

# An extracellular residue determines the agonist specificity of V<sub>2</sub> vasopressin receptors

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**Abstract** The specific V<sub>2</sub> agonist 1-deamino [8-D-arginine]-vasopressin (dDAVP), used for treatment of central diabetes insipidus, binds to vasopressin V<sub>2</sub> receptors from human, bovine and rat kidney with an affinity that is similar to that of the natural hormone vasopressin. In contrast, the V<sub>1</sub> receptors and the porcine V<sub>2</sub> receptor do not tolerate a D-arginine in position 8 of vasopressin. By site directed mutagenesis of the cloned bovine and porcine V<sub>2</sub> receptors we identified a residue (Asp-103) in the first extracellular loop of vasopressin receptors which is responsible for high affinity binding of dDAVP.

**Key words:** Vasopressin; Receptor subtype; Specificity; Central diabetes insipidus

## 1. Introduction

The neurohypophyseal nonapeptide arginine vasopressin (AVP) is the most important hormone regulating water reabsorption in the mammalian kidney. The antidiuretic effect is mediated through the adenylate cyclase coupled V<sub>2</sub> receptor located in the basolateral membrane of collecting ducts [1]. The hormonal induced increase of the cyclic AMP level results in the activation of cAMP dependent protein kinases. The final step of vasopressin action is the exocytic insertion of water channels into the apical membrane of collecting ducts [2,3] (for a recent review see [4]).

The insufficiency of AVP caused by malfunction of AVP biosynthesis in the hypothalamo-neurohypophyseal system results in the syndrome of central hypothalamic diabetes insipidus (CDI) which is physiologically characterized by primary polyuria [5]. V<sub>2</sub> receptor specific agonists and antagonists have been developed for treatment of disturbances in the vasopressin-regulated salt and water balances [6,7]. In 1968 the use of dDAVP (1-deamino [8-D-arginine]vasopressin) or desmopressin (Minirin) for therapy of CDI was reported for the first time [8] and has been successfully used for this indication ever since. This nonapeptide was obtained by two modifications of vasopressin [9]: deamination of the hemicycstine in position 1

prolongs and enhances the antidiuretic effect and substitution of L-Arg to D-Arg in position 8 reduces pressor activity in rat bioassays [10].

At present, the molecular basis for the interaction of the V<sub>2</sub> receptor subtype with such specific ligands like dDAVP is unknown, although receptor cloning has shown that V<sub>1</sub> and V<sub>2</sub> receptors belong to the G-protein coupled receptor family with seven transmembrane helices [11–16]. V<sub>1a</sub>, V<sub>1b</sub> and V<sub>2</sub> receptors have different ligand specificities and second messenger systems [17,18]. Recently, an extracellular agonist binding site in the renal V<sub>2</sub> vasopressin receptor has been localized by a protein chemical approach [19]. Applying photoaffinity labeling with a radioactive agonist and sequencing of peptide fragments, amino acid residues in the second extracellular domain (first extracellular loop) of the bovine V<sub>2</sub> receptor were identified as part of the hormone binding site. These results provide a basis for further studies of the relationship between structure and function of the V<sub>2</sub> receptor by site directed in vitro mutagenesis of the cloned V<sub>2</sub> receptor. The present work was performed to identify residues involved in hormone binding especially those which determine the high specificity for the V<sub>2</sub> agonist dDAVP.

## 2. Materials and methods

### 2.1. Construction and screening of a cDNA library

Messenger RNA was isolated from bovine kidneys by the guanidinium-thiocyanate method [20]. Poly(A)-RNA was purified by oligo(dT)-cellulose chromatography. The cDNA library was constructed by ligation of 1.3–8 kb sized cDNA, synthesized by Superscript reverse transcriptase (GibcoBRL), to  $\lambda$ gt 10-EcoRI-arms (Stratagene). A <sup>32</sup>P-labeled probe from the full-length porcine V<sub>2</sub> receptor-DNA [14] was taken for screening of the  $\lambda$ gt 10 cDNA library. Hybridization and washing was performed as previously described [14]. The inserts from recombinant phages were recloned into pGEM-7Zf(+) (Promega) and sequenced in both directions with the Sequenase version 2.0 kit (USB). The only full length clone was sequenced completely by application of the exonuclease III mung bean nuclease approach [21] and proper oligonucleotides.

### 2.2. Mutagenesis

Wild type cDNA of the bovine and porcine V<sub>2</sub> receptor [14] was recloned in the mutagenesis plasmid pALTER-1 (PROMEGA). The mutants were constructed using a modified protocol from PROMEGA for oligonucleotide-mediated, site-directed mutagenesis. Mutagenesis was confirmed by DNA sequencing.

### 2.3. Expression in COS cells

The wild type bovine and porcine V<sub>2</sub> receptor cDNA and their mutants were subcloned into the expression vector pCDM8 [22] (Invitrogen) using BstXI-adapters. COS.M6 cells were transfected with the resulting plasmids using the DEAE-dextran method [14]. 72 h after transfection cells were removed from dishes by PBS/1 mM EDTA treatment, collected by centrifugation at 1200 × g and resuspended in 5 mM Tris-HCl pH 7.4, 1 mM EDTA. Then they were homogenized with a Teflon-potter at 3000 rpm. Membranes were collected by centrifugation at 45,000 × g. The pellet was washed three times by centrifuga-

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The novel nucleotide sequence data published here have been deposited with EMBL sequence data bank under Accession No. X83741.

**Abbreviations:** AVP, [8-arginine]vasopressin; CDI, central diabetes insipidus; dDAVP, 1-deamino [8-D-arginine]vasopressin; DAVP, [8-D-arginine]vasopressin; dAVP, 1-deamino [8-arginine] vasopressin; LVP, [8-lysine]vasopressin; OT, oxytocin.

tion and resuspension in 15 mM HEPES pH 7.4, 1.5 mM MgCl<sub>2</sub>. Membranes were frozen in aliquots at -80°C. The V<sub>2</sub> receptor density for wild types and mutants was in all cases higher than 2.5 pmol per mg protein.

#### 2.4. Binding assay

Saturation binding studies were performed at 30°C for 30 min in a total volume of 300 µl containing 26 µg membrane protein, 50 mM EPPS pH 8.3/5 mM MgCl<sub>2</sub> and various concentrations of [<sup>3</sup>H]AVP (411–736 GBq/mmol; NEN). Non-specific binding was determined in the presence of 300-fold excess of non-radioactive AVP. Bound [<sup>3</sup>H]AVP was separated from free ligand by filtration through Whatman GF/F filters. The same conditions were used for displacement experiments. In a total volume of 100 µl 10 nM [<sup>3</sup>H]AVP was displaced by several non-radioactive ligands in various concentrations. The binding data were analysed using the Ligand program [23] (Biosoft). Peptide ligands were obtained from Bachem, Heidelberg, the non-peptide V<sub>2</sub> antagonist OPC-31260 was obtained from Otsuka America Pharmaceutical, Inc., MD.

### 3. Results and discussion

The protein chemical identification of an extracellular agonist binding site in the renal V<sub>2</sub> receptor has been recently performed on the V<sub>2</sub> receptor protein isolated from bovine kidney membranes [19]. Using these results as a basis for further structure activity studies, site directed in vitro mutagenesis should be first performed on the V<sub>2</sub> receptor cDNA from the same species. As no full length bovine V<sub>2</sub> receptor cDNA has yet been cloned, a λgt10 cDNA library from bovine kidney medulla was constructed and screened with the <sup>32</sup>P-labeled full-length porcine V<sub>2</sub> receptor cDNA [14]. Analysis of 19 independent recombinant bacteriophages showed different cDNA-sizes for each clone ranging between 500 bp and 1.6 kb. Only one clone represented a full-length cDNA. It was 1443 nucleotides long and had an open reading frame of 1110 nucleotides.

The bovine V<sub>2</sub> receptor cDNA predicts a protein of 370 amino acids with a calculated M<sub>r</sub> of 40,236 in the absence of post-translational modifications (Fig. 1a). The amino acid sequence identity with the human, rat and pig V<sub>2</sub> receptors is 85%, 82% and 88% respectively, with the human and rat V<sub>1a</sub> receptors only 39%.

The binding properties of the cloned bovine V<sub>2</sub> receptor were examined after transient expression in COS M6 cells. Dose-dependent binding of [<sup>3</sup>H]AVP gave an apparent dissociation constant (K<sub>d</sub>) of 0.5 nM. Competition binding experiments confirmed that the cloned receptor has the typical pharmacological profile of the V<sub>2</sub> subtype (Table 1). The most competent inhibitor of [<sup>3</sup>H]AVP was the nonpeptide specific V<sub>2</sub> antagonist OPC-31260 [7], (K<sub>i</sub> = 0.2 nM), followed by AVP itself, (K<sub>i</sub> = 1.3 nM), the specific V<sub>2</sub> agonist dDAVP (K<sub>i</sub> = 3.4 nM),

[Lys<sup>8</sup>]vasopressin (LVP) (K<sub>i</sub> = 4.4 nM) and oxytocin (K<sub>i</sub> = 96 nM).

For further definition of the V<sub>2</sub> receptor agonist binding site, residues for in vitro mutagenesis were selected based on the protein chemical labeling results on the one hand, and differences in amino acid sequence and ligand selectivity of vasopressin receptors on the other hand. The sequence of the 18 amino acids in the first extracellular loop is conserved within the cloned V<sub>2</sub> receptors with only one substitution in each V<sub>2</sub> receptor (Fig. 1b). With respect to the affinity for the specific V<sub>2</sub> agonist dDAVP, there exist differences between V<sub>2</sub> receptors from different species which might be related to structural differences in their first extracellular loop: dDAVP binds to V<sub>2</sub> receptors from the human kidney [12,24], the rat kidney [13,25] and the bovine kidney (Table 1) with an affinity that is similar to that for AVP. In contrast, the affinity of the porcine V<sub>2</sub> receptor, which has [Lys<sup>8</sup>]vasopressin as the natural ligand, is for dDAVP more than 200-fold lower than for AVP [26]. A similar difference in affinity between dDAVP and AVP has been described for the V<sub>1a</sub> receptor from the human [27] and the rat liver [11] and for the V<sub>1b</sub> from the rat pituitary receptor [28]. Whereas V<sub>2</sub> receptors with high affinity for dDAVP have an aspartic acid residue in position 103, this residue is replaced in the porcine V<sub>2</sub> receptor by tyrosine. A tyrosine residue is also present in the corresponding position of the cloned V<sub>1a</sub> and V<sub>1b</sub> receptors (Fig. 1b).

These observations and the location of Asp-103 between the two photoaffinity-labeled residues Thr-102 and Arg-106 led us to the hypothesis that aspartic acid in position 103 of V<sub>2</sub> receptors is involved in hormone binding, especially in high affinity binding for the V<sub>2</sub> agonist dDAVP. To test this hypothesis, we exchanged by site directed in vitro mutagenesis Asp-103 through tyrosine yielding the mutant bD103Y. Following this single mutation, the affinity for dDAVP decreased more than 40-fold as compared to the wild type (Table 1, Fig. 2a and d), whereas the high affinity for AVP or for the specific V<sub>2</sub> antagonist OPC-31260 was retained. The reduction in affinity for dDAVP as compared to that for AVP could mainly be due to either the replacement of L-arginine in position 8 of AVP by the D-isomer in dDAVP or to the lack of an α-amino group in dDAVP. To test the influence of these substitutions separately, we determined the K<sub>i</sub> values of the two analogues with one substitution: 1-deamino [Arg<sup>8</sup>] vasopressin (dAVP) and [D-Arg<sup>8</sup>]vasopressin (DAVP). Binding experiments showed (Table 1, Fig. 2) that the bovine and porcine wild type V<sub>2</sub> receptors (Fig. 2a and b) and the mutant bD103Y (Fig. 2d) bind dAVP with the same or even slightly higher affinity than AVP. In contrast, the replacement of L-arginine by its D-isomer in

Table 1  
K<sub>i</sub> values (nM) of wildtype (wt) and mutated V2-receptors<sup>a</sup>

| Ligands   | bV2R (wt) | bD103Y | pV2R (wt) | pY102D                | pY102N | bR106L |
|-----------|-----------|--------|-----------|-----------------------|--------|--------|
| AVP       | 1.31      | 1.87   | 2.46      | 7.13                  | 15.95  | 1.38   |
| dDAVP     | 3.36      | 138.91 | 44.63     | 40.14                 | 41.03  | 2.65   |
| dAVP      | 0.36      | 0.94   | 1.25      | 3.76                  | 5.55   | n.d.   |
| DAVP      | 0.92      | 40.81  | 72.67     | 6.82                  | 10.47  | n.d.   |
| OPC-31260 | 0.19      | 0.34   | 2.46      | 3.07                  | 9.61   | n.d.   |
| LVP       | 4.43      | 3.21   | 3.02      | 84.95                 | 33.36  | 5.05   |
| OT        | 95.94     | 94.52  | 310.65    | < 10,000 <sup>b</sup> | 864.18 | 62.46  |

<sup>a</sup> K<sub>i</sub> values were calculated from displacement experiments as shown in Fig. 2.

<sup>b</sup> No complete displacement was achieved.

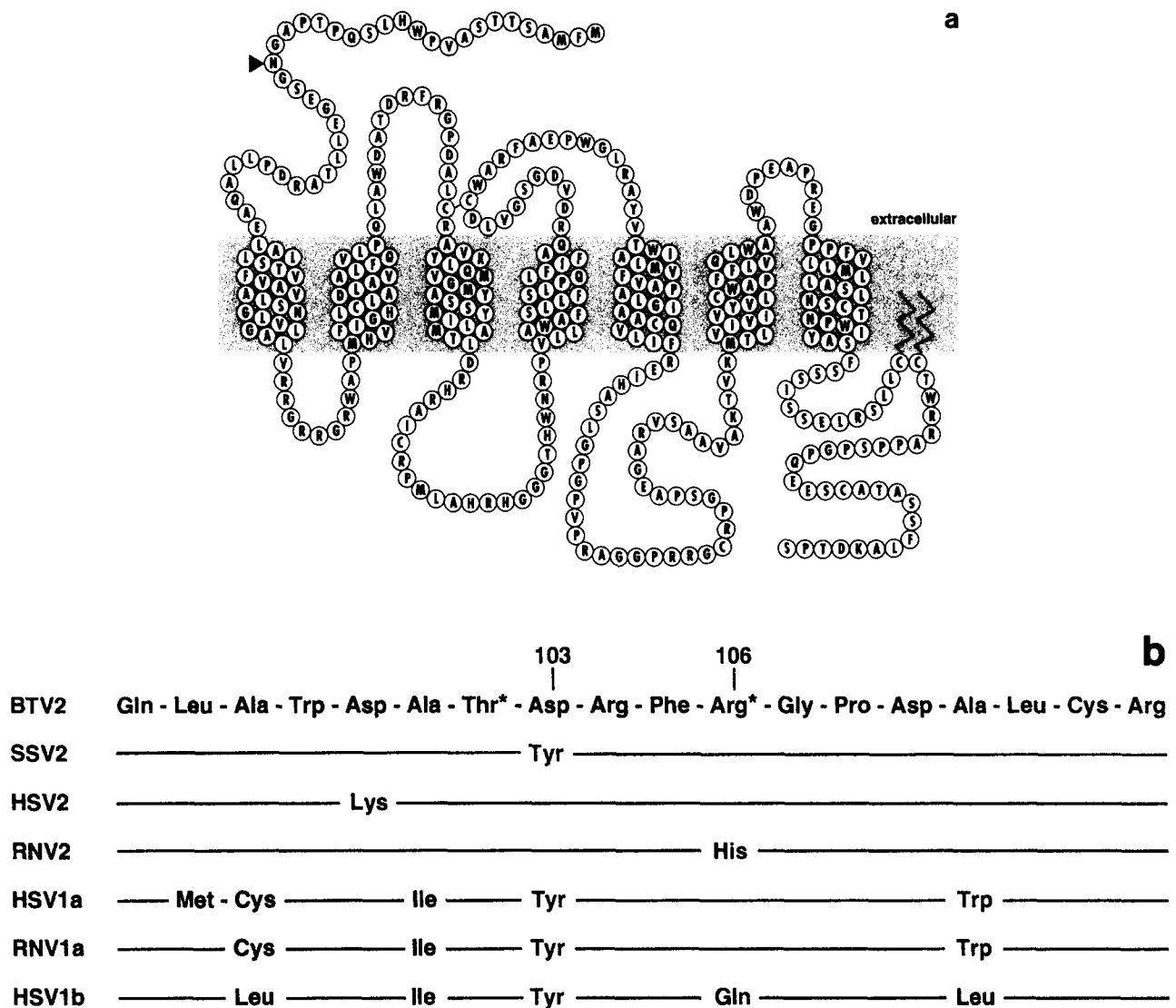


Fig. 1. (a) Deduced amino acid sequence and schematic model of the bovine V<sub>2</sub> receptor. (b) Comparison of the putative first extracellular loop from cloned V<sub>2</sub> and V<sub>1</sub> receptors. Transmembrane regions were predicted by hydrophobicity analysis [30]. ▲ marks an N-glycosylation site. Positions of site-directed in vitro mutagenesis (103 and 106) are indicated. Asterisks indicate the residues identified by photoaffinity labeling. BT, bovine; SS, porcine; HS, human; RN, rat.

DAVP leads to a loss of affinity of the porcine V<sub>2</sub> receptor and the mutant bD103Y. This reduction in affinity for DAVP is lower than that for dDAVP indicating an influence of the protonated  $\alpha$ -amino group on the conformation of the ligand. This effect has been previously reported for AVP [29]. Regression analysis of log  $K_i$ -values of the seven ligands (Table 1) determined for the mutant bD103Y versus log  $K_i$ -values for the porcine V<sub>2</sub> receptor showed a correlation coefficient of 0.96, whereas for the two wild-type V<sub>2</sub> receptors a value of 0.65 was found. These results provide evidence that by single exchange of Asp-103 through tyrosine the differences in ligand specificity of the two V<sub>2</sub> receptors and especially the difference in affinity for ligands with D-arginine in position 8 like dDAVP and DAVP are determined.

For verification of this observation we mutated the corresponding Tyr-residue (Tyr-102) in the porcine V<sub>2</sub> receptor into the corresponding Asp-residue found in the human, bovine and rat V<sub>2</sub> receptor, which indeed turned the pharmacology of the

mutant pY102D to a 10-fold higher affinity state for dDAVP and DAVP, whereas the affinity for AVP slightly (3-fold) decreased (Table 1, Fig. 2b and c). Regression analysis of six log  $K_i$  values determined for the mutant pY102D against the corresponding log  $K_i$  values of wild type bovine V<sub>2</sub> receptor yielded a correlation coefficient of 0.94. The results of orthologues mutagenesis of both V<sub>2</sub> receptors demonstrate that the amino acid residue in position 103, respectively 102 located in the first extracellular loop of V<sub>2</sub> receptors determines the high affinity binding for the specific V<sub>2</sub> agonist dDAVP. The increase in the free energy of binding by introducing aspartic acid in this position could be due to the formation of a salt bridge between Asp-103 and D-arginine of the ligand. Alternatively, the weaker binding affinity for dDAVP and DAVP of receptors containing a tyrosine in this position could be explained by steric hindrance due to the Tyr residue which may inhibit ion-ion binding of D-arginine in dDAVP but not of L-arginine in AVP with another acidic residue in the loop region. To discriminate

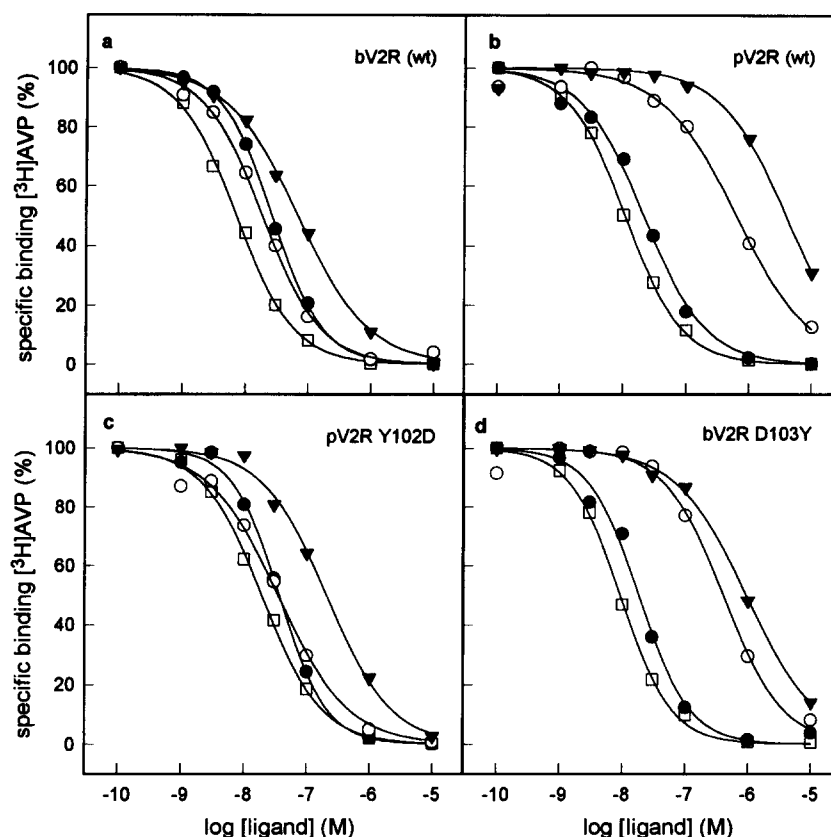


Fig. 2. Binding of AVP, dDAVP and analogues to wild type and mutant  $V_2$  receptors. Membranes from COS.M6 cells expressing wild type (a) and the bD103Y mutant (d) of the bovine  $V_2$  receptor were incubated with 10 nM [ $^3$ H]AVP and increasing concentrations of AVP (●), dDAVP (▼), dAVP (□), and DAVP (○). Values of 100% corresponds to binding of [ $^3$ H]AVP found at the lowest concentration ( $10^{-10}$  M) of non-labeled ligand. The same procedure was performed with membranes from COS.M6 cells expressing wild type (b) and the pY102D mutant (c) of the porcine  $V_2$  receptor. Data were analysed by the LIGAND program. The data shown are the means of duplicate values and are representative of two independent experiments.

between these two possibilities we mutated Tyr-102 in the porcine  $V_2$  receptor to an asparagine residue. This replacement again resulted in an increase of the affinity for the ligands with D-arginine in position 8 dDAVP and DAVP relative to that for AVP. Regression analysis of the log  $K_i$  values (Table 1) determined for the mutant pY102N against the  $K_i$  values for wild type bovine  $V_2$  receptor yielded a correlation coefficient of 0.96. These results demonstrate that replacement of Tyr-102 by either Asp or Asn in the porcine  $V_2$  receptor turns the pharmacology to the higher affinity state for dDAVP. All mutated and wildtype receptors were able to stimulate adenylate cyclase in COS cells (results not shown).

The results of the photoaffinity labeling experiments suggested that Arg-106 is close to the hormone binding site. We decided to substitute Arg-106 in the bovine  $V_2$  receptor by a non-charged leucine residue, whereas all  $V_2$  and  $V_{1a}$  receptors exhibit in this position a positively charged amino acid. Binding experiments with the corresponding mutant bR106L exclude that a positively charged amino acid in position 106 is required for high affinity binding for any of the tested ligands to the  $V_2$  receptor (Table 1).

In conclusion, the high affinity of renal  $V_2$  receptors for the selective  $V_2$  agonist dDAVP can be abolished by substitution of Asp-103 against Tyr which is found in the corresponding position of  $V_1$  receptors. This mutation does not affect the affinity for the natural hormone AVP or a specific non-peptide

$V_2$  antagonist. In the case of the porcine  $V_2$  receptor, which does not tolerate D-arginine in position 8 of vasopressin analogues, high affinity for dDAVP can be induced by replacement of the corresponding Tyr-102 in the porcine receptor against Asp or Asn. The observations suggest that steric requirements imposed by a D-arginine in position 8 of dDAVP and by the extracellular tyrosine in vasopressin receptors prevent their high affinity interaction. A single extracellular residue obviously can determine the agonist specificity of receptor subtypes. The results should provide the basis for a more detailed analysis of interaction between ligands and the vasopressin receptor by new mutagenesis experiments and for the development of specific  $V_2$  agonists with further improved properties.

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