The Effect of Eight V2 Vasopressin Receptor Mutations on Stimulation of Adenylyl Cyclase and Binding to Vasopressin*

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We previously identified six V2 vasopressin receptor mutations in five unrelated nephrogenic diabetes insipidus (NDI) families. In order to elucidate the effect of these mutations on the function of the V2 vasopressin receptor, we introduced these six and two additional, naturally occurring mutations into the V2 vasopressin receptor gene by in vitro mutagenesis. Five of the mutants (two frameshift, one nonsense, and two missense) failed to stimulate adenyl cyclase due to their inability to bind vasopressin under the experimental conditions. In contrast, ligand binding and cAMP accumulation were normal for two other mutations, a A61V missense mutation and an in-frame deletion of four amino acids (Arg-247 to Gly-250), suggesting that they are not the cause of NDI in these families. The deletion mutation was found in a family in conjunction with a second mutation, R181C, which yielded a much reduced ligand-binding capacity. The $K_0$ of R181C was at least 26 times higher than that of the wild type. Further characterization by an immunofluorescent assay showed that the R181C mutant receptor is expressed and distributed on the cell surface in a manner similar to that of the wild type. This finding indicates that the inability of this mutant to stimulate adenyl cyclase is caused by the reduced capacity for vasopressin binding and that the R181C mutation is responsible for NDI in this family.

NDI is a rare X-linked disorder in which the kidney is insensitive to the antidiuretic hormone, vasopressin. The gene responsible for NDI, the V2 vasopressin receptor, has been cloned and mapped to Xq28 (1, 2). More than 17 mutations in the V2 vasopressin receptor gene have been identified from different NDI families (3-7), and all of these have arisen independently.

Sequence analysis suggests that the V2 vasopressin receptor belongs to the large family of G-protein-coupled receptors which contain seven transmembrane domains. Its signal transduction pathway is via stimulation of the G$_i$/adenyl cyclase complex (1). Although the structure of bacterial rhodopsin, a protein homologous in sequence, has been determined (8), additional information about structure-function relationships in this receptor family has been obtained by the introduction of various mutations and analysis of their functions. A few of these receptors contain naturally occurring mutations in the genes for G-protein-coupled receptors, such as rhodopsin.

growth hormone-releasing hormone receptor (9), and melanocyte-stimulating hormone receptor (10). Analysis of the large variety of mutations in the V2 vasopressin receptor in NDI should augment the understanding of the relationship between the function and structure. Thus far, the effects of only a few mutations in the V2 vasopressin receptor have been characterized (11). Here we report the results for both cAMP accumulation and binding to vasopressin in eight naturally occurring mutants of the V2 vasopressin receptor, as well as the immunofluorescence localization.

EXPERIMENTAL PROCEDURES

Molecular Cloning—The wild type V2 vasopressin receptor gene was generated from human genomic DNA by polymerase chain reaction with primers based on the sequences from the 5'-and 3'-flanking region of the gene, as described previously (3). Nucleotides coding for a 9-amino acid epitope (EEEEYMPME) (12) were added either at the 3' end of the 3' primer or the 5' end of the 5' primer as a tag for protein detection. The polymerase chain reaction product was gel-purified and cloned into pBluescript (Stratagene, La Jolla, CA) and then subsequently into pcDNAI (Invitrogen, San Diego, CA). In vitro site-directed mutagenesis was performed as described by Kunkel (13). The mutations were verified by sequencing. The supercoiled DNA used for transfection was prepared with Qiagen columns (Qiagen, Chatsworth, CA).

Cell Culture and Transient Transfection—A COS-7 cell line was purchased from the cell culture facility at UCSF. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS in a 5% CO$_2$ incubator throughout the experiments. 15 μg of DNA were transfected into the COS-7 cells (10$^5$ cells/60-mm dish) in the presence of 400 μg/ml DEAE-dextran and 0.1 mg/mL chloroquine for 2.5 h in non-supplemented Dulbecco's modified Eagle's medium. Cells were incubated with PBS containing 100 mM Na$_2$SO$_4$ for 2 min at room temperature, washed twice with PBS, and then maintained in growth medium.

Western Blotting Assay—Transfected cells were lysed with lysis buffer and denatured by boiling for 5 min. The proteins were separated on a 10% acrylamide gel in Tris-glycine buffer. After electrophoresis, the membranes were incubated first with mouse monoclonal antibody (OxxyPharm) against the epitope tag and then with rabbit horseradish anti-mouse antibody. The signals were detected by using an ECL kit (Amersham Corp.).

cAMP Accumulation Assay—Cells were split 24 h after the transfection into six wells in a 24-well plate and incubated in the growth medium with 1 μCi of [3H]adenine overnight. Cells were washed twice with 1 ml of assay medium (20 mM HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate) and incubated with 0.5 ml of assay medium containing 1 μM 1-methyl-3-isobutylxanthine with or without 10^{-6}M vasopressin (Boehringer Mannheim) at 37 °C for 30 min. Reactions were then stopped by adding 0.5 ml of 0.5 v trichloroacetic acid. Intracellular accumulation of cAMP was determined as described by Wong (14).

Ligand Binding Assay—The assays were carried out as described by Birnbaumer et al. (15). 24 h after transfection, cells were split into six-well plates at a density of 0.8 × 10$^5$ cells/well and incubated overnight in growth medium. Cells were then washed once with cold assay medium (2% glucose and 1% bovine serum albumin in PBS) and incubated with a corresponding dilution of [3H]vasopressin (73 Ci/mmol, DuPont NEN) in the absence or presence of 10 μM cold vasopressin for 2 h at 4 °C. Competition assays were carried out in 20 μM [3H]vasopressin with a corresponding dilution of cold vasopressin. Unbound ligand

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1 The abbreviations used are: NDI, nephrogenic diabetes insipidus; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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was removed with two rinses of 1 ml of assay medium. Cells were lysed with 1 ml of 0.1 M NaOH at 37 °C for 30 min. The liquid from each well was transferred to 4 ml of scintillation fluid and counted in a scintillation counter.

**Localization of the V2 Vasopressin Receptor in Fixed Cells by Immunofluorescence Microscopy**—After transfection, COS-7 cells were replated in the growth medium on glass coverslips overnight. The cells on the coverslips were then fixed separately with either freshly made 4% paraformaldehyde in PBS or methanol for 20 min. For both permeabilization and blocking, fixed cells were treated with 0.5% Nonidet P-40 in a blocking buffer containing 10% goat serum or 5% nonfat dry milk for 20 min. Mouse monoclonal antibody against the epitope tag was diluted 1 to 500 in the blocking buffer and incubated for 1 h. FITC-conjugated rabbit anti-mouse antibody was diluted 1 to 100 in the same buffer and incubated for 20 min. The treated coverslips were examined and photographed under a Zeiss fluorescent microscope (18).

**RESULTS**

Fig. 1 shows the proposed structure of the V2 vasopressin receptor and the positions of the eight mutations analyzed in this study. The effects of four missense mutations (2, 5a, 6, and 7), one nonsense mutation (4), two frameshift mutations (3 and 8), and one in-frame deletion (5b) were examined. Mutations 2-6 were found previously by analyzing six unrelated NDI families (see Ref. 3); no mutation was identified in the family 1 and two mutations (5a and 5b) in that study were found in family 5. Mutations 7 and 8 were newly detected in this study by single-strand conformational polymorphism analysis (3) of two additional unrelated NDI families. The eight mutations were introduced separately into an epitope-tagged wild type receptor and the positions of the eight mutations analyzed in NDI families. In contrast, the A61V substitution (7) had no effect on cAMP production, and in repeated experiments the deletion mutant 5b accumulated cAMP at the same level as the control (data not shown).

The R181C mutant showed a consistent decrease of cAMP accumulation to one-third to one-half that of the control. For this mutant, an additional experiment was carried out in order to assess its ability to stimulate adenylyl cyclase at more physiological levels of vasopressin. As shown in the dosage response curve in Fig. 2b, this mutant is dramatically less responsive to vasopressin in comparison to the control, with a greatly increased EC$_{50}$.

In order to determine whether the mutants’ inability to stimulate adenylyl cyclase was the result of impaired ligand-binding ability, ligand binding assays were performed, as shown in Fig. 3. All five mutants that did not accumulate cAMP upon addition of vasopressin in the previous test showed no vasopressin binding up to 50 nM. Although this result was predicted for the nonsense and frameshift mutations (3, 4, and 8), the finding suggests that the amino acid substitution in mutations 2 and 6 also have profoundly debilitating effects on the protein. Although 5a, the R181C mutant did not bind to the ligand under the same physiological concentrations, competition assays indicated it did bind at a much higher concentration, with a $K_{d}$ of 131 nM. The $K_{d}$ for the wild type receptor is 5 nM. All other mutants (2, 3, 4, 6, and 8) did not show any binding in the same competition experiments.

Western blots indicated that the mutant V2 vasopressin receptor proteins were present in all of the cell lysates in which mutations did not interrupt the 3' epitope tag’s expression. The expression levels were the same as the wild type receptor, as shown in Fig. 4. The broad band right below the 69-kDa marker was determined to be the monomeric receptor protein, based on the study of bovine V2 vasopressin receptor (24).

To determine whether the results from the binding assays were due to a decreased level of the receptor expressed on the plasma membrane or to their abnormal binding ability, we examined the expression and distribution of these mutants in transfected COS-7 cells by fluorescent microscopy. The 3' tag was used for the detection of mutants 2, 4, and 5a, which...
V2 Vasopressin Receptor Mutations

FIG. 2. cAMP accumulation assays. In a, cAMP accumulation was assayed in response to the presence of vasopressin at a concentration of 10−8 M. The basal level of CAMP accumulation without vasopressin was also measured. Eight mutants were assayed, as well as the control line, transfected with the wild type gene, and a line transfected with the vector only. The accumulation is presented as the ratio of cAMP to the sum of ATP plus cAMP for each sample to offset the difference in cell populations. Repeated experiments indicate 5b has the same level as the wild type receptor. In b, cAMP accumulation was assayed for varying concentrations of vasopressin. Dosage response of the R181C mutant is presented together with the wild type receptor and a negative control (frameshift mutant).

FIG. 3. a, binding assays of eight V2 vasopressin receptor mutants. The specific [3H]vasopressin bound to the V2 vasopressin receptor is measured as a function of vasopressin concentration. Both total and nonspecific binding were measured as indicated under "Experimental Procedures," and the specific bindings present here were after subtracting nonspecific binding from the total binding. ⊗, wild type receptor; ⊙, 2; ○, 3; ■, 4; ▲, 5a; □, 5b; △, 6; □, 7; □, 8. b, competition binding curve of the wild type receptor and 5a mutant.

DISCUSSION

Our experimental data show that six of eight mutations identified in the V2 vasopressin receptor genes of seven unrelated patients with nephrogenic diabetes insipidus lead to an absence or reduction of vasopressin binding ability and, consequently, blocking the V2 vasopressin receptor signaling. Since all mutant receptors are expressed on the membrane of the cell, this result suggests the altered capacity to bind vasopressin is contained point mutations, and 5' tag for mutants 3, 6, and 8, which contained frameshifts and a stop codon. Under the experimental conditions, both with formaldehyde and methanol fixation, the mutants and normal control have the same expression level on the cell surface when stained with FITC-conjugated antibody against the epitope tag. Fig. 5 shows an example of the R181C mutant and control receptor expression patterns with formaldehyde fixation.
the cause for their dysfunctions. These findings clearly indicate that mutations in this gene are responsible for nephrogenic diabetes insipidus in some families.

These experiments have also allowed us to dissect the cause for ND1 in one family having two mutations, an R181C substitution and an Arg-247 to Gly-250 in-frame deletion in the vasopressin receptor gene. Previously, we hypothesized that the R181C substitution was the likely cause, rather than the deletion of 4 amino acids. Our data show that the small deletion in the third cytoplasmic loop has no negative effect on cAMP accumulation. This result agrees with previous deletion studies of the β-adrenergic receptor which demonstrated that only the amino acid residues at either ends of the third loop are important for interaction with the G-protein (17). The finding is also consistent with a recent report of an ND1 family bearing only the R181C point mutation (25).

Previously we suggested that the substitution of a cysteine for Arg-181 in the second extracellular loop could disrupt an essential disulfide bond between the first and second loops. In bovine rhodopsin 2 cysteines (Cys-110 and Cys-187) in these loops have proven essential for protein folding by forming a disulfide bridge between the two loops. Mutants with a serine substitution at these two positions were found to be unable to bind ligand due to both reduced expression on the cell surface and altered binding capacity (18). With the exception of human mas oncogene (19) and rat cannabinoid receptor (20), cysteines at these positions are present in all reported members of the G-protein-coupled receptor family, including the V2 vasopressin receptor.

A substitution mutation, Y178C, has been described in human rhodopsin and is analogous to the R181C mutation of the V2 vasopressin receptor. However, in contrast to the substitution in the vasopressin receptor, the Y178C mutant in human rhodopsin was found to be transported inefficiently to the plasma membrane as seen by immunolocalization (21). If indeed the disulfide bridge is disrupted by the R181C mutation, then our finding suggests that the correct formation of the intradiscal disulfide bond is important for ligand recognition of this receptor and may be in the majority of the G-protein-coupled receptors. This is done presumably by directing the interaction of the transmembrane domains (18).

Two other substitution mutations, Y128S and P286R, yield non-functional proteins. Pro-286 is conserved in almost all the G-protein-coupled receptors. The P286R mutation does not appear to affect its expression level on the cell surface. However, substitution of the corresponding Pro with Ala in the muscarinic m3 receptor shows a much reduced expression (22). Tyr-128 is present in all neurohypophysial hormone receptors, and presumably it is important for the receptor function (26).

The A61V mutation, found in family 7 in this study, did not affect vasopressin binding or stimulation of adenylate cyclase activity. This finding suggests that this mutation, which results in the substitution of one hydrophobic amino acid for another within a transmembrane domain, is not the cause of nephrogenic diabetes in this patient, but rather is a rare polymorphism. It is possible that the causative mutation, such as one affecting the promoter of the V2 vasopressin receptor gene, was overlooked in our mutation screen. Previously we reported a family (family 1 in Ref. 3) with two affected boys where we failed to detect a mutation in the V2 vasopressin receptor gene and ruled out mutations in the vasopressin receptor gene by genetic linkage. Thus it is possible that nephrogenic diabetes insipidus in this patient, the only affected individual in his family, is due to a mutation in a different gene. Advances in our

![Fig. 4. Western blots of eight V2 vasopressin receptor mutants. The blots were probed with mouse monoclonal antibody against the epitope tag.](image)

![Fig. 5. Immunofluorescence assay of the wild type receptor (a) and 5a (R181C) mutant (b). Transfected cells were fixed with freshly made 4% paraformaldehyde in PBS. The receptor antigen was detected using mouse monoclonal antibody and FITC-conjugated antimouse antibodies. Immunofluorescent signal was visualized using a Zeiss fluorescent microscope.](image)
understanding of molecules in the renal tubule, such as the recent discovery of the gene encoding the renal tubule water channel (23), will allow this question to be answered.

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