Nephrogenic Diabetes Insipidus

A V2 VASOPRESSIN RECEPTOR UNABLE TO STIMULATE ADENYLYL CYCLASE*

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The coding region of the human vasopressin type 2 receptor gene bears mutations in the individuals affected with congenital nephrogenic diabetes insipidus, a disease characterized by the inability of the kidney to concentrate urine in response to vasopressin. Although it is assumed that the mutations result in loss of receptor function, proof of this hypothesis is lacking. We introduced one of these naturally occurring point mutations leading to a single amino acid change (Arg137 → His) into wild type cDNA. The mutant protein was expressed, and the functional properties of the receptor were examined. The mutant receptor exhibited an unaltered binding affinity for vasopressin compared to the wild type but failed to stimulate the G/i adenyl cyclase system. These data provide biochemical proof that the mutant receptor is the cause of the disease.

The V2R belongs to the family of hormone receptors that are coupled to effector systems by G proteins (Birnbaumer et al., 1992b). Its function is to mediate the antidiuretic activity of vasopressin in the renal collecting duct cells (Orloff and Handler, 1967), which it does by stimulating the G/i adenyl cyclase system. Congenital nephrogenic diabetes insipidus (CNDI) is a disease characterized by the inability of the kidney to concentrate urine in response to vasopressin (Culpepper et al., 1989). The inability of newborn CNDI patients to concentrate urine properly may cause severe dehydration resulting in mental retardation, inadequate growth or even death. The disease shows a sex-linked recessive inheritance and was mapped to the X chromosome by genetic linkage (Kambouris et al., 1988; Knoers et al., 1988). Cloning of the human V2R cDNA (Birnbaumer et al., 1992b) enabled us to map the V2R gene to the vicinity of the CNDI locus (Seibold et al., 1992). We also showed that CNDI patients have mutations in the V2R gene (Rosenthal et al., 1992). To date, 16 distinct mutations occurring in patients with CNDI have been reported (Pan et al., 1992; van den Ouwendal et al., 1992; Davies, 1992; Birch et al., 1993). Six mutations lead to truncated forms of the receptor protein; none of these mutant receptors was expected to have biological activity. Ten mutations result in substitution of single amino acids, of which six are predicted to modify the extracellular portion of the receptor, three in transmembrane segments, and one at the junction of the third transmembrane segment and the second intracellular loop of the receptor. Although it is assumed that these single amino acid changes lead to receptor inactivation, biochemical proof of receptor inactivation has not yet been obtained. In the present study we concentrated on the last of the mutations mentioned above referred to as Q2 (Birch et al., 1992, 1993) and sought to determine whether it indeed impairs receptor function, and if so, which aspect of receptor function would be affected. The Q2 mutation is a G → A transition in codon 137 of the V2R resulting in the change of an arginine that is highly conserved in G protein-coupled receptors to histidine (R137H; Fig. 1). Because of the location near the plasma membrane/cytoplasm interface we hypothesized that the Q2 V2R mutant might have normal or close to normal ligand binding but impaired signaling activity. We report here that the mutant receptor binds AVP with an affinity very similar to that of the wild type receptor but fails to stimulate adenyl cyclase.

MATERIALS AND METHODS

Radiochemicals—[3H]Arginine vasopressin, specific activity 60 Ci/mmol, was purchased from Du Pont-New England Nuclear; [α-32P]ATP was from the NICHD Center for Population Research and Studies in Reproductive Biology at Baylor College of Medicine; [3H]cyclic 3',5'-AMP was from ICN Biochemicals, Irvine, CA.

Construction of phV2R-Q2—The human cDNA encoding the human V2R (Birnbaumer et al., 1992b) was excised from the Bluescript plasmid with EcoRI and cloned into the replicative form of M13mp18. Site-directed mutagenesis was performed following the method of Nakanayama and Eckstein (1986) using reagents purchased as a kit from Amersham Corp. An antisense oligonucleotide of 27 bases that contained an A instead of G at the position corresponding to nucleotide 410 of the open reading frame was annealed to the single-stranded cDNA. Following in vitro mutagenesis, the complete cDNA was sequenced by the method of Sanger et al. (1977) to verify that only the desired mutation had been introduced. The modified cDNA (Q2 cDNA) was excised with EcoRI, and the “sticky” ends were filled with the Klenow fragment of DNA polymerase I and ligated into the expression vector pKNH (Birnbaumer et al., 1992b).

Transient Expression in COS.M6 Cells—COS.M6 cells were grown in DMEM (high glucose), supplemented with 10% heat-inactivated FBS, penicillin (50 units/ml) and streptomycin (50 µg/ml). Subconfluent cells were plated at a density of 1.0–2.0 × 10⁶ cells/100-mm dish and transfected by a modification of the DEAE-dextran method of Luthman and van der Eb (1973) as described (Birnbaumer et al., 1992). After 3–4 h at 37 °C, cells were harvested with a rubber policeman, and assayed for adenylyl cyclase activity.

Stable Expression in Ltk- Cells—Ltk- cells kept subconfluent were transfected by the calcium phosphate precipitation technique of Graham and van der Eb (1973) as described (Birnbaumer et al., 1990b). Cells grown in MEM medium containing 10% FBS, penicillin (50 units/ml) and streptomycin (50 µg/ml) were plated the day before transfection (1–2 × 10⁶ cells/plate) and exposed for 18 h to the DNA-calcium phosphate coprecipitate containing 2.5 µg of plasmid DNA. Thereafter...
cells were treated with 25% glycerol in HBSS at 37 °C for 1 min and returned to fresh medium. The next day cells were trypsinized, diluted with a selection medium containing G-418 (400 μg/ml), and distributed into the wells of two 96-well microtiter plates (2000–4000 cells/well), using a COSTAR transplate device. G418-resistant clones were picked (after 16–18 days) and expanded in 6-well plates. V2R-expressing HTB-2 cells (Birnbaumer et al., 1992b) were cultured in MEMα medium (Life Technologies, Inc.), containing 0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 10% heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Hormone Binding to Intact Cells—Cells were plated in 12-well plates at a density of 1.0 × 10⁶ cells/well. Binding assays were performed the following day as described (Birnbaumer et al., 1992a).

Adenylyl Cyclase Activity in Cell Homogenates—Adenylyl cyclase activity was assayed as described (Birnbaumer et al., 1992a).

RESULTS

Transient Expression in COS.M6 Cells—Equal amounts of wild type and Q2 cDNAs cloned into the expression vector pKNIH were transfected in parallel into COS.M6 cells as described under “Materials and Methods.” The binding of [3H]AVP to intact cells and the determination of adenylyl cyclase activity in cell homogenates was done 48 h after transfection.

The results are summarized in Table I. Receptors were present on the surface of cells bearing the mutant as well as the wild type cDNA. The number of receptors per cell was approximately 14 times lower for the Q2 mutant than for the wild type receptor. In contrast, the Kd values for AVP deduced from saturation binding experiment (not shown), did not differ significantly (6.7 and 10.4 nM for the Q2 and wild type receptor, respectively). Stimulation of adenylyl cyclase activity by the endogenous adrenergic receptor was similar in cells bearing the wild type or the mutant cDNA (Table I). However, only cells transfected with wild type cDNA showed an increase in adenylyl cyclase activity when challenged with 100 nM AVP. The stimulation of adenylyl cyclase activity measured in COS.M6 cells after transient expression is only 2–3-fold over basal. Thus, it was possible that the assay failed to detect a weak stimulation of adenylyl cyclase activity by the reduced number of Q2 receptors. Since the V2R expressed in stably transfected L cells shows a robust stimulation of adenylyl cyclase (Birnbaumer et al., 1990b), we decided to develop L cells expressing the Q2 mutant receptor to analyze the correlation between receptor abundance and stimulation of adenylyl cyclase activity.

Stable Expression in Ltk- Cells—Since the mutant receptor protein is successfully transported to the plasma membrane in COS.M6 cells the stable transformants expressing the receptor were identified by binding assays (Liao et al., 1988). G418-resistant clonal cells transfected with the mutant cDNA were plated in duplicate in 12-well plates and single point binding assays were performed; one for total binding and one for nonspecific binding in the presence of 10 μM unlabeled AVP. We identified three cell lines expressing the Q2 mutation that were expanded and cloned by limiting dilution. The Q2-3 cell line was chosen for further experimentation because it expressed the highest number of mutant receptors (see below); the other lines contained 8,000 (Q2-6) and 15,000 (Q2-24) sites per cell.

Saturation binding of [3H]AVP to Q2-3 cells and to HTB-2

| Transfected cDNA | Wild type | Q2 mutant |
|------------------|-----------|-----------|
| [3H]AVP binding (pmol AVP/mg) | 1,700,000 ± 162,000 | 122,200 ± 8,500 |
| Adenylyl cyclase (pmol CAMP/min/mg) | | |
| Basal | 80.0 ± 3.0 | 75.0 ± 2.5 |
| AVP (100 nM) | 225.0 ± 5.0 | 72.0 ± 3.0 |
| Isoproterenol (10 μM) | 225.0 ± 7.0 | 210.0 ± 6.0 |

Table I

The human V2R, Q2 mutant receptor expressed in COS.M6 cells: [3H]AVP binding and adenylyl cyclase activity

Values reported are the average ± half the range of results obtained in two experiments in each of which incubations were carried out in duplicate.

Fig. 2. Saturation binding of [3H]AVP to Q2-3 and HTB-2 cells and analysis of data according to Scatchard. The [3H]AVP saturation binding assays were carried out at 0 °C. Upper left panel, [3H]AVP binding to Q2-3 cells. ●, total binding; ▲, binding in the presence of 10 μM AVP; ○, specific binding. Lower left panel, Scatchard analysis. Upper right panel, [3H]AVP binding to HTB-2 cells. ●, total binding; ▲, binding in the presence of 10 μM AVP; ○, specific binding. Lower right panel, Scatchard analysis.
cells (expressing the wild type receptor), was determined in the same experiment (Fig. 2). The Q2 receptor exhibited an affinity for vasopressin ($K_D = 8.4 \pm 0.5$ nM) that was comparable to that of the wild type receptor ($K_D = 6.4 \pm 0.2$ nM) as determined in three experiments; values were similar to those observed in COS cells. The Q2-3 cell line expressed 25,000 to 30,000 receptors per cell. This value is close to the number of wild type receptors present on the surface of the HTB-2 cells (15,000–20,000 sites per cell).

Because of the similar receptor abundance of Q2-3 and HTB-2 cells (Birnbaumer et al., 1992b), we compared the adenylyl cyclase response to vasopressin in these two cell lines. As illustrated in Fig. 3, the mutant receptors were unable to trigger adenylyl cyclase stimulation even at very high concentrations of hormone, at which the receptor is fully occupied. In the same assay, the adenylyl cyclase activity of the HTB-2 cells was stimulated 15-fold. The responses of adenylyl cyclase to forskolin and prostaglandin $E_1$ (acting through endogenous prostaglandin receptors), were similar in both cells. Similar results were obtained with the Q2-6 and Q2-24 cells (data not shown).

It is worth mentioning that the adenylyl cyclase activity of the LV2.E2 cell, an $L$ cell expressing only 5,000 receptors per cell (Birnbaumer et al., 1992b), is stimulated 7-fold by AVP. The present data identifies the biochemical defect of the Q2 mutant receptor as failure to activate $G_A$ rather than an alteration of the affinity for AVP.

**DISCUSSION**

The present report proves that the Q2 mutation is indeed responsible for the CNBD phenotype. Although the mutant receptor shows normal binding properties, it is unable to stimulate the $G_A$/adenylyl cyclase system. We also observed in the COS cells a remarkable difference in the expression of the wild type versus the Q2 receptor. The low number of mutant receptors could be due to decreased stability of the mutant RNA, although we consider this unlikely. It seems more likely that the $\text{Arg} \rightarrow \text{His}$ change at codon 137 interferes with proper folding of the nascent protein giving rise to a “mis-folded” receptor, which is less efficiently translocated across the endoplasmic reticulum and the Golgi apparatus. The modification of protein structure may also interfere with appropriate insertion into the plasma membrane or result in an increased degradation rate in the endoplasmic reticulum or Golgi cisternae. Because the mutant receptor has retained AVP binding activity with an affinity similar to that of the wild type, we assume that protein half-life rather than its insertion into the plasma membrane is altered. In either case we expect the affected members of the Q2 families to have reduced number of receptor protein on the surface of the corresponding kidney cells. Regardless of these considerations we expect the Q2 receptor to have a cytoplasmic conformation different from that of the wild type, and as a consequence, to lack the ability to contact $G_A$ or to activate the GDP/GTP exchange. The identification of the stably transfected cells expressing the mutant receptor was done by binding, a less sensitive assay than the measurement of AVP-responsive adenylyl cyclase that we have used for the identification of cells expressing the wild type receptor (Birnbaumer et al., 1992b). Thus, the screening procedure selected for cells with a high number of receptors. The majority of the G418-resistant clones expressed the Q2 mutant receptor at much lower numbers than the three cell lines reported here.

The Q2 mutation is of interest not only because of its localization and biochemical consequences but also because it alters an $\text{Asp} \rightarrow \text{Glu}$ motif conserved in many G protein-coupled receptors (Savarese and Fraser, 1992), including, among others, neurotransmitter receptors, peptide hormone receptors, glycoprotein receptors, and light and odor receptors. Six receptors lacking this double amino acid motif form a subfamily including the receptors for parathyroid hormone, calcitonin, and the glucagon-related peptides glucagon, glucagon-like peptide I, vasoactive intestinal peptide, and secretin (Thorens, 1992; Jelinek et al., 1993).

This motif has received attention already in two G protein-coupled receptors: rhodopsin and the $\beta$-adrenergic receptor ($\beta$-AR). Studies with the $\beta$-AR addressed the effect of substituting the Asp of the Asp-Arg motif at codons 130–131. The $\text{Asp}^{130} \rightarrow \text{Gly}$ mutant constructed by Dixon et al. (1988) showed an unaltered EC$_{50}$ value for stimulation of adenylyl cyclase by (-)-isoproterenol, and a presumably unaltered affinity for agonist. However there was a 50% reduction in the maximal stimulation of the $G_A$/adenylyl cyclase system, probably reflecting a reduced ability of the mutant receptor to couple to $G_A$. The $\text{Asp}^{130} \rightarrow \text{Glu}$ mutant (Fraser et al., 1988) had decreased affinity for agonist and retained some of the effects of GTP on agonist binding, but it had totally lost the ability to stimulate the $G_A$/adenylyl cyclase system. The data indicated that the mutant interacts with $G_A$ (i.e. it forms a ternary complex) but does not promote nucleotide exchange on the $G_A$ a-subunit.

Working with bovine rhodopsin Khorana and collaborators constructed a $\text{Glu}^{134}, \text{Arg}^{135} \rightarrow \text{Arg}$ mutant. The mutant retained its ability to bind retinal and supported light-induced retinal isomerization but failed to interact with transducin, as was evident from the failure to be stabilized as metarhodopsin-II by transducin, akin to the induction of the high affinity state of the $\beta$-AR, and from the failure to stimulate the GTPase activity of transducin (Franke et al., 1990). The same results were obtained with $\text{Arg}^{135} \rightarrow \text{Gln}$ mutant; retinal bound normally, but interaction with transducin did not occur (Franke et al., 1990).

In two cases of individuals with autosomal dominant retinitis pigmentosa, Sung et al. (1991a) identified changes in the codon $\text{Arg}^{135}$ of human opsin. The naturally occurring mutants, $\text{Arg}^{135} \rightarrow \text{Leu}$ and $\text{Arg}^{135} \rightarrow \text{Trp}$, did not bind retinal (Sung et al., 1991b), in contrast to the bovine opsin mutants mentioned above.

![Fig. 3. Adenylyl cyclase activity of homogenates of HTB-2 and Q2-3 cells. Left, adenylyl cyclase activity of homogenates measured in the presence of increasing concentrations of AVP. Right, adenylyl cyclase activity of homogenates measured without (basal) or with 10 μM forskolin or 10 μM prostaglandin $E_1$. Activities were determined in triplicate; bars indicate the range of the results. The experiment was repeated three times with similar results.](image-url)
The present data and that obtained with the various opsin and β-AR mutants demonstrate not only that the Asp-Glu-Arg motif is important for proper receptor function, but also that the degree of impairment of receptor function of the mutant proteins is strongly dependent on the identity of the substituting amino acid. In the case of the V2R, Arg^{137} → His did not alter recognition of the hormone but impaired the ability of the receptor to activate Gs, thus causing CNDI.

REFERENCES

Bicht, D. G., Hendy, G. N., Lonergan, M., Arthurs, M.-F., Ligier, S., Paussova, Z., Kluge, R., Zingg, H., Saenger, P., Oppenheimer, E., Hirsh, D. J., Gilgenkrantz, S., Salles, J.-P., Oberle, I., Maudel, J.-L., Gregory, M. C., Fujiwara, T. M., Morgan, K., and Serwer, C. R. (1992) Am. J. Hum. Genet. 51, 898–1102

Bicht, D. G., Arthurs, M.-F., Lonergan, M., Hendy, G. N., Paradis, A. J., Fujiwara, T. M., Morgan, K., Gregory, M. C., Rosenthal, W., Antaramian, A., and Birnbaumer, M. (1993) J. Clin. Invest., in press

Birnbaumer, L., Abramowicz, J., and Brown, A. M. (1990) Biochim. Biophys. Acta 1031, 163–224

Birnbaumer, M., Hinrichs, M. V., and Themmen, A. P. N. (1990b) Mol. Endocrinol. 4, 245–254

Birnbaumer, M., Antaramian, A., Themmen, A. P. N., and Gilbert, S. (1992a) J. Biol. Chem. 267, 11783–11788

Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brebot, P., and Rosenthal, W. (1993b) Nature 365, 233–235

Culpepper, R. M., Hebert, S. C., and Andreoli, T. E. (1991) in The Metabolic Bases of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th Ed., pp. 103–106 McGraw-Hill, New York

Davies, K. (1992) Nature Genetics 2, 105–106

Dixon, R. A. F., Sigal, I. S., and Strader, C. D. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 487–497

Franke, R. R., Koenig, B., Sakmar, T. P., Khorana, H. G., and Hoffman, K. P. (1990) Science 250, 123–125

Franke, R. R., Sakmar, T. P., Graham, R. M., and Khorana, H. G. (1992) J. Biol. Chem. 267, 14767–14774

Fraser, C. M., Chung, F. Z., Want, C.-D., and Venter, J. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5478–5482

Graham, F. L., and Van der Eb, A. J. (1973) Virology 52, 456–467

Jelinek, L., Jak, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bench, P. A., Knüpfer, J. L., Sheppard, P. O., Sprecher, C. A., O’Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A., and Kindsvogel, W. (1993) Science 259, 1614–1616

Kambouris, M., Doushy, S. R., Trofatter, J. A., Conneally, P. M., and Hodes M. E. (1988) Am. J. Med. Genet. 29, 239–246

Karnik, S. S., Sakmar, T. P., Chen, H.-B., and Khorana, H. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8458–8463

Knaus, N., vd. Hayden, H., v. Oost, B. A., Montens, L., Willems, J., and Ropers, H. H. (1988) Nephron 50, 187–190

Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132

Liao, C.-F., Themmen, A. P. N., Jobo, R., Barberis, C., Birnbaumer, M., and Birnbaumer, L. (1988) J. Biol. Chem. 264, 7328–7337

Luthman, H., and Magnusson, G. (1983) Nucleic Acids Res. 11, 1295–1308

Nakamaye, K., and Eckstein, F. (1986) Nucleic Acids Res. 14, 9679–9688

Orloff, J., and Handler, J. S. (1987) Am. J. Med. 82, 757–768

Pao, Y., Metzenberg, A., Das, S., and Gitschier, J. (1992) Nature Genetics 2, 103–106

Rosenthal, W., Antaramian, A., Arthurs, M.-F., Lonergan, M., Hendy, G. N., Birnbaumer, M., and Bicht, D. G. (1992) Nature 359, 233–235

Sanger, F., Nicklen, S., and Coulson, A. B. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467

Savage, T. M., and Fraser, C. M. (1992) Biochem. J. 283, 1–19

Seibold, A., Brabet, P., Rosenthal, W., and Birnbaumer, M. (1992) Am. J. Hum. Genet. 51, 1078–1093

Sung, C.-H., Davenport, C. M., Hennessy, J. C., Maumenee, I. H., Jacobson, S. G., Heckenlively, J. R., Nowakowski, R., Fishman, G., Gours, P., and Nannya, J. (1991a) Proc. Natl. Acad. Sci. U. S. A. 88, 6481–6485

Sung, C.-H., Schneider, B. G., Agarwal, N., Pappas, M., and Nannya, J. (1991b) Proc. Natl. Acad. Sci. U. S. A. 88, 8840–8844

Thorens, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8641–8645

van den Ouweland, A. M. W., Drenth, J. C. F. M., Verdijk, M., Koers, N. V. A. M., Montens, L. A. H., Becchi, M., and van Oost, B. A. (1992) Nature Genetics 2, 99–102