK$^-$ and CHO cells expressing the OTR at similar levels (B_{max} values reported in Table 2) no significant differences in the binding affinities of [^{125}I]OTA, OT and AVP were observed (data not shown). These binding properties indicate that the cloned human OTR may correspond to one of the high affinity binding sites for OT characterised in the human myometrium, in particular to the site which has been shown to bind OT, AVP and AVT with comparable affinity [21].

The cloned human OTR therefore appears to be an unselective receptor for OT and AVP when only the binding affinities of these two peptides are considered. However, it is known that OT and AVP induce very different responses on uterine contractility and PLC activation [21–24]. Differences in the capability of OT and AVP to activate the human OTR may contribute to determining OT versus AVP selectivity, a possibility that has never been investigated in details. To elucidate this point, we determined the efficacies of OT and AVP on the human OTR by performing dose-response curves of InsPs accumulation in LTK$^-$, CHO and COS7 cells (Fig. 1A–C). No difference in maximal OT stimulation was observed in the three cell types used. Under our experimental conditions, OT induced a maximal 3–5-fold increase in total InsPs production when applied at a concentration of 10 μM. AVP maximal response at the same concentration (10 μM) was always less than 60% that of maximal OT stimulation in both the transiently and stably transfected cells (Table 2), suggesting that AVP can be considered a partial agonist on the cloned human OTR. Our data indicate that neither the type of transfection nor the cell line used affect the intrinsic activity of AVP on the human OTR. However, it has been recently shown that the efficacy of agonists in transfected cells may be greatly influenced by the level of receptor expression [25–27]. A low level of receptor expression might lead to a molecule being considered a partial agonist, whereas the greater number of receptors expressed in vivo might lead it to behave as a full agonist. To avoid this problem, we selected cells that express a large number of receptors (0.5–2 pmol/mg

Table 2
Coupling properties of the wild-type and mutant OTRs

Human OT receptor	OT EC_{50} (nM)	AVP EC_{50} (nM)	AVP intrinsic activity	Receptor density (pmol/mg)
WT in CHO	12.9 ± 4.2 (n = 5)	316 ± 95 (n = 5)	49 ± 3.4% (n = 5)	0.99 ± 0.03 (n = 2)
WT in LTK$^-$	6.3 ± 0.8 (n = 7)	75 ± 34 (n = 2)	58 ± 0.5% (n = 2)	0.53 ± 0.14 (n = 4)
WT in COS7	4.8 ± 0.8 (n = 7)	81 ± 19 (n = 4)	59 ± 2.0% (n = 7)	1.73 ± 0.30 (n = 6)
Y509F in COS7	0.45 ± 0.2 (n = 4)	47 ± 5.4 (n = 3)	87 ± 4.9% (n = 7)	0.85 ± 0.39 (n = 3)
F609Y in COS7	2.9 ± 0.69 (n = 4)	115 ± 65 (n = 4)	88 ± 4.3% (n = 7)	1.47 ± 0.35 (n = 5)

The EC_{50} values of OT and AVP were obtained by analysing the dose-response curves of InsP production (shown in Fig. 1) with a non-linear regression equation (GraphPad Prism). To evaluate the intrinsic activity of the peptides, the levels of total InsP accumulation induced by OT and AVP, at the concentration of 10^{-5} M, were determined in parallel in several independent experiments. For each determination, AVP intrinsic activity was calculated as the % of the maximal OT stimulation obtained. Receptor density was determined in saturation experiments using the specific antagonist [^{125}I]OTA for the wild-type OTR and the F609Y mutant and with [^{3}H]AVP for the Y509F mutant; the values are expressed as pmol/mg of membrane proteins. All of the values are expressed as means ± S.E.M.

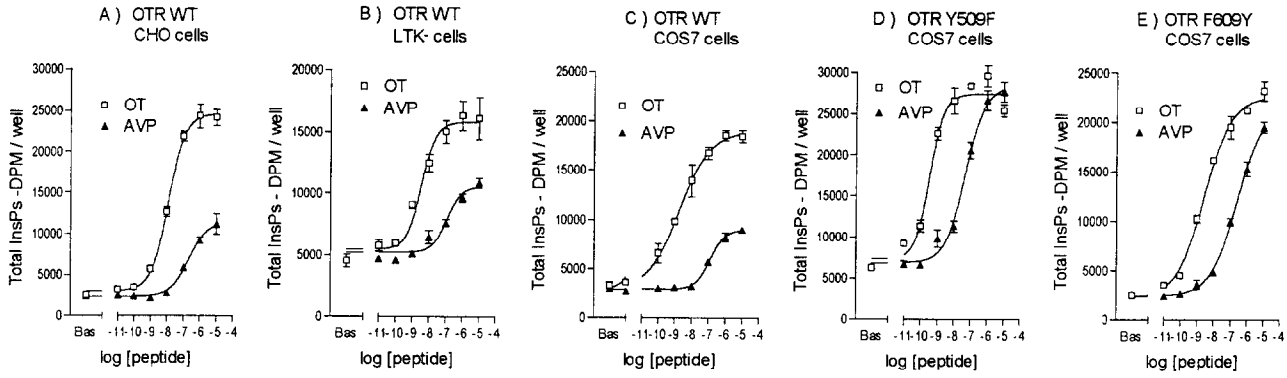

Fig. 1. Analysis of second messenger responses of wild-type and mutant OTRs. For the wild type OTR, the accumulation of inositol phosphates induced by increasing doses of OT and AVP was measured in stably transfected CHO cells (A), stably transfected LTK$^-$ cells (B) and transiently transfected COS7 cells (C). The coupling efficiency of OTR mutants Y509F and F609Y to PLC was assayed in transiently transfected COS7 cells (D, E). The curves are representative of at least three independent assays performed in triplicate. Each point represents the total amount of inositol phosphates (total InsP) and is expressed as DPM/well. Fitting was obtained by means of non-linear regression (GraphPad Prism Software). To evaluate the intrinsic activity of the peptides, the levels of total InsPs accumulation induced by OT and AVP at a concentration of 10^{-5} M were determined in parallel in several independent experiments. For each determination, AVP intrinsic activity was calculated as the % of the maximal OT stimulation obtained. The mean values ± S.E.M. of the intrinsic activity obtained for AVP on the wild-type and mutant OTRs are shown in Table 2.

of proteins), which is approximately equal to the number of OTR present in the human uterus at term parturition (0.6 pmol/mg of proteins) [21].

The OT EC_{50} values in COS7 and LTK$^-$ cells (Table 2) were comparable with those obtained in human cells expressing endogenous OTRs [23,28]. However, slightly higher EC_{50} values were observed in CHO cells, probably because of differences in the transduction components present in this cell type, as has already been observed for the human V1a receptor [5]. Our data indicate that AVP is less potent than OT in all of the three cell lines considered; we found that a 100 times higher concentration of AVP was necessary to elicit the same response as that induced by OT, a result also obtained on the cloned human OTR expressed in *Xenopus* oocytes [29]. Thus, although OT and AVP have a very similar binding affinity, their EC_{50}s differ by a factor of at least 10: a lower EC_{50} for AVP is to be expected if this analogue is only a partial agonist of the human OTR [30].

In conclusion, these data indicate that AVP is a partial agonist of the human OTR, a pharmacological property that may contribute to its selective physiological action in vivo.

3.2. *Molecular modelling of the human OTR*

In order to identify the interactions that may contribute to determine the agonist selectivity of the human OTR, we constructed a three-dimensional model of the receptor and docked the two ligands OT and AVP. The OTR model was based on our previous work on the V1a vasopressin receptor in which we analysed the hydrogen bonding and ionic interactions between the peptide agonists and several transmembrane receptor residues. Our results strongly supported the binding of OT and AVP to a pocket located in the upper part of the receptor transmembrane domains [13].

Since it is known that substitutions in peptide positions 2 and 3 greatly affect the efficacy of the neurohypophyseal peptides, we made a molecular analysis of the interactions of these residues within the binding pocket of the human OTR. In our model, a network of aromatic-aromatic and hydrophobic-aromatic interactions can be observed between Tyr^2 and Ile^3 of OT and Tyr^{509}, Phe^{609}, Phe^{616}, Trp^{613}, Phe^{616} and Phe^{617} of the OTR (Fig. 2). (To facilitate the comparison, the residues have been numbered as previously defined [31]: the left digit indicates the transmembrane domain number, and the following two digits indicate the rank of the residue in this domain.) Interestingly, most of these aromatic receptor residues, located at the bottom of the agonist binding pocket, are conserved in a large number of opsin-like G protein coupled receptors (GPCRs), suggesting that the

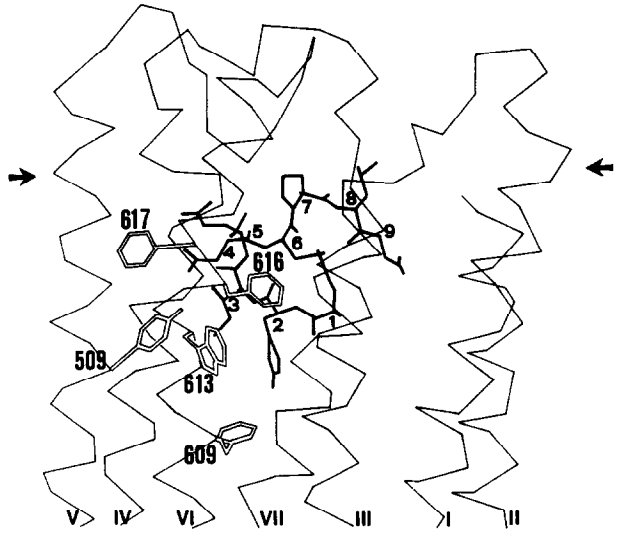

Fig. 2. Schematic presentation of oxytocin docked in the human OTR. The extracellular regions and the two thirds of the transmembrane domains of the receptor are shown from a direction parallel to the cell membrane; the arrows indicate the limit of the lipid bilayer. Transmembrane domains (numbered I–VII) are arranged anticlockwise; only the Cα-carbon backbone of the receptor is shown, with the exception of the side chains of residues 509, 609, 613, 616 and 617. Receptor residues are labelled according to the following rule: the left digit indicates the number of the transmembrane α-helix, the next two digits indicate the rank of the residue in this transmembrane region (e.g. Phe^{509} is the 9th residue of helix 5). Oxytocin is positioned inside the putative agonist binding pocket; all the side chain residues of the peptide (numbered 1–9) are shown.

	TMR5								509											
human OTR	I	T	W	I	T	L	A	V	**Y**	I	V	P	V	I	V	L	A	T	C	Y	G	L
rat OTR	V	T	W	I	T	L	A	V	**Y**	I	V	P	V	I	V	L	A	A	C	Y	G	L
human V1a	V	T	W	N	T	G	G	I	**F**	V	A	P	V	V	I	L	G	T	C	Y	G	F
rat V1a	V	T	W	N	T	S	G	V	**F**	V	A	P	V	V	V	L	G	T	C	Y	G	F
human V1b	V	T	W	T	T	L	G	I	**F**	V	L	P	V	T	M	L	T	A	C	Y	S	L
rat V1b	I	T	W	T	T	M	A	I	**F**	V	L	P	V	A	V	L	T	A	C	Y	G	L
human V2	V	T	W	I	A	L	M	V	**F**	V	A	P	T	L	G	I	A	A	C	Q	V	L
rat V2	V	T	W	I	A	L	M	V	**F**	V	A	P	A	L	G	I	A	A	C	Q	V	L
fish AVT	I	T	W	I	T	V	G	I	**F**	L	I	P	V	V	I	L	M	I	S	Y	G	L
fish IST	I	T	W	I	S	L	T	I	**Y**	I	I	P	V	A	I	L	G	G	C	Y	G	L
LSCPR1	I	T	W	V	F	V	A	N	**Y**	V	I	P	F	L	L	L	A	F	C	Y	G	R

	TMR6								609											
human OTR	M	T	F	I	I	V	L	A	**F**	I	V	C	W	T	P	F	F	F	V	Q	M	W
rat OTR	M	T	F	I	I	V	L	A	**F**	I	V	C	W	T	P	F	F	F	V	Q	M	W
human V1a	M	T	F	V	I	V	T	A	**Y**	I	V	C	W	A	P	F	F	I	I	Q	M	W
rat V1a	M	T	F	V	I	V	S	A	**Y**	I	L	C	W	A	P	F	F	I	V	Q	M	W
human V1b	M	T	F	V	I	V	L	A	**Y**	I	A	C	W	A	P	F	F	S	V	Q	M	W
rat V1b	M	T	F	V	I	V	L	A	**Y**	I	A	C	W	A	P	F	F	S	V	Q	M	W
human V2	M	T	L	V	I	V	I	V	**Y**	V	L	C	W	A	P	F	F	L	V	Q	L	W
rat V2	M	T	L	V	I	V	V	V	**Y**	V	L	C	W	A	P	F	F	L	V	Q	L	W
fish AVT	M	T	L	V	I	V	L	A	**Y**	I	V	C	W	A	P	F	F	I	V	Q	M	W
fish IST	M	T	F	V	I	V	L	A	**Y**	I	V	C	W	T	P	F	F	F	V	Q	M	W
LSCPR1	L	T	L	T	V	V	L	C	**Y**	L	F	C	W	A	P	F	F	V	V	Q	M	W

Fig. 3. Comparison of the amino acid sequences of transmembrane regions 5 and 6 of the human and rat oxytocin receptors (OTR) [2,3], V1a vasopressin receptors [4,5], V1b vasopressin receptors [6,7] and V2 vasopressin receptors [8,9]; as well as those of the teleost fish *Catostomus commersoni* receptors for Arg-vasotocin (AVT) and Isotocin (IST) [10,11], and the mollusc *Lymnaea stagnalis* receptor for Lys-conopressin (LSCPR1) [12]. Residues at position 509 and 609 in the OT/AVP receptor subtypes are printed in bold and shadowed.

aromatic cluster may play a key role in regulating the agonist-receptor interactions, as has previously been proposed [31].

Since residues 509 and 609 are not conserved between OT and AVP receptors (Fig. 3), similar, but not identical interactions can be predicted between residues 2 and 3 of the peptide agonists and the aromatic cluster present in the different receptor subtypes. We proposed that residues 509 and 609 may thus play a pivotal role in regulating agonist efficacy in the human OTR and this hypothesis was experimentally investigated.

3.3. *Mutations Y509F and F609Y convert AVP from a partial to a full agonist of human OTR*

Residues 509 and 609 were subjected to site-directed mutagenesis in the human OTR, and the pharmacological properties of the mutant receptors were assayed in transiently transfected COS7 cells.

Two preliminary mutations, in which the two residues were substituted by alanine residues, led to receptors with no measurable binding affinity for either agonists or antagonists; furthermore, no agonist-stimulated production of InsPs was observed (data not shown). These findings indicate that these two residues are critical in determining the folding and/or functional properties of the receptor. Their important functional role is further supported by the finding that natural point mutations at these two positions have been identified in the vasopressin V2 receptors of two families of patients with X-linked nephrogenic diabetes insipidus [32,33]. A number of mutations have recently been described in patients suffering from this pathological condition, including some which have already been proved to cause a loss of V2 receptor function (see [34] for review); the two natural mutations involving residues 509 and 609 also certainly deserve careful functional investigation.

We then produced two receptor mutants in which residues 509 and 609 were replaced by the amino acids present in the vasopressin receptor subtypes: Tyr^{509} was converted into a Phe (Y509F mutant) and Phe^{609} into a Tyr (F609Y mutant). Substitutions with aromatic residues are less likely to perturb the folding or overall structure of the receptor dramatically and, in fact, the expression of the receptor mutants was comparable with that observed for the wild-type OTR, as deduced from the B_{max} values determined in saturation experiments (Table 2).

As shown in Table 1, both of the receptor mutants maintained their ability to bind all of the tested agonists and antagonists: in the case of mutant Y509F, there was a slight increase in the affinity for some agonists (OT, Phe^3-OT, Phe^2Orn^8-VT, Thr^4Gly^7-OT), whereas the affinity of the specific OTR antagonist OTA was decreased by a factor of 38. In the case of the F609Y mutant, there was no change (OT) or a slight reduction in the binding affinities for some agonists (AVP, Phe^3-OT, AVT, Phe^2Orn^8-VT, Thr^4Gly^7-OT), whereas the OTA affinity remained unchanged. These data indicate that residues 509 and 609 do not contribute to the high affinity binding of these analogues to the human OTR.

Since our data indicate that AVP is a partial agonist of the human OTR, and that this property does not depend on the

cell line expressing the receptor, we performed dose-response curves of total InsPs accumulation only in the transiently transfected COS7 expressing the mutant Y509F and F609Y receptors (Fig. 1D,E). With both mutants, the intrinsic activity of OT itself was not significantly affected: it produced a maximal 3–5-fold increase in total InsPs over baseline, the same degree of stimulation as that observed with the wild-type receptor. In addition, the dose-response curve extended over an agonist concentration range of two orders of magnitude, suggesting that the reaction obeys the classical mass law action and that the system is not saturated. However, the intrinsic activity of AVP increased dramatically, becoming respectively 87% ($n=7$; $P<0.001$) and 88% ($n=7$; $P<0.001$) of the maximal OT stimulation in the Y509F and F609Y OTR mutants (Table 2). Our data indicate that the intrinsic activity of AVP is greatly modified in the two mutated receptors.

It is now generally accepted that GPCRs exist as a population of metastable conformers whose intrinsic dynamics and distribution are modulated by various factors: the presence of proline residues in the α-helical transmembrane domains; the length of the connecting loops, which may possibly even allow the sub-domains a certain relative motion; and finally the network of side chain–side chain interactions between adjacent transmembrane domains (see [35–38] and references therein). The ligands may have a preferential affinity for a given pre-existing side-chain network (conformational selection theory), or they may be regarded as agents capable of perturbing the system by triggering a reorganisation of the network of interactions (conformational induction theory) ([39] and references therein); it is most probable that the two phenomena coexist.

Our mutagenesis data do not make it possible to distinguish between the two theories, although we can suggest that mutation Y509F is more likely to affect the spontaneous equilibrium between the inactive (R) and active receptor (R*) states. In this mutation, we observed a generalised increase in affinity for almost all of the agonists tested, with a parallel decrease in the affinity for the specific OTR antagonist OTA; we also observed 10-fold lower EC_{50} values for OT which probably reflect the 3-fold increase in the affinity of this analogue. Similarly, a slight decrease in the EC_{50} of AVP was observed, even if in this case no significant change in AVP affinity was measured. Unfortunately, we were not able to detect any significant and reproducible increase in its basal activity, a finding that would have strongly supported this hypothesis.

In the case of mutation Y609F, a decrease in the affinity for some agonists was observed, with no change in either the potency or intrinsic activity of OT itself. Similarly, the affinity of the specific antagonist [^{125}I]OTA remained unchanged. In this case, a direct interactions between residue 609 and the aromatic residues 2 and/or 3 of the peptides may contribute towards the intrinsic efficacy of the ligand-receptor complex, an hypothesis that will need further studies to be experimentally validated.

A limited number of direct ligand-receptor contacts have been shown to modulate the coupling efficacy of GPCRs: these include hydrogen bonding interactions, as in muscarinic receptors [40]; ionic interactions, as in β-adrenergic and angiotensin II receptors [41,42]; and now the aromatic-hydrophobic interactions described here for the OT/AVP receptors. It can be presumed that a few residues will be found to play a general role in stabilising active conformations in different receptor subtypes, whereas specific peptide–receptor interactions regulating agonist efficacy will be found in each receptor family. It is worth mentioning that aromatic residues are present at positions 509 and 609 in most rhodopsin-like GPCRs; we therefore think that these residues may modulate the process of receptor activation in various members of the GPCR family.

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