Diverse vasopressin V2 receptor functionality underlying partial congenital nephrogenic diabetes insipidus

Mia Faerch,1,3 Jane H. Christensen,1,3 Søren Rittig,1 Jan-Ove Johansson,4 Niels Gregersen,2 Francis de Zegher,5 and Thomas J. Corydon3

1Department of Pediatrics, and 2Research Unit for Molecular Medicine, Aarhus University Hospital, Skejby; 3Department of Human Genetics, Aarhus University, Aarhus, Denmark; 4Department of Endocrinology, Sahlgrenska University Hospital, Göteborg, Sweden; and 5Department of Pediatrics, University of Leuven, Belgium

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The antidiuretic hormone arginine vasopressin (AVP) is, in part, responsible for maintaining proper water homeostasis. The hormone is secreted by the neurohypophysis in response to rising plasma osmolality. AVP executes its antidiuretic effect on the collecting ducts in the kidney, by binding to the G protein-coupled vasopressin V2 receptor located on the basolateral membrane of the principal cells (16). Binding of AVP activates the V2 receptor and leads to conformational changes. These changes lead to activation of adenylyl cyclase and thereby the formation of intracellular cAMP from ATP. cAMP binds to the regulatory subunits of cAMP-dependent protein kinase A, and the resulting phosphorylation cascade leads to exocytotic insertion of aquaporin-2 (AQP2) water channels in the luminal membrane of the principal cells (21). Water is transported through the water channels as a result of the difference in osmotic pressure between the lumen and the interstitium. Water exits the cells through AQP3 and AQP4 water channels located on the basolateral membrane (16).

Variations in the AVP receptor 2 (AVPR2) gene encoding the V2 receptor are responsible for 90% of all congenital nephrogenic diabetes insipidus (CNDI) cases. Variations in the AVPR2 gene encoding the AQP2 water channels are responsible for the remaining 10%. CNDI caused by these variations is transmitted either autosomal recessive or dominant, whereas CNDI caused by AVPR2 variations is X-linked (5, 18).

To date, at least 211 AVPR2 gene variations have been recognized to cause X-linked CNDI. By functional analysis, variations have been categorized into five classes according to their apparent effect on the V2 receptor biosynthesis. The most common type (up to 70% of all variations investigated) causes impaired intracellular trafficking of the variant V2 receptor (class II) (16). The remaining types result in either defects in the synthesis of stable mRNA (class I), failure to generate an increase in intracellular cAMP (class III), reduced ligand binding capacity (class IV), or problems with exo- or endocytosis of the receptor (class V) (12).

To date, only seven of all known variations identified in the AVPR2 gene cause a partial CNDI phenotype, namely those predicting the following amino acid substitutions: p.Asp85Asn, p.Arg104Cys, p.Arg106Cys, p.Gly201Asp, p.Pro322Ser, and p.Ser329Arg (1, 6, 13, 14, 19, 23) (Fig. 1). Knowledge is limited about the molecular mechanisms underlying this unique phenotype, which is characterized by an ability to concentrate urine close to normal at very high levels of plasma AVP or 1-deamino-8-D-AVP (dDAVP) concentrations. Expression of p.Asp85Asn and p.Pro322Ser variant V2 receptors in cell lines demonstrated practically identical number of functional receptors (Bmax) at the cell surface compared with that of wild-type (WT), but reduced ligand binding affinity as well as decreased cAMP production capacity (1, 23). Expression of p.Gly201Asp and p.Arg104Cys variant receptors showed low Bmax values compared with that of the WT. Furthermore, the p.Arg104Cys variant V2 receptor showed a slight reduction in cAMP activation capacity but an increase in binding affinity (14), whereas the p.Arg201Asp variant V2 receptor demonstrated normal receptor functionality (23).

We previously reported that the p.Ser329Arg substitution of the V2 receptor causes a partial CNDI phenotype (13). In the...
present study, we characterized the p.Arg104Cys and p.Ser329Arg variant V2 receptors by transient expression in human embryonic kidney (HEK-293) cells to determine whether they share common functional properties reflecting their potential to cause a partial clinical phenotype.

**MATERIALS AND METHODS**

**Expression constructs.** A GeneStorm hORF expression vector, pcDNA 3.1/GS, containing the 1,215-bp coding region of a human AVPR2 cDNA and two in-frame 3′ epitope tags (V5 and 6xHis; Invitrogen, Carlsbad, CA) was corrected for a discrepancy from the normal genomic sequence by QuikChange Mutagenesis (Stratagene, La Jolla, CA). A Ndel-AvrII fragment containing the entire AVPR2 coding region as well as the two epitope tags was excised from the resulting construct and cloned into an empty pcDNA3.1 vector (Invitrogen). The g.742C→T (p.Arg104Cys) or the g.1525C→G (p.Arg137Gly) substitutions described previously (13, 14) and a novel variation g.841C→G (p.Arg137Gly) causing a complete phenotype were introduced by QuikChange Mutagenesis (Stratagene). To verify the presence of the variations and exclude other variations in the expression constructs, the nucleotide sequence of the AVPR2 coding regions as well as the flanking regions was determined by sequencing in both directions using a BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

**Cell culture and transfection.** HEK-293 cells (American Type Culture Collection, Manassas, VA) were cultured in either 10-cm2-slide flasks for confocal laser-scanning microscopy (CLSM), 25-cm2 tissue culture flasks for Western blotting analysis, or 75-cm2 tissue culture flasks for Northern blotting. HEK-293 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen) containing 10% fetal calf serum (Invitrogen), 10,000 IU/ml penicillin, and 1% streptomycin (both Leo Pharma, Copenhagen, Denmark). The cells were maintained in a 5% CO2 atmosphere at 37°C. Transfection was performed by lipofection with FuGENE 6 transfection reagent (Roche, Basel, Switzerland) (11). Cells were cultured in either 10-cm2-slide flasks for confocal laser-scanning microscopy (CLSM), 25-cm2 tissue culture flasks for Western blotting analysis, or 75-cm2 tissue culture flasks for Northern blotting analysis. For ligand binding, cAMP analysis, and competition assay, 24-well tissue culture test plates were used.

**Northern blotting.** Northern blotting analysis was performed almost as previously described (8). Total RNA was isolated according to the RNAsol protocol (LifeTecnologies, Gibco BRL, Gaithersburg, MD) and 10 μg (per lane) were separated on a 1% agarose formaldehyde gel (according to standard procedures) and blotted to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Hercules, CA). A gel-purified 906-bp 

**Immunostaining and microscopy.** For immunostaining and CLSM, cells were fixed for 1 h in a formaldehyde solution (4%) and permeabilized in 70% ice-cold ethanol. The V2 receptors were detected using mouse monoclonal anti-V5 antibody (1:500; Invitrogen) as the primary antibody. Mitochondrial electron transfer flavoprotein (ETF) activity was performed by using STORM 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics).

**Western blotting.** Western blotting analysis was performed almost as previously described (10). In brief, cells were harvested, washed twice in PBS, and lysed in lysis buffer [50 mM Tris-HCl, pH 7.8 containing 5 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, 1 mg/ml soybean trypsin inhibitor, 1% Triton X-100, and Complete Mini (Roche)] for 30 min at 4°C. Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories). Twenty micrograms of protein (per lane) were separated on a 12% polyacrylamide gel (according to standard procedures) and blotted to a Zeta-Probe nylon membrane (Bio-Rad). Detection was performed according to the SuperSignal West Dura Extended Duration Substrate protocol (Pierce Biotechnology) using mouse monoclonal anti-V5 antibody (1:500; Invitrogen) as the primary antibody. Mitochondrial electron transfer flavoprotein (ETF) was detected concomitantly as a loading control using rabbit polyclonal anti-ETF antibody (kind gift from lector, PhD P. Bross, Research Unit for Molecular Medicine, Aarhus University Hospital, Denmark). Visualization of radioactivity was performed using STORM 840 PhosphorImager (Molecular Dynamics).
and slides were mounted using antifade solution (9). Visualization was performed by CLSM utilizing a Leica-DMRXE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Ligand binding analysis.** Forty-eight hours after transfection, cells were washed three times with ice-cold n-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.0 mM Na2HPO4, 0.9 mM CaCl2, 0.5 mM MgCl2, pH 7.4) and incubated on ice for 2 h in binding buffer consisting of ice-cold n-PBS, 2% BSA, and varying amounts of [3H]AVP (PerkinElmer Life Sciences, Waltham, MA) either in the presence or absence of 10 μM unlabeled AVP (Sigma, St. Louis, MO) (18). The incubation solution was transferred by aspiration to scintillation vials containing 3.5 ml of ULTIMA GOLD MV scintillation fluid to determine the amount of free radioactivity on a QuantaSmart/TriCarb β-counter (Pachard Instrument, Meriden, CT). Cells were washed quickly three times with ice-cold n-PBS and incubated overnight at 37°C with 0.2 ml 0.1 N NaOH to extract bound [3H]AVP (18). A 50-μl aliquot was removed to measure the protein concentration by the DC Protein Assay (Bio-Rad Laboratories). The radioactivity in the remaining solution was determined as described above. Bmax and Kd values were calculated with the SigmaPlot 10.0 software (Systat Software, San Jose, CA).

**cAMP assay.** For cAMP assay, an additional transfection with cDNA containing a novel AVPR2 gene variation (g.841C>G) was performed to assess the transcription capability of the AVPR2 gene variation (g.841C>G) as associated with a complete form of CNDI was done as a positive control. Forty-eight hours posttransfection, media were aspirated, and 0.2 ml of preincubation buffer (DMEM containing 0.1 μM adenine and 1 μCi/ml [2,8-3H]adenine) were added. Cells were incubated for 48 h at 37°C to allow the incorporation of [2,8-3H]adenine into the intracellular ATP pool. Cells were washed twice in 37°C warm DMEM to remove extracellular [2,8-3H]adenine. Reaction buffer consisting of 1 mM isobutylmethylxanthine added to 37°C DMEM was mixed with varying amounts of unlabeled AVP or dDAVP and applied to each well (20). After 40 min of incubation, the reaction buffer was aspirated. Cells were washed twice in n-PBS before the addition of lysis buffer consisting of 5% trichloroacetic acid containing 1 mM ATP and 1 mM CAMP. An amount of 0.05 μCi [14C]cAMP was used as the recovery standard (20). Isolation of cAMP was carried out according to the one column separation method (2). The amount of soluble receptor protein detected in cells expressing Arg104Cys-V2R was slightly lower compared with WT-V2R. The total amount of bound radioactivity associated with the Ser329Arg-V2R was clearly diminished (Fig. 3) (15). The total amount of soluble receptor protein detected in cells expressing Arg104Cys-V2R was slightly lower compared with WT-V2R, while the amount of protein detected in cells expressing the Ser329Arg-V2R was clearly diminished (Fig. 3). No V2 receptor protein was detected in lysates derived from cells transfected with empty pcDNA3.1 vector (Fig. 3, compare lanes 1–3 with lane 4). The loading control, mitochondrial ETF, which was detected concomitantly with the V5 epitope, revealed no significant differences in the amounts of loaded protein between lanes 27.

**RESULTS**

**Transcription and translation.** Northern blotting analysis performed to assess the transcription capability of the AVPR2 cDNA constructs revealed no significant difference in expression levels of AVPR2-mRNA in HEK-293 cells transfected with plasmid DNA encoding WT, p.Arg104Cys, or p.Ser329Arg V2 receptor proteins (WT-V2R, Arg104Cys-V2R, or Ser329Arg-V2R; Fig. 2A). The levels of AVPR2-mRNA correlated positively to the levels of GAPDH-mRNA (Fig. 2B), which supported the results. Furthermore, no AVPR2-mRNA was detected in the RNA from cells transfected with empty pcDNA3.1 vector (Fig. 2A). To determine whether the transcripts were translated into stable proteins, Western blot analysis was carried out using an anti-V5 antibody recognizing a V5 epitope translated into the COOH terminal of each of the different V2 receptor proteins. The analysis revealed anti-V5 antibody-specific bands with relative mobilities ranging from 45 to 50 kDa consistent with varying degrees of glycosylation of the V2 receptor in HEK-293 cells expressing WT-V2R (Fig. 3) (15). The total amount of soluble receptor protein detected in cells expressing Arg104Cys-V2R was slightly lower compared with WT-V2R, while the amount of protein detected in cells expressing the Ser329Arg-V2R was clearly diminished (Fig. 3). No V2 receptor protein was detected in lysates derived from cells transfected with empty pcDNA3.1 vector (Fig. 3, compare lanes 1–3 with lane 4). The loading control, mitochondrial ETF, which was detected concomitantly with the V5 epitope, revealed no significant differences in the amounts of loaded protein between lanes 27.

**Cellular localization.** To investigate whether the two implicated variations had any effect on the cell surface expression of the V2 receptor, localization in transiently transfected HEK-293 cells was evaluated by immunostaining and CLSM. V2 receptors were detected by anti-V5 antibody and fluorescent-labeled secondary antibody. As shown in Fig. 4, cells expressing WT-V2R display a clear and pronounced cell surface expression. The Arg104Cys-V2R was mainly localized on the cell surface; however, a proportion of the protein was localized in intracellular compartments (Fig. 4). In general, less anti-V5-positive cells were detected in cells expressing the Ser329Arg-V2R proteins. The Ser329Arg-V2R was localized mainly in a diffuse, intracellular and perinuclear pattern with reduced cell surface expression compared with WT-V2R (Fig. 4). An ER-specific antibody recognizing calreticulin was used to investigate possible colocalization of variant V2 receptor protein with the ER. As shown in Fig. 4 (column 3), there is no significant colocalization between Arg104Cys-V2R and ER, while a ma-
major fraction of the Ser329Arg-V2R protein shows colocalization to the ER.

Pharmacological and functional characterization. To investigate the pharmacological properties of expressed variant V2 receptors, whole cell saturation binding assays were carried out on transiently transfected HEK-293 cells using [3H]AVP as the radioligand in the presence or absence of excess (10 μM) unlabeled AVP (Fig. 5). B_max for cells expressing WT-V2R was 2.5-fold higher than B_max for cells expressing Ser329Arg-V2R and 14.8-fold higher than B_max for cells expressing Arg104Cys-V2R (Fig. 5). The difference between the AVP binding affinity (1/K_d) between WT-V2R and Ser329Arg-V2R was not as pronounced as the difference between WT-V2R and Arg104Cys-V2R, the latter having a fourfold increased ligand binding affinity compared with WT.

To investigate the ability of the V2 receptors that reach the cell surface to stimulate the adenylate cyclase to produce cAMP, a cAMP assay was performed on transiently transfected HEK-293 cells. After incubation with [2,8-3H]adenine, cells were stimulated with increasing amounts of unlabeled AVP or dDAVP. The degree of stimulation of adenylate cyclase was determined by measuring the amount of radioactive cAMP produced (Fig. 6). AVP stimulation revealed a maximum cAMP production (V_max) for cells expressing Ser329Arg-V2R that was 6.8-fold lower than for cells expressing WT-V2R, while the maximum cAMP production in cells expressing Arg104Cys-V2R was only 1.7-fold lower than WT-V2R (Fig. 6A). The amount of agonist required to induce 50% effect (E_{50}) was the same for WT-V2R and Arg104Cys-V2R, whereas cells expressing Ser329Arg-V2 needed a threefold larger amount of agonist to reach E_{50}. As a positive control, cells were transfected with plasmid DNA containing AVPR2 cDNA with a novel AVPR2 variation (g.841C>A/p.Arg137Gly) identified to cause a complete CNDI phenotype (own observation, unpublished data). The ability of cells expressing Arg137Gly-V2R to produce cAMP was as low as cells transfected with empty pcDNA3.1 vector.

Maximum amounts of cAMP produced by cells expressing WT-V2R as well as Arg104Cys-V2R and Ser329Arg-V2R were analyzed in relation to stimulation with dDAVP. The results showed no difference in the maximum production of cAMP produced by WT-V2R and Ser329Arg-V2R. However, Arg104Cys-V2R showed a 1.5-fold increased cAMP production compared with WT-V2R.

Fig. 3. Western blot analysis of whole cell lysates from transiently transfected HEK-293 cells expressing V5-tagged V2 receptor proteins. Blots were probed concomitantly with mouse monoclonal antibody directed against the V5 epitope tag and rabbit polyclonal anti-electron transfer flavoprotein (ETF) antibody. Lane 1, lysate from cells expressing WT-V2R. Lane 2 and 3, lysate from cells expressing Arg104Cys-V2R and Ser329Arg-V2R, respectively. Lane 4, lysate from cells transfected with empty pcDNA3.1.

Fig. 4. Immunofluorescence staining and confocal laser-scanning microscopy of HEK-293 cells expressing V5-tagged WT-, Arg104Cys-, and Ser329Arg-V2R after permeabilization. Column 1 represents the V2R (green color). Column 2 shows endoplasmatic reticulum tagged by an anti-calreticulin antibody (red color). Column 3 displays the merging of the 2 images. Cells transfected with empty pcDNA3.1 vector were analyzed in parallel as a control (data not shown). Nuclear DNA was stained using To-Pro-3 iodide (pseudo-colored blue). The results shown are representative of cells examined in several separate experiments.
cAMP (V_{max}) compared with stimulation with AVP, whereas there was a 10-fold difference in EC_{50} for both Arg104Cys-V2R and Ser329Arg-V2R compared with WT-V2R, indicating that 10 times as much dDAVP are needed for the two variant receptors to reach the same level of response as for WT-V2R (Fig. 6B).

Displacement binding analysis was carried out to investigate whether there is a difference in the binding affinity for dDAVP between cells expressing WT-V2R and those expressing Arg104Cys-V2R and Ser329Arg-V2R (Fig. 7). For this purpose, identical amounts of [3H]AVP were added to each cell culture well in the presence of increasing amounts of unlabeled AVP or dDAVP. The results revealed that dDAVP was 2.1 times less potent than AVP in displacing [3H]AVP from cells expressing the WT-V2R and 6.8 times less potent in cells expressing Ser329Arg-V2R (Fig. 7), whereas it was 21.7 times less potent in the case of Arg104Cys-V2R, as illustrated by a considerable shift to the right of the curve representing the displacement of dDAVP (Fig. 7).

DISCUSSION

The two missense variations resulting in Arg104Cys and Ser329Arg substitutions in the V2 receptor protein are distinct in that patients expressing either of these were initially misdiagnosed as familiar neurogenic diabetes insipidus because of their ability to concentrate urine following administration of AVP or dDAVP. In the present study, we demonstrate that despite no difference in the clinical phenotype of patients carrying the two variations, the molecular mechanisms affecting receptor functionality differ considerably according to the localization of the affected amino acid residues within the V2 receptor protein.

To investigate the mechanisms involved, HEK-293 cells were transiently transfected with AVPR2 cDNA containing the two missense variations. Northern blotting analysis established that 1) similar amounts of AVPR2-mRNA were produced in cells expressing variant and WT AVPR2 cDNA and 2) that the transfection frequency of the three AVPR2 cDNA constructs is comparable (Fig. 2). We conducted the experiments at least three times using plasmid DNA from at least two separate preparations, thus, we believe that the presented findings are not influenced by fluctuation in the transfection frequency. Western blotting analysis showed that at steady state the total amount of mature V2 receptor protein produced was only marginally lower in cells expressing Arg104Cys-V2R compared with WT-V2R, while reduced amounts of receptor protein were observed in cells expressing Ser329Arg-V2R (Fig. 3). The amount of Ser329Arg-V2R variant protein detected by Western blotting varied between experiments but was always
clearly diminished compared with cells expressing WT-V2R and Arg104Cys-V2R.

As aforementioned, impaired trafficking of the variant V2 receptor is believed to be the most common cause of CNDI (16) based on observations of retention of receptor protein in the ER and middle Golgi compartments in cell expression studies (4). To assess the intracellular localization of the variant receptors, cells were immunostained with an antibody recognizing an ER resident protein (calreticulin) concurrently with the V2 receptor-specific antibody (Fig. 4). The results show no significant colocalization of the V2 receptor with ER in cells expressing either WT-V2R or Arg104Cys-V2R. In contrast, the Ser329Arg variant V2 receptor shows mainly colocalization with ER. However, it should be stressed that a proportion of the Ser329Arg-V2R shows normal trafficking and cell surface localization. This finding correlates well with the results of the Western blotting showing a lower amount of fully processed, mature protein is present in cells expressing this variation (Fig. 3). It could be speculated that ER-retained immature variant Ser329Arg-V2R protein could be in an insoluble form, which is not detected by Western blotting, and/or that it is subject to increased ER-associated degradation (24). The fact that the number of anti-V5-positive cells expressing the Ser329Arg-V2R protein is lower than WT-V2R is in concordance with the observations by Western blotting. Since equal transcription levels were observed (Fig. 2), reduced amounts of total V2R protein and lower number of positive stained cells (Fig. 4) may thus not be explained by different transfection frequency but rather by differences in intracellular trafficking of Ser329Arg-V2R.

Ligand binding analysis revealed a reduction in maximal binding capacity ($B_{\text{max}}$) for both Arg104Cys-V2R and Ser329Arg-V2R (Fig. 5). $B_{\text{max}}$ for Ser329Arg-V2R was 2.5-fold lower than that of WT-V2R, and binding affinity ($K_d$) was equal to that of the WT receptors (Fig. 5). This is indicative of fewer functional receptors reaching the cellular surface, whereas those reaching the surface have preserved binding capacity. The curve derived from cells expressing Arg104Cys-V2R was only slightly steeper than the curve derived from cells expressing an empty pcDNA3.1 vector, and $B_{\text{max}}$ was reduced 14.8-fold compared with $B_{\text{max}}$ for WT-V2R. This reduction in $B_{\text{max}}$ indicates that the number of receptors that bind the ligand under saturated conditions is reduced. This pronounced reduction in $B_{\text{max}}$ can hardly be explained by the slightly reduced levels of Arg104Cys-V2R compared with WT-V2R indicated by the Western blotting and CLSM analysis. In support of this notion, Ser329Arg-V2R has a higher binding capacity compared with Arg104Cys despite that significantly lower amounts of total protein were observed by Western blotting.

Since the binding affinity of the Arg104Cys variant receptors is increased compared with WT receptors, we suggest that the etiology behind the development of clinical symptoms in patients carrying this variation could be ascribed to a reduction in the ability of the ligand to reach the binding site in the V2 receptor because of conformational changes. The binding pocket for AVP in the V2 receptor is constituted of the first extracellular loop and the adjacent transmembrane domains. The presence of a disulfide bond between the two conserved cysteine residues present in extracellular loop 1 and 2 (cysteine-122 and cysteine-192) is required to maintain the integrity of the receptor structure. The eventual formation of new alternative disulfide bonds due to the Arg104Cys substitution could lead to conformational changes making the pocket less accessible for the hormone (17, 25). The assembly of V2 receptor protein and establishment of disulfide bonds in the ER could be heterogeneous and some receptors may bind the ligand better than others.

A cAMP assay was performed to evaluate the ability of the receptors to stimulate the adenylate cyclase upon binding AVP. Evidently, the maximum amount of cAMP produced by cells expressing Arg104Cys-V2R is only 1.7-fold less than that of cells expressing WT-V2R even though the number of receptors that bind the ligand is reduced (compare Fig. 6 with Fig. 5). This could be due to the improved binding affinity of Arg104Cys-V2R leading to prolonged receptor activation. In contrast, cells expressing Ser329Arg-V2R give rise to a 6.8-fold lower production of cAMP than that of WT-V2R. This could reflect that the Ser329Arg substitution at the intracellular COOH terminal interferes with coupling and activation of the G protein. The exact amino acid residues in the V2 receptor responsible for G protein coupling have not been identified. Several studies

**Fig. 7. Displacement binding analysis of [3H]AVP.** Intact HEK-293 cells expressing WT-V2R, Arg104Cys-V2R, or Ser329Arg-V2R were incubated for 2 h with [3H]AVP (2 nM) and increasing amounts of AVP (●) or dDAVP (○). Calculated $K_i$ values for AVP and dDAVP were, respectively, 34.9 and 74.7 nM for WT-V2R, 10.1 and 220.1 nM for Arg104Cys-V2R, and 10.2 and 69.0 nM for Ser329Arg-V2R. Data represent mean values of duplicates and are representative of 2 independent experiments.
have been performed on different G protein-coupled receptors to define G protein selectivity (27). It seems that the important residues are dispersed widely at different positions with no common motif. This is probably because the 2nd and 3rd intracellular loops and the COOH terminal are organized together in a three-dimensional structure that binds the G protein (3, 17). By interfering with the coupling of the G protein, the Ser329Arg substitution could potentially cause inhibition of the intracellular receptor signaling, resulting in a diminished production of cAMP upon stimulation. As a positive control of the cAMP assay, a novel variation (p.Arg137Gly) associated with a complete clinical phenotype (own observations, unpublished data) was investigated. The maximum cAMP produced by Arg137Gly-V2R was at the level of cells expressing an empty pcDNA3.1 vector, indicating that the cAMP assay indeed could reveal differences between variations. Overall, the results concerning the Arg104Cys-V2R confirm the results previously published by Inaba et al. (14).

The initial evaluation of the proband carrying the p.Ser329Arg variation gave suspicion of a difference in the renal response to dDAVP compared with endogenous AVP (13). Based on that displacement binding analysis was performed to reveal whether dDAVP was more potent than AVP in displacing \(^{[3}H\)AVP from cells expressing Arg104Cys-V2R and Ser329Arg-V2R compared with WT-V2R. This was, however, not the case as dDAVP was 6.8-fold less potent than AVP in displacing \(^{[3}H\)AVP from the Ser329Arg-V2R (Fig. 7), whereas it was 21.7 times less potent in the case of the Arg104Cys-V2R (Fig. 7). This is consistent with the fact that the Arg104Cys-V2R demonstrated higher affinity for AVP than Ser329Arg-V2R and WT-V2R as assessed in the ligand binding studies. A reduction in the binding affinity for dDAVP is coherent with the hypothesis that the Arg104Cys variation establishes new disulfide bonds interfering with the ligand selectivity. In this context, it is very interesting that residue Asp103, also located at the first extracellular loop, has been reported to be associated with increased binding affinity for dDAVP (26). An aspartic acid to tyrosine substitution at position l03 led to a 40-fold decrease in the binding affinity for dDAVP compared with WT while the high affinity for AVP was preserved. To assert that residue Arg104 possesses the same ability as Asp103 is reasonable.

The phenotype in patients expressing the Arg104Cys-V2R as well as the Ser329Arg-V2R proteins has been studied by extensive clinical investigations (13) (Robertson GL, Rittig S, Johansson J-O, Kamderis K, Faerch M, unpublished data). Both patient groups presented with slight polyuria; basal conditions revealed normal plasma osmolalities with an exceptional elevation in plasma AVP levels. During fluid deprivation, a severe increase in plasma osmolality further increased plasma AVP levels demonstrating a natural ability to produce AVP on osmotic stimulation. An increased plasma osmolality during fluid deprivation and hypertonic saline infusion led to urine osmolality just below normal range. This confirmed a diminished effect of the V2 receptor even at high levels of antiidiuretic hormone. Infusion of AVP was carried out and revealed that both patient groups had the ability to concentrate urine close to normal levels when plasma levels of AVP were exceedingly high (~60 pg/ml). Furthermore, infusion of dDAVP was carried out on patients carrying the Ser329Arg variation with the same results as the infusion of AVP. These results suggest that markedly elevated concentrations of AVP (or dDAVP) produce efficient responses, implicating that the receptor efficiency can be improved (13).

In conclusion, our results indicate that a heterogeneous molecular background underlies the partial forms of CNDI. The Arg104Cys substitution, residing in the ligand binding domain of the receptor, affects AVP binding, whereas the ability of the receptor to initiate intracellular signaling is only slightly affected. The AVP binding of the Ser329Arg-V2R is also reduced, but not to the same extent as the Arg104Cys-V2R. Despite this, the Ser329Arg-V2R has diminished ability to initiate intracellular signaling, in line with the localization of the substitution in the receptor signal transduction domain. Comprehensive knowledge of receptor functionality in patients with CNDI could be of great value in future treatments, particularly, if a decision between strategies involving either rescue of intracellular retained functional receptors or drug-induced insertion of AQP2 water channels in the apical membrane of the principal cells has to be made.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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