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Original Article

Effect of mutations in putative hormone binding sites on V2 vasopressin receptor function

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Abstract

The vasopressin V2 receptor belongs to the large family of the G-protein coupled receptors and is responsible for the antidiuretic effect of the neurohypophyseal hormone arginine vasopressin (AVP). Based on bioinformatic studies it seems that Ala300 and Asp297 of the V2 vasopressin receptor (V2R) are involved in receptor binding. Ala300Glu mutation resulted in lower energy while Asp297Tyr mutation resulted in higher energy in AVP-V2R docked complex rather than the wild type. Therefore we hypothesized that the Ala300Glu mutation results in stronger and Asp297Tyr mutation leads to weaker ligand-receptor binding. Site directed mutagenesis of Asp297Tyr and Ala300Glu was performed using nested polymerase chain reaction. After restriction enzyme digestion, the inserts were ligated into the pcDNA3 vector and Escherichia *coli* XL1-Blue competent cells were transformed using commercial kit and electroporation methods. The obtained colonies were analyzed for the presence and orientation of the inserts using proper restriction enzymes. After transient transfection of COS-7 cells using ESCORT^{TM} IV transfection reagent, the adenylyl cyclase activity assay was performed for functional studies. The cell surface expression of V2R was analyzed by indirect ELISA method. Based on the obtained results, the Ala300Glu mutation of V2R led to reduced levels of cAMP production without a marked effect on the receptor expression and the receptor binding. Effect of Asp297Tyr mutation on cell surface expression of V2R was the same as the wild type receptor. Pretreatment with 1 nM vasopressin showed an increased level of Asp297Tyr mutant receptor internalization as compared to the wild type receptor, while the effect of 100 nM vasopressin was similar in the mutant and wild type receptors. These data suggest that alterations in Asp297 but not Ala300 would affect the hormone receptor binding.

Keywords: V2 vasopressin receptor; Mutation; Nested PCR; COS-7 cells; cAMP; Binding

INTRODUCTION

Arginine vasopressin (AVP), the antidiuretic hormone, is a cyclic nonapeptide that modulates various physiological functions such as water reabsorption, blood volume, blood pressure, cellular proliferation and adrenocorticotropic hormone secretion. The antidiuretic effect of AVP is mediated by the vasopressin V2 receptor (V2R), a member of the large family of G protein-coupled receptors (1-4). V2R is a 41-kDa seven-transmembrane protein of 371 residues (5).

In the collecting ducts of the kidney, AVP binds to the V2R, thereby activating the Gs/adenylyl cyclase system. The subsequent rise in intracellular cAMP levels induces protein kinase A (PKA) to phosphorylate aquaporin 2. The effects of PKA activation vary with cell type. For example in the kidney's principal cells, activation of this kinase leads to the exocytosis of aquaporin 2 to apical membrane, synthesis of aquaporin 2 and stimulating phosphorylation of aquaporin 2 (4,6-7). Some mutant of the V2 receptors such as V2R R113W were found to affect cell

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surface expression (20% of wild type), ligand binding (20-fold decrease in affinity) and signaling (8, 9). Although D85N is expressed normally on the cell surface, binding to arginine vasopressin was lowered by 6-folds, which together with a 20-fold decrease in coupling efficiency, resulted in a 50-fold increase in EC50 (8,10). G12E, A61V and ΔR247-G250, were found to have normal ligand binding and signaling suggesting that they may not be the cause of nephrogenic diabetes insipidus in patients having these mutations (8,11). In the human V_2 vasopressin receptor, the substitution of the aspartate at position 136 by alanine leads to agonist-independent activation of this mutant V2 receptor (12). The G12E mutant was functionally the same as the wild type V_2 receptor in both cAMP stimulation and binding affinity (13). Mutant receptors G185C and R202C were efficiently transported to the plasma membrane but were defective in ligand binding (14). By site directed mutagenesis of the cloned bovine and porcine V_2 receptors a residue (Asp-103) in the first extracellular loop of vasopressin receptors was identified which was responsible for high affinity binding of desmopressin (dDAVP) (15). In contrast to the wild-type receptor, the naturally occurring mutant R337X failed to confer specific [^{3}H] AVP binding to the transfected cells (16). Y128S, R181C, ΔR202, R202C, and P286R, are defective in ligand binding (8,11,17-18).Mutant receptors (C112S, C112A and C192S, C192A) were non-functional and located mostly in the cell's interior. The conserved cysteine residues of the V2 receptor are thus not only important for the structure of the ligand binding domain but also for efficient intracellular receptor transport (14).

Residual binding capacity for the AVP could be detected for the T204N mutant but was 10-fold lower than the wild-type receptor. Stimulation of the transfected cells with 1 mM AVP showed that the T204N mutant was able to activate the adenylyl cyclase pathway. In contrast, the Y205C mutant was almost inactive and stimulation of the V206D mutant slightly increased the cAMP accumulation (19). The conserved cysteine residues Cys-341 and Cys-342 were replaced by serine residues, yielding the single (C-341S and C-342S) and double (C-341S, C-342S) mutants. Functional expression in stably transfected Ltk−cells showed that the affinities of the three mutant receptors for arginine vasopressin were not altered (20).

Some mutations might affect multiple aspects of receptor function. Because previous researchers were not able to detect the exact site of the ligand–receptor binding, in this study the amino acid substitution in the predicted binding domain was investigated. Since there are differences between function, ligand binding and cell surface expression of wild type V2 receptor and that of the mutant V2 receptor, therefore functional assay of the predicted binding site of V2 vasopressin receptor hormone was the main purpose of this study. Based on our previous bioinformatics studies it seems that Ala300 and Asp297 of the V2 vasopressin receptor are involved in the receptor binding domain. The docking study of AVP to its receptor showed that Ala300Glu substitution possess positively inducing effect in ligand binding while Asp297Tyr substitution possess negatively inducing effect in ligand binding. Therefore, in the present study we investigated the effect of these mutations on V2 receptor function.

MATERIALS AND METHODS

Correlate–ELA Direct Cyclic AMP Enzyme Immunoassay Kit was from Assay Designs, Inc. USA. Gel Extraction Kit was from Qiagen, Germany. PureLink® HiPure Plasmid DNA Purification Kit was from Invitrogene, Life Technologies, USA. Primers were procured from Sigma-Aldrich, Germany.

Anti-Mouse Peroxidase, Anti HA (12CA5), Forskolin, Isobutyl-1-methylxanthine, O-phenylenediamine, AVP and bovine serum albumin (BSA) lyophilized powder ≥96% were purchased from Sigma, USA.

*Bbr*PI restriction enzyme was from Roche, Germany. 10X High Fidelity polymerase chain reaction (PCR) buffer, MgCl2, dNTP, Taq DNA polymerase, DNA weight marker, RNase A enzyme, FastDigest *Eco*RI, FastDigest *Xba*I, FastDigest *Hind*III, dNTP MIX 10 mM, Lambda DNA, TransformAid™

Bacterial Transformation Kit, High Fidelity PCR Enzyme Mix, GeneJET Plasmid Miniprep Kit were all from Fermentase, Poland.

Escherichia coli XL1-Blue (E. coli XL1-Blue) and COS7 Cells were from Pasteur Institute of Iran.

Construction of plasmids containing mutant V2 vasopressin receptors

Specific primers were designed for mutants (a; Ala300Glu and b; Asp297Tyr) using WDNASIS program V.2.5. Primer sequences are shown in Table 1.

The mutant V2 receptor inserts were synthesized using nested polymerase chain reaction. The first PCR was carried out in a reaction mixture containing 50 ng cDNA of the wild type V2R in pcDNA3 vector (as template), 10X High Fidelity PCR buffer with 15 mM MgCl2, dNTP, Taq DNA polymerase, 2.5 µM of each sense and anti-sense of a or b with outer and inner (NPFI) primers in V2R. PCR cycles are shown in Table 2

PCR carried out in a termocycler (MyCycler™ thermal cycler system with gradient option, Bio-Rad laboratories, California, USA). The products of the first PCR were gel electrophoresed and the size of the bands was confirmed. Subsequently, a mixture of the first PCR products (as template) with NPFI and RII (mutation 'a') and NPFI and RI (mutation 'b') primers were used for the nested PCR with the same thermal condition described above (21-23).

The PCR products were gel electrophoresed and visualized on a UV trans-luminator by staining the DNA with ethidium bromide.

After sequencing, the PCR products having the desired mutations were selected. pcDNA3 vector containing the wild type V2R and the selected PCR products were digested by *Eco*RI and *Xba*I restriction enzymes, followed by gel extraction of the digested products by QIAquic kit (Qiagen Co, Germany). The inserts were ligated to the pcDNA3 vector with a molar ratio of 3:1 respectively.

The digested vector was treated with alkaline phosphatase to prevent self-ligation of the two ends of the linear vector. Transformations were carried out using either a commercial kit (TransformedAid™, Bacterial Transformation Kit) method or electroporation (Gene Pulser Xcell™ Electroporation system, BIO-RAD, United Kingdom) method. After transformation, the obtained colonies were used for plasmid preparation. One colony for each mutation containing the correct plasmid (orientation of the insert was confirmed by *Eco*RI and *Xba*I double digestion, *Eco*RI and *Bbr*pI double digestion, and *HindIII* single digestion) was grown overnight and large quantities of this plasmid was obtained using PureLink® HiPure Maxi prep Plasmid DNA Purification Kits (Invitrogene, Life Technologies, USA) (24,25).

Table 1. Primer sequences; NPFI and Reverse (Ala300Glu mutant), RII and Forward (Ala300Glu mutant), NPFI and Reverse (Asp297Tyr mutant) and RII and Forward (Asp297Tyr mutant) primers were used in the 1st PCR round.

Desired Primer	Anti-sense primer sequence (Reverse)	Sense primer sequence (Forward)
Ala300Glu	(5'-CCTTCCAGAGAGGTTCCTCCGGGTCCCA-3')	(5'-TGGGACCCGGAGGAACCTCTCTGGAAGG-3')
Asp297Tyr	(5'-AGAGGTGCCTCCGGGTACCACGCGGCCCACAG-3')	(5'-CTGTGGGCCGCGTGGTACCCGGAGGCACCTCT-3')
Inner in pcDNA3	RI primer: (5'-ggtcaaggaag gcacgg-3')	NPFI primer: (5'-gtggatagcggtttgact-3')
Outer in pcDNA3	RII primer: (5'-gttctttccgcctcaga-3')	N/A*

*N/A: Not Available

Table 2. PCR cycling conditions.

PCR Phase	Temperature (˚C)	Time (min)	Number of cycle
Initial denaturation	94	5	1
Denaturation	94	1	
Annealing	55	2	35
Extension	72	3	
Final extension	72	20	1

Cell culture and transfection

COS-7 cells (African green monkey kidney fibroblast cell line) were cultured in 25 cm^2 flasks. Cells were grown in medium containing dulbecco's modified eagle's medium (DMEM), 10% fetal bovine serum (Gibco, BRL Life Technologies, Scotland), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified CO2 incubator (5% CO2, 37 ºC) (22,26). The cells were transiently transfected according to the manufacturer's instructions by using ESCORT^{TM} IV Transfection Reagent (Sigma, USA). Briefly, the DNA /liposome complex was prepared in serum free DMEM in 1 ml total volume at room temperature for 15 min. Subsequently, 1 ml of DMEM containing 20% fetal bovine serum was added to the above complex (final FBS concentration was 10%). The culture medium was removed from the growing cells and immediately 2 ml of the DNA/liposome/DMEM solution was added to the freshly aspirated 35 mm dishes at 80% confluence and incubated overnight. Subsequently, the medium was aspirated and replaced with 2 ml medium containing 10% serum. Transfected cells were incubated for another 24 h before using for expression and cyclase assays.

The enzyme-linked immunosorbent assay

For assessment of the receptor expression, the enzyme-linked immunosorbent assay (ELISA) was performed by using an antibody against N-terminal HA epitope of V2R and measuring optical density in microplates using an ELISA plate reader. ELISA was used to assay the receptor expression at the appropriate times (24 to 72 h post-transfection). COS7 cells were transiently transfected with the wild type or mutant V2R (the receptor containing the HA epitope at the N terminus). Briefly, 24 h after transfection, cells were placed at a density of 0.1×10^5 cells per well in a 96-well plate and incubated over night at 37 ºC. Plates were washed twice with phosphate buffered saline (PBS), and then fixed with 10% buffered formalin. After adding PBS: 2% bovine serum albumin (BSA), they were incubated for 1 h at 37 ºC. Afterwards, 1 mg 12CA5 monoclonal antibody (diluted in PBS:1% BSA solution) (anti-HA epitope)/100 ml media was added and cells were incubated for 2 h at 37 ºC. Finally, anti-mouse IgG:HRP diluted in PBS:1% BSA was added and the plates were incubated at 37 °C for 1 h. The enzymatic reaction was induced by H2O2 and O-phenylenediamine (Sigma, USA) and then the reaction was stopped by addition of 3 M HCl. The optical density was measured at 492 nm in a microplate reader (STAT FAX, USA). Un-transfected cells or cells transfected without any plasmids were used as negative control. Additionally, similar experiments were performed after the cells were exposed to different concentrations (1, 10, and 100 nM) of arginine vasopressin (AVP, Sigma, Germany) for 20 min at 37 °C.

Adenylyl cyclase activity assay

Functional characterization of the wild type and two mutated V2Rs was investigated by performing adenylyl cyclase activity assay.

Forty eight hours after transfection, the cells were exposed to1, 10 and 100 nM vasopressin, 2 mM isobutylmethylxanthine or 100 µM forskolin (all from Sigma, Germany) for 20 min at 37 °C (21,22,26,27). After rinsing with Hanks buffer and lysis with 0.1 M HCl, cAMP was measured using direct cyclic AMP Enzyme Immunometric Assay kit (Assay Designs, USA) for the quantitative determination of cAMP concentration in cells. The procedure was performed according to the manufacturer's instructions using polyclonal antibody against cAMP.

Statistical analysis

The data are presented as the mean ± SEM. Statistical comparisons were performed using one-way analysis of variance (ANOVA). *P* value <0.05 was considered as statistically significant.

RESULTS

In the first round of PCR, two pieces of 1250 and 350 bp DNA were produced. In the second round of PCR, 1700 and 1600 bp DNA products were obtained containing the desired mutations. The second PCR products were sequenced by Faza Pajoh Company, Iran (Fig. 1).

AGGCTGGCCAGCAACATGAGTAGCACAAAGGGCGCCCCTTCCAGAGAGGTTC*CTCCGGGTCCCACGCGGCCCACAGCTGCACCAGGAAGAAGGGTGCCCA
TGGGCACCCTTCTTCCTGGTGCAGCTGTGGGCCGCGTGGGACCCGGAGGAA*CCTCTCTGGAAGGGGCGCCCTTTGTGCTACTCATGTTGCTGGCCAGCCT

CAGCAACATGAGTAGCACAAAGGGCGCCCCTTCCAGAGGTGCCTCCGGGTAC*CACGCGGCCCACAGCTGCACCAGGAAAAAGGGTGCCCAGCACAGCACA
TGTGCTGTGCTGGGCACCCTTCTTCCTGGTGCAGCTGTGGGCCGCGTGGTA*CCCGGAGGCACCTCTGGAAGGGGCGCCCTTTGTGCTACTCATGTTTGCTG

Fig. 1. DNA sequence of the second PCR products. The segments of the second PCR products that contained the desired mutations are shown with an asterisk. GAA and TAC are the codons of glutamic acid and tyrosine, respectively.

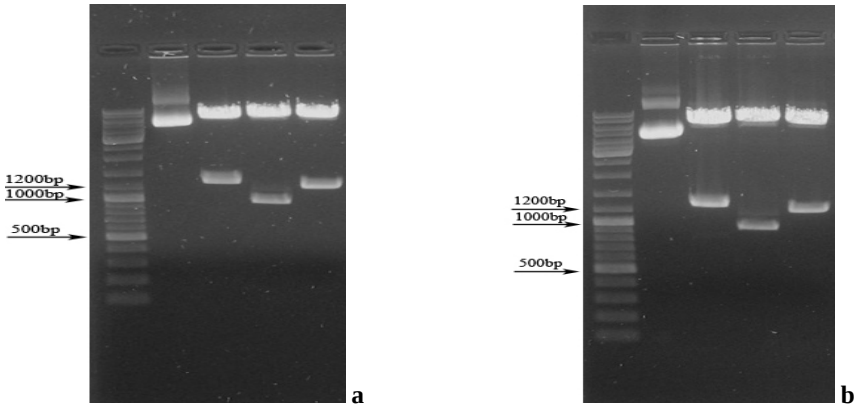

Fig. 2. Digestion of the recombinant plasmids (pcDNA3, Ala300Glu and Asp297Tyr mutant V2Rs). Plasmid DNAs were isolated using Maxiprep Kit and after digestion, these products were electrophoresed on a 0.8% agarose gel. a; shows plasmids containing Ala300Glu mutation, b; demonstrates Asp297Tyr mutation; In each figure from left to right are molecular weight marker, undigested recombinant plasmids, a band of 1200 bp obtained by *Xba*I-*Eco*RI digestion, a band of 800 bp obtained by *Eco*RI-*Bbr*pI digestion and a band of 1100 bp obtained by *Hind*III digestion.

For vector preparation, pcDNA3 containing the wild type V2R was digested by *Eco*RI and *Xba*I restriction enzymes which removed the V2 receptor DNA (1200 bp) out of the pcDNA3 plasmid (5400 bp). The confirmed second PCR products also were digested by *Eco*RI and *Xba*I enzymes and then the inserts were ligated into the vector. The orientation of the insert was confirmed by restriction enzyme digestion (*Eco*RI and *Xba*I double digestion, *EcoRI* and *Bbr*pI double digestion as well as *Hind*III single digestion). The size of the obtained bands showed the cloning and correct orientation of the inserts (Fig. 2).

The efficiency of the transient expression of V2R was examined by transfection of the pEGFP-N1 plasmid which has been optimized for brighter fluorescence and higher expression.

There was no significant difference in receptor expression between the wild type and mutant receptors. The results of ELISA for receptor expression assay are shown in Fig. 3. There were no significant differences between cell surface expression of the wild type and mutants receptors. Binding assays were also performed by adding different concentrations (1 and 100 nM) of vasopressin (Sigma, Germany) for 20 min at 37 °C and then the ELISA protocol was performed as described previously (Fig. 4). The effect of each mutation on receptor binding showed that in the presence of AVP (1 nM), cell surface presence of Ala300Glu mutant of V2R was the same as the wild type while Asp297Tyr mutant of V2R was significantly reduced. At high AVP concentration (100 nM) no significant differences between the wild type and mutant receptors was observed.

Adenylyl cyclase activity assay

Transiently transfected COS7 cells expressing the wild type and mutated V2Rs (Ala300Glu and Asp297Tyr) were exposed to 100 nM of AVP for 20 min, and the ability of the receptors to mediate AVP stimulation of adenylyl cyclase activity was assessed in cell lysates (Fig. 5). The functional assays indicated that Ala300Glu mutant form of V2R significantly reduced cAMP production but cAMP production of the Asp297Tyr mutant form of V2R was similar to that of wild type V2R.

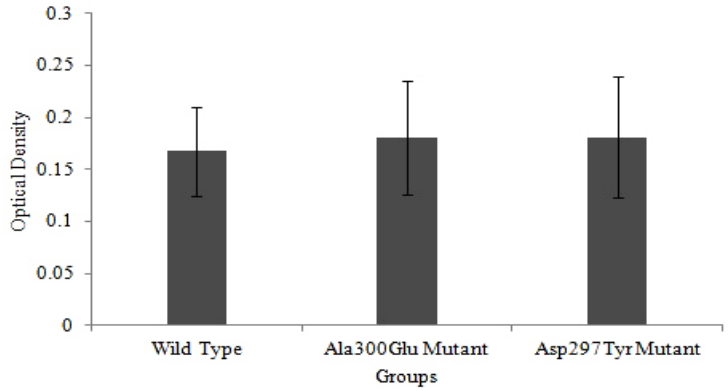

Fig. 3. The effect of mutations on cell surface V2 receptor expression in COS-7 cells by ELISA assay. The cells were transfected with a plasmid containing the wild type cDNA of V2R (WT) or containing mutant cDNA of V2R (Ala300Glu and Asp297Tyr mutants). Cells were placed at a density of 5×10^5 per well in a 96-well plate and the amount of DNA for each plasmid was 1000 ng. Data represent the optical density (OD) as the mean ± SEM, (n=3).

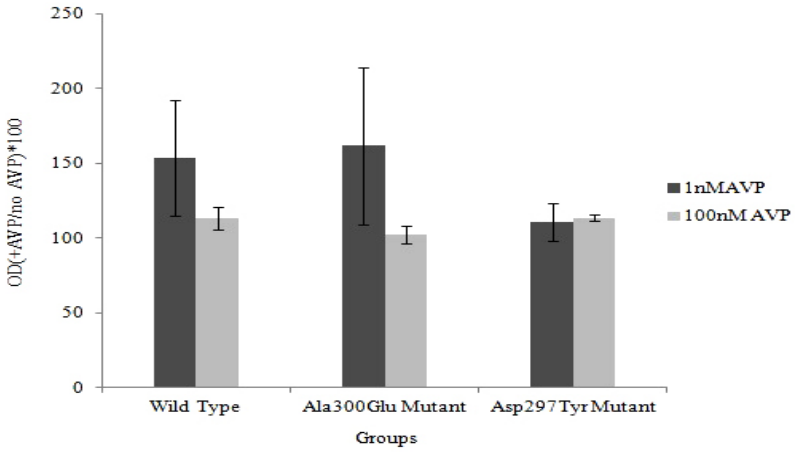

Fig. 4. Cell surface V2R detected in the presence of different concentrations of AVP. Cells were placed at a density of 5×10^5 per well in a 96-well plate and the amount of DNA for each plasmid was 1000 ng. Data represent the optical density (OD) as the mean ± SEM (n=3). Black columns show the percentage of OD in the presence of 1 nM AVP/OD in the absence of AVP (as Control) and gray columns show the percentage of OD in the presence of 100 nM AVP/OD in the absence of AVP (as Control).

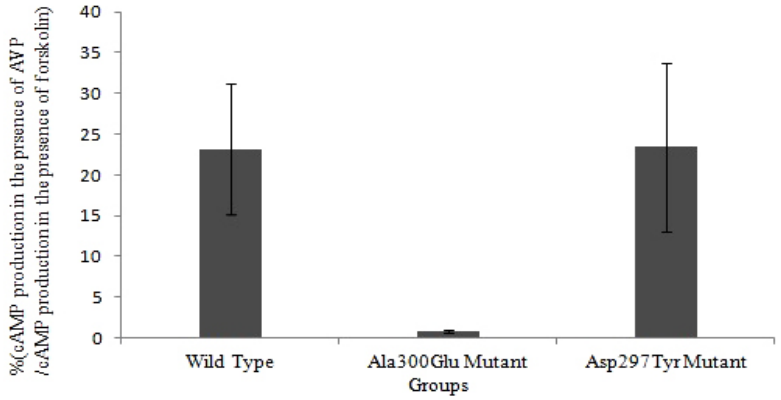

Fig. 5. cAMP concentration (pmol/ml) measured after addition of AVP to the cells. Transiently transfected cells with a plasmid containing the wild type , Ala300Glu and Asp297Tyr mutants were placed at a density of 5×10^5 per well in a 96-well plate (the amount of DNA for each plasmid was 1000 ng). Forty eight hours after transfection, cells were exposed to AVP (final concentration of 100 nM) for 20 min at 37 ºC. Data represent the mean optical density (OD) ± SEM (n=3).

DISCUSSION

The main goal of our study was to conduct experiments to compare the in silico data with those of laboratory experiments about V2 receptor amino acid residues potentially important in ligand binding.

Based on our studies using bioinformatic methods, Ala300Glu mutation resulted in lower energy but Asp297Tyr mutation resulted in higher energy in AVP-V2R docked complex as compared to the wild type. According to the results, it is possible that the Ala300Glu mutation would lead to an appropriate ligand-receptor binding, while Asp297Tyr mutation leads to an improper binding.

In this research, the results of ELISA assay showed that there were no significant differences between cell surface expression of the wild type and mutants receptors.

The effect of each mutation on receptor binding showed that in the presence of ligand (1 nM AVP), cell surface presence of Ala300Glu mutant form of V2R was the same as the wild type and Asp297Tyr mutant form of V2R was significantly reduced. In other words, mutation of Asp297Tyr showed more internalization as compared to the wild type receptor. This suggests that possibly the binding of AVP to this receptor is better and more pronounced as compared to the wild type receptor.

At high AVP concentration (100 nM) there were no significant differences in binding assay.

The functional assays indicated that Ala300Glu mutant form of V2R significantly reduced cAMP production without marked effect on receptor expression and receptor binding. But cAMP production of the Asp297Tyr mutant form of V2R was the same as the wild type V2R on receptor expression and cAMP production. According to the receptor reserve phenomenon the activation of all receptors are not needed to get the maximum response and also elimination of some receptors from the cell surface does not necessarily decrease the function of the receptors.

According to the previous studies, the receptor amino acid residues, potentially important in ligand binding, are mainly in the TM3-TM7 helices (28). Also by generating chimeric hV_2Rs in which the first and the second extracellular loops were replaced by the corresponding loops of the mV_2R, a high-affinity binding to the hV_2R was obtained (29).

Although the results of our in silico studies indicate that the binding domain residues were mainly located in β-turn and γ-turn adjacent to the TM7 helices.

Whereas the cell surface expression of Ala300Glu V2R was the same as the wild type receptor, the function was reduced greatly meaning that either the binding site was affected by the mutation or the receptor interaction with Gs/adenylyl cyclase system was impaired. Pretreatment with AVP produced a slight change in the cell surface presence of V2R when compared to the wild type. Collectively, the data indicate that possibly this mutation mainly affected the receptor interaction with Gs/adenylyl cyclase system.

With regard to Asp297Tyr mutation, since cell surface expression and function of the receptor was the same as the wild type, it can be deduced that this mutation does not affect the receptor interaction with Gs/adenylyl cyclase system. Also pretreatment with AVP caused slight change in the cell surface presence of V2R as compared to the wild type (1 nM AVP). Therefore, it is possible that this mutation can affect receptor binding.

Based on our results, it seems that mutation 300 does not have a pronounced effect on receptor binding while mutation 297 seems to have a positive effect on receptor binding.

CONCLUSION

In conclusion, this study, to some extent, confirms our bioinformatics data about binding domain. This means that D297 residue is involved in binding site but amino acid substitution in the determined binding site did not confirm the bioinformatics data about free energy of the protein complex (hormone-receptor). Further experiments with more mutations are needed to investigate the binding site of V2 vasopressin receptor.

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