An extracellular residue determines the agonist specificity of V_2 vasopressin receptors

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Received 11 January 1995; revised version received 2 February 1995

Abstract The specific V_2 agonist 1-deamino [8-D-arginine]-vasopressin (dDAVP), used for treatment of central diabetes insipidus, binds to vasopressin V_2 receptors from human, bovine and rat kidney with an affinity that is similar to that of the natural hormone vasopressin. In contrast, the V_1 receptors and the porcine V_2 receptor do not tolerate a D-arginine in position 8 of vasopressin. By site directed mutagenesis of the cloned bovine and porcine V_2 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_2 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_2 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 hemicystine 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_2 receptor subtype with such specific ligands like dDAVP is unknown, although receptor cloning has shown that V_1 and V_2 receptors belong to the G-protein coupled receptor family with seven transmembrane helices [11–16]. V_{1a}, V_{1b} and V_2 receptors have different ligand specificities and second messenger systems [17,18]. Recently, an extracellular agonist binding site in the renal V_2 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_2 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_2 receptor by site directed in vitro mutagenesis of the cloned V_2 receptor. The present work was performed to identify residues involved in hormone binding especially those which determine the high specificity for the V_2 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 λgt 10-*Eco*RI-arms (Stratagene). A ^{32}P-labeled probe from the full-length porcine V_2 receptor-DNA [14] was taken for screening of the λgt 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_2 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_2 receptor cDNA and their mutants were subcloned into the expression vector pCDM8 [22] (Invitrogen) using *Bst*XI-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 $1200 \times g$ 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 $45{,}000 \times g$. 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.

SSDI 0014-5793(95)00150-6

tion and resuspension in 15 mM HEPES pH 7.4, 1.5 mM $MgCl_2$. Membranes were frozen in aliquots at −80°C. The V_2 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 $MgCl_2$ and various concentrations of [^{3}H]AVP (411–736 GBq/mmol; NEN). Non-specific binding was determined in the presence of 300-fold excess of non-radioactive AVP. Bound [^{3}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 [^{3}H]AVP was displaced by several non-radioactive ligands in various concentrations. The binding datas were analysed using the Ligand program [23] (Biosoft). Peptide ligands were obtained from Bachem, Heidelberg, the non-peptide V_2 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_2 receptor has been recently performed on the V_2 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_2 receptor cDNA from the same species. As no full length bovine V_2 receptor cDNA has yet been cloned, a λgt10 cDNA library from bovine kidney medulla was constructed and screened with the ^{32}P-labeled full-length porcine V_2 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_2 receptor cDNA predicts a protein of 370 amino acids with a calculated M_r of 40,236 in the absence of post-translational modifications (Fig. 1a). The amino acid sequence identity with the human, rat and pig V_2 receptors is 85%, 82% and 88% respectively, with the human and rat V_{1a} receptors only 39%.

The binding properties of the cloned bovine V_2 receptor were examined after transient expression in COS M6 cells. Dose-dependent binding of [^{3}H]AVP gave an apparent dissociation constant (K_d) of 0.5 nM. Competition binding experiments confirmed that the cloned receptor has the typical pharmacological profile of the V_2 subtype (Table 1). The most competent inhibitor of [^{3}H]AVP was the nonpeptide specific V_2 antagonist OPC-31260 [7], (K_i = 0.2 nM), followed by AVP itself, (K_i = 1.3 nM), the specific V_2 agonist dDAVP (K_i = 3.4 nM), [Lys^8]vasopressin (LVP) (K_i = 4.4 nM) and oxytocin (K_i = 96 nM).

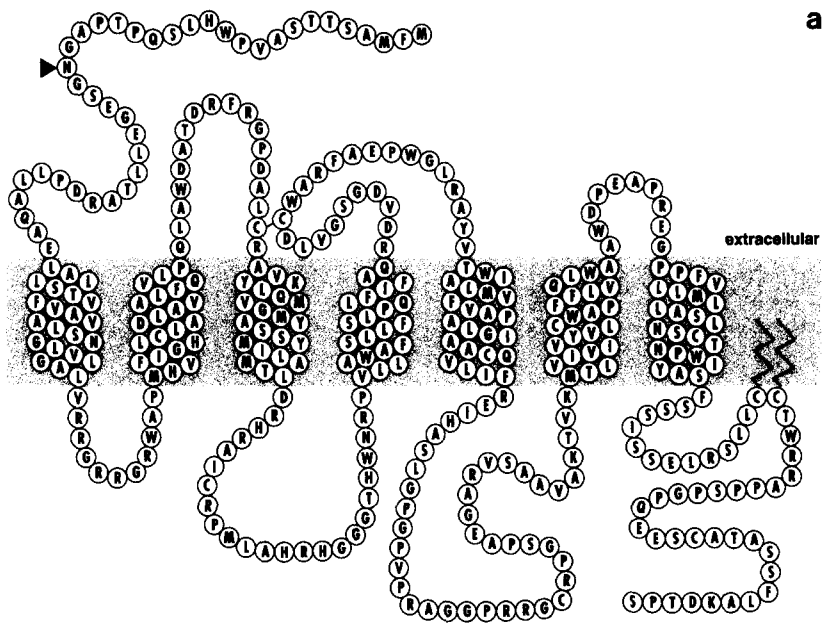
For further definition of the V_2 receptor agonist binding site, residues for in vitro mutagenesis were selected based on the protein chemical labeling results on the one hand, and differences in amino acid sequence and ligand selectivity of vasopressin receptors on the other hand. The sequence of the 18 amino acids in the first extracellular loop is conserved within the cloned V_2 receptors with only one substitution in each V_2 receptor (Fig. 1b). With respect to the affinity for the specific V_2 agonist dDAVP, there exist differences between V_2 receptors from different species which might be related to structural differences in their first extracellular loop: dDAVP binds to V_2 receptors from the human kidney [12,24], the rat kidney [13,25] and the bovine kidney (Table 1) with an affinity that is similiar to that for AVP. In contrast, the affinity of the porcine V_2 receptor, which has [Lys^8]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_{1a} receptor from the human [27] and the rat liver [11] and for the V_{1b} from the rat pituitary receptor [28]. Whereas V_2 receptors with high affinity for dDAVP have an aspartic acid residue in position 103, this residue is replaced in the porcine V_2 receptor by tyrosine. A tyrosine residue is also present in the corresponding position of the cloned V_{1a} and V_{1b} 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_2 receptors is involved in hormone binding, especially in high affinity binding for the V_2 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_2 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_i values of the two analogues with one substitution: 1-deamino [Arg^8] vasopressin (dAVP) and [D-Arg^8]vasopressin (DAVP). Binding experiments showed (Table 1, Fig. 2) that the bovine and porcine wild type V_2 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_i values (nM) of wildtype (wt) and mutated V2-receptors[a]

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[b]	864.18	62.46

[a] K_i values were calculated from displacement experiments as shown in Fig. 2.
[b] No complete displacement was achieved.



b

	103	106
BTV2	Gln - Leu - Ala - Trp - Asp - Ala - Thr* - Asp - Arg - Phe - Arg* - Gly - Pro - Asp - Ala - Leu - Cys - Arg	
SSV2	Tyr	
HSV2	Lys	
RNV2		His
HSV1a	Met - Cys — Ile — Tyr	Trp
RNV1a	Cys — Ile — Tyr	Trp
HSV1b	Leu — Ile — Tyr	Gln — Leu

Fig. 1. (a) Deduced amino acid sequence and schematic model of the bovine V_2 receptor. (b) Comparison of the putative first extracellular loop from cloned V_2 and V_1 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_2 receptor and the mutant bD103Y. This reduction in affinity for DAVP is lower than that for dDAVP indicating an influence of the protonated α-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_2 receptor showed a correlation coefficient of 0.96, whereas for the two wild-type V_2 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_2 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_2 receptor into the corresponding Asp-residue found in the human, bovine and rat V_2 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_2 receptor yielded a correlation coefficient of 0.94. The results of orthologues mutagenesis of both V_2 receptors demonstrate that the amino acid residue in position 103, respectively 102 located in the first extracellular loop of V_2 receptors determines the high affinity binding for the specific V_2 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

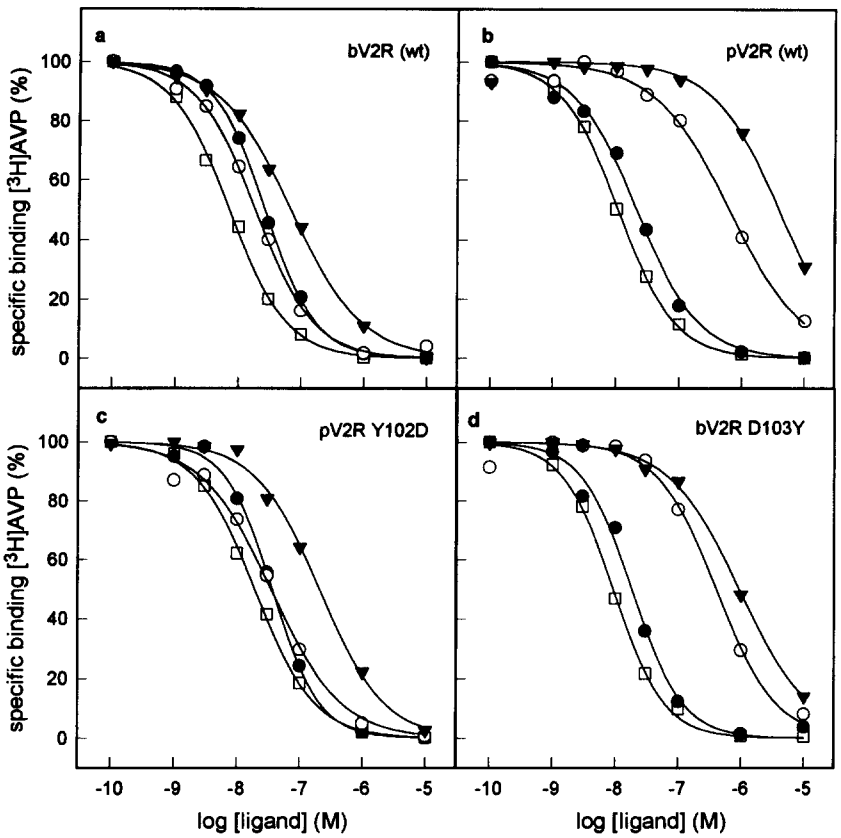


Fig. 2. Binding of AVP, dDAVP and analogues to wild type and mutant V_2 receptors. Membranes from COS.M6 cells expressing wild type (a) and the bD103Y mutant (d) of the bovine V_2 receptor were incubated with 10 nM [^{3}H]AVP and increasing concentrations of AVP (●), dDAVP (▼), dAVP (□), and DAVP (○). 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 programm. 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.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 169). We thank Dr. Aida Akhundova for preparation of mRNA from bovine kidney, Dr. Gisela Fobo, Max-Planck-Institute of Biochemistry, Munich, for the sequence alignments, Otsuka America Pharmaceutical, Inc. Maryland for the V_2 antagonist OPC-31260.

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