A Low-Affinity Vasopressin V2-Receptor Gene in a Kindred with X-Linked Nephrogenic Diabetes Insipidus

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ABSTRACT

In this study, a mutation in vasopressin Type 2 receptor (V2R) in a patient with hereditary nephrogenic diabetes insipidus (NDI) has been identified and characterized. The sequencing of the V2R gene from the patient revealed that there was a missense mutation (TAT to TGT) resulting in the substitution of 205Tyr for Cys in the putative third extracellular domain. The expression analysis in COS cells showed that the binding affinity of the mutant receptor ($K_D = 19.8 \text{nM}$) for arginine vasopressin was much lower than that of the wild-type receptor ($K_D = 1.8 \text{nM}$) so that intracellular cAMP production stimulated by arginine vasopressin was impaired in cells with the mutant V2R. From these results, it was concluded that the single amino-acid substitution of V2R is responsible for this familial disease.

Key Words: Nephrogenic diabetes insipidus, cAMP, G-protein-coupled receptor, transfection, COS cell

Hereditary nephrogenic diabetes insipidus (NDI) is characterized by unresponsiveness to the antidiuretic action of arginine vasopressin (AVP) in renal collecting ducts. The action of AVP is mediated by the Type 2 vasopressin receptor (V2R) that is coupled to adenyl cyclase via GTP-binding protein. Ligand binding results in the activation of adenyl cyclase and the generation of intracellular cAMP. The increased levels of cAMP activate protein kinase A, and the subsequent phosphorylation cascade increases the water permeability of the luminal membrane of the collecting duct cells by recruiting preformed water channels into their apical surface.

In the last 5 yr, the understanding of the genetics and pathogenesis of hereditary NDI has progressed extensively. The genes for V2R (1,2) and vasopressin-sensitive water channel aquaporin-2 (AQP2) (3), both of which are candidate genes for hereditary NDI, have been cloned and characterized. A considerable number of reports regarding the mutations in the genes have been reported in hereditary NDI patients. NDI is now classified in two distinct entities: (1) X-linked NDI secondary to V2R mutations (4–16), and (2) autosomal recessive NDI secondary to AQP2 mutations (17,18). Plasma cAMP responses to 1-desamino [8-D-arginine] vasopressin could be used to distinguish a prereceptor defective mechanism (V2R mutations) from a postreceptor defective mechanism (AQP2 mutations).

The sequence of the V2R cDNA predicts a polypeptide of 371 amino acids that has seven transmembrane, four extracellular, and four cytoplasmic domains (1.2). The gene contains three exons and two small introns (4). Some of the mutations lead to truncated forms of the receptor protein, and other mutations result in substitutions of a single amino acid. In the former, the mutant receptor is not expected to have biologic activity. In the latter, there is no guarantee that the single amino-acid change really leads to receptor inactivation. There have been, however, only a few reports concerning the structure-function relationship of a V2R mutant (7,15,19).

In this report, we identified the V2R gene disorder in a Japanese patient with Type 1 hereditary NDI. The mutation causes a single amino-acid change located at the end of the second extracellular loop, which is considered to be important for hormone binding. We characterized the mutant V2R by AVP binding and cAMP production activity with COS cells expressing the V2R.

METHODS

Subject

A 46-yr-old Japanese man (Figure 1, indicated by arrow) was found to have NDI on the basis of clinical manifestations with past history of polyuria and polydipsia. Because his parents had been well aware of his need for large amounts of water, no severe dehydration episodes during infancy were observed and his mental state was normal. The urine volume was 10.5 L/day and the urine osmolality was 293 mosmol/kg on admission. Five U of pitressin did not cause any change in urine output, urine osmolality, or urinary and plasma cAMP concentration. Because many members of his family complained of similar symptoms since birth, it was strongly suggested that the disease was inherited in an X-linked recessive pattern (Figure 1). To see if the cause of
were amplified by polymerase chain reaction (PCR). The PCR

The three fragments of the V2R gene, which overlapped each

we investigated the V2R gene of the patient.

subjects are indicated by symbols with a slash. Half-filled
circles show putative carrier females. The patient is indicated

by an arrow.

this familial disease was the mutant gene encoding the V2R,
we investigated the V2R gene of the patient.

Genomic V2R DNA Amplification

Genomic DNA was isolated from peripheral blood mononuclear cells with a DNA isolation kit (Stratagene, La Jolla, CA). The three fragments of the V2R gene, which overlapped each other and covered the full length of the coding region for V2R, were amplified by polymerase chain reaction (PCR). The PCR primers used are three combinations as follows: 5'-AGAGGCTGAGTCCGCACATCA-3' (sense primer, corresponding to nucleotides +286 to +307) and 5'-GGTGGTGCAGGACTCACGTCTTCATTGG-3' (antisense primer, corresponding to nucleotides +411 to +431), 5'-CACCCATAGCTGCAGATACTFCACGGC-3' (antisense primer, corresponding to nucleotides +1191 to +1211), and 5'-CATCTGCAGATACTFCACGGC-3' (antisense primer, corresponding to nucleotides +1191 to +1211). Wild-type V2R cDNA was cloned into pCR®-vector plasmid. Positive clones were completely sequenced by the dideoxy chain termination method as described above.

Site-directed mutagenesis was performed with the Transformer® Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) with mutagenesis primer (5'-GTCGCACCTGTGTCACGCTG-3', corresponding to nucleotides +676 to +697) phosphorylated at the 5' end. The substituted DNA sequences were confirmed by sequencing. The wild-type and mutant V2R cDNA were excised from pCR®-vector plasmid with BamHI and XbaI and introduced in a sense orientation into the expression vector pSVL (Pharmacia, LKB Biotechnology Inc., Piscataway, NJ).

Transient Expression in COS cells

COS cells were grown in Dulbecco's modified essential medium (DME; Sigma, St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/mL streptomycin, and 50 units/mL penicillin at 37°C in a 95% air/5% CO2. In a subconfluent state, the cells planted on 10-cm dishes were transfected with calcium phosphate coprecipitation method with 10 μg of plasmid DNA/plate. Equal amounts of wild-type and mutant V2R cDNA cloned into pSVL were transfected in parallel into COS cells. Twenty-four h after transfection, the cells were seeded at a density of 1.0 × 106 cells/well in 24-well multiplate for binding and cAMP production assay.

Binding Assay

Twenty-four h after subculture, the AVP binding assay was performed. Each well was washed twice with DMEM without any additives, and then 0.5 mL of DMEM with 1% of BSA containing the appropriate dilutions of [3H]AVP was added. The incubations were carried out for 2 h at 4°C. Non-specific binding was determined under the same conditions in the presence of 1 μM of unlabeled AVP. At the end of the incubation, the wells were washed twice with DMEM without any additives, and 0.5 mL of 0.1 N NaOH was added to extract the bound radioactivity. After neutralization by 1 N HCl, radioactivity was measured in 5 mL of scintillation fluid (ACSII, Amersham, Arlington Heights, IL).
cAMP Production in Intact Cells

Twenty-four h after subculture, cAMP accumulation in transfected COS cells was measured. Each well was washed twice with DMEM without any additives. DMEM (0.5 mL) was then added with 1% BSA and 0.5 mM 1-isobutyl-3-methylxanthine containing the appropriate dilutions of AVP, and incubated at 37°C for 10 min. After the end of the incubation, the reactions were stopped by removing the medium and adding 0.25 mL of 0.1 N HCl to each well. To extract intracellular cAMP, 0.25 mL of 0.1 N HCl was added and incubated for 20 min at 37°C. The quantity of cAMP was determined with the cAMP detection ELISA kit (Amersham).

Materials

[3H] AVP (20 Ci/mmol) and [α-32P] ATP (3000 Ci/mmol) were purchased from Amersham. Unlabeled AVP and 1-isobutyl-3-methylxanthine were from Sigma. Other chemicals were of the highest purity available from commercial sources.

RESULTS

Mutational Analysis

To see if there was an abnormality in the V2R gene in the patient, we completely sequenced the PCR fragments for the V2R gene from the patient. Two nucleotides different from the reported sequence were identified. One was found to be a transition of 998G to A without amino-acid change (data not shown). This silent substitution has already been described in previous reports (11,21). Another was a substitution of 685A for G, resulting in a missense mutation (TAT to TGT), with exchange of Y205I to Cys (Y205C, Figure 2). Sequencing of the V2R gene of the patient’s mother showed the sequences of both the wild-type and the mutant gene, indicating that the asymptomatic mother is the carrier of the mutant gene found in the patient.

The change of amino acid was located at the end of the putative second extracellular loop in the V2R (Figure 3). It has been suggested that the first and the second extracellular loops form the three-dimensional structure to which AVP binds (5). If the cause of hereditary NDI in the patient is the amino-acid substitution of Y205C, the mutant V2R may have a low affinity for AVP. So, we next investigated the functional property of the mutant V2R in COS cells that expressed either the wild-type or the mutant V2R.

AVP Binding Assay

Saturation binding of [3H]-AVP to cells that expressed the wild-type V2R or to cells that expressed the mutant was determined in the same experiment (Figure 4). From the Scatchard analysis, the number of receptors (approximately 8000 sites/cell) was approximately equal on the surface of cells that bore the mutant as well as the wild-type cDNA. The wild-type receptor exhibited an affinity (KD = 1.8 nM) for AVP.

Figure 2. DNA sequence analysis of V2R in a healthy man and the patient. In the V2R gene of the patient, a substitution of 685A for G was detected, resulting in a missense mutation (TAT to TGT) with Y205C.

Figure 4. Saturation binding of [3H]AVP to COS cells that expressed wild-type (A) or mutant (B) V2R. Results were the means of three experiments. Closed circles, total binding; open circles, nonspecific binding. Each curve shows specific binding. The inset panel shows analysis according to Scatchard.
comparable with that in the previous study (1). The mutant receptor, however, showed much lower affinity for AVP (K_D = 19.8 nM), indicating that the single amino-acid substitution (Y205C) of V2R impairs the binding affinity to AVP.

CAMP Production in the Stimulation of AVP

Because the mutant receptor has much lower affinity for AVP, cAMP production by AVP stimulation would be depressed in cells with the mutant receptor. To confirm this issue, we examined the effects of AVP on CAMP production in the transfected COS cells. In COS cells that expressed the wild-type V2R, intracellular CAMP production increased in a dose-dependent manner from 0.1 nM AVP and reached plateau at 1 nM AVP for 10 min of incubation (Figure 5). In contrast, the cells with mutant V2R accumulate cAMP minimally in response to high concentrations over 10 nM AVP.

DISCUSSION

In this report, we investigated the pathogenesis of hereditary NDI found in a Japanese kindred. Because of the typical X-linked transmission pattern and the unresponsiveness of plasma cAMP to pitressin, it was strongly suggested that the cause of NDI was the mutation of V2R gene. We analyzed the V2 receptor gene and detected a missense mutation at codon 685. We also identified that the patient's mother as a gene carrier. The missense mutation induced the amino-acid substitution of Y205C in the second extracellular loop. The same mutation has already been described (5), but there is no relationship between the families in the two studies. We characterized this mutation by using COS cells expressing the mutant V2R. The mutant receptor showed extremely lower affinity for AVP and less production of cAMP, suggesting that this mutation in V2R is responsible for NDI.

Although there is a little information available for the structure-function relationships of V2R, we can speculate about those relationships from the data of other G protein-coupled receptors. The first and the second extracellular loops are considered to form the three-dimensional structure to which AVP may bind (5). Especially, extracellular disulfide-bonded cysteines have been shown to play an important role in forming the ligand-binding site in many G protein-coupled receptors. For example, bovine rhodopsin has ten cysteines, of which four are in the transmembrane, three are in the cytoplasmic domain, and three are in the intradiscal domain. Only intradiscal 110Cys and 187Cys are shown to be essential for the correct tertiary structure of the protein (22). Similar results were obtained in β2-adrenergic receptor (23) and Type 1 angiotensin II receptor (24). In the V2R, the disulfide bond is considered to be formed between 112Cys and 193Cys. Some of the mutations located in the first and second putative extracellular loops may interact with the disulfide-bond, which results in the change of the binding affinity to AVP. There have already been some reports concerning the structure-function relationships of V2R. Either the R113W mutant (19) or the R181C mutant (15) located in the first or second extracellular loop has been shown to have much lower affinity for AVP. They suggested that these mutations could disrupt the disulfide-bond between the first and second extracellular loops. We assume that the amino-acid change in our patient (Y205C) may form a new disulfide bond (i.e., 112Cys-205Cys, 192Cys-205Cys, or 193Cys-205Cys) in error. It is likely that an abnormally formed extracellular disulfide bond may depress the affinity for AVP.

It is possible that this mutation causes not only a reduction in binding affinity, but also dysfunction of the intracellular signal transduction system such as the adenylyl cyclase-protein kinase A system. Rosenthal et al. have shown that a single amino-acid change of the V2R located near the plasma membrane/cytoplasm interface, which is highly conserved in G protein-coupled receptors, causes an inability to stimulate adenylyl cyclase without changing the binding affinity for vasopressin (7). In that case, the mutant receptor cannot respond to a high dose of ligand in the V2 receptor. In contrast, the mutant of the hormone-binding domain of the β2-adrenergic receptor can respond to a high dose of isoproterenol and increase the intracellular cAMP concentration, even in the mutant β2-adrenergic receptor unable to form a disulfide bond in the extracellular loop (23). As shown in Figure 5, the cAMP level in the COS cells that expressed the mutant V2R (Y205C) seemed to be increased in a dose-dependent manner, although the magnitude of the increase was much less. This suggests that the Gs/adenylate cyclase system may work well in the mutant receptor in spite of lower binding affinity to AVP.

Figure 5. Dose dependency of intracellular cAMP accumulation in COS cells that expressed wild-type (closed circles) or mutant (open circles) V2R. Results were means ± SE of three experiments.
ACKNOWLEDGMENTS

This research was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. We thank Dr. Joseph S. Handler (The Johns Hopkins University, Baltimore, MD) for his helpful advice.

REFERENCES