Identification and Characterization of an Activating F229V Substitution in the V2 Vasopressin Receptor in an Infant with NSIAD

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ABSTRACT

Gain-of-function mutations in the gene encoding the V2 vasopressin receptor (V2R) cause nephrogenic syndrome of inappropriate antidiuresis. To date, reported mutations lead to the substitution of arginine 137 by either a cysteine or leucine (R137C/L). Here, we describe a 3-month-old hyponatremic infant found to have a phenylalanine 229 to valine (F229V) substitution in V2R. Characterization of this substitution in vitro revealed that it leads to high constitutive activity of the receptor, compatible with spontaneous antidiuresis. In contrast to R137C/L mutant receptors, F229V receptors do not undergo spontaneous desensitization, which results in sustained, high basal activity. Notably, the V2R-selective inverse agonists tolvaptan and satavaptan completely silenced the constitutive signaling activity of the F229V mutant receptor, indicating that this substitution does not lock the receptor in an irreversible active state. Thus, inverse agonists might prove to be effective therapies for treating patients with this or other spontaneously activating mutations that do not lock the V2R in its active state. These results emphasize the importance of genetic testing and the functional characterization of mutant receptors for patients with nephrogenic syndrome of inappropriate antidiuresis because the results might inform treatment decisions.


The vasopressin type 2 receptor (V2R) plays a central role in the control of water homeostasis by the kidney. Its activation by arginine-vasopressin (AVP) leads to water reabsorption, an event requiring V2R-promoted cAMP production.1 Inactivating mutations in the V2R gene (AVPR2) cause nephrogenic diabetes insipidus, an X-linked disease characterized by polyuria and polydipsia,2 whereas activating AVPR2 mutations are responsible for the nephrogenic syndrome of inappropriate antidiuresis (NSIAD). Patients with NSIAD have reduced free water excretion and concentrated urine despite hyponatremia and low or undetectable circulating AVP levels.3 Such low AVP levels distinguish NSIAD from the syndrome of inappropriate antidiuretic hormone secretion (SIADH), which is usually associated with elevated serum AVP levels.3 In all previously described NSIAD cases, substitution of arginine-137, located at the bottom of transmembrane domain 3 (TM3) (Figure 1A), by either a cysteine or a leucine (R137C/L) induces the spontaneous activation of the V2R3–5 that is responsible for the inappropriate antidiuresis in the absence of elevated AVP. The increased V2R activity is reflected by elevated basal cAMP levels observed in cells expressing the NSIAD mutants compared with the wild-type (WT) receptor.3,4,6

In this study, we report the case of a 3-month-old male infant that presented with an episode of apnea associated with a 1 week history of an upper respiratory infection due to respiratory syncytial virus. Initial evaluation revealed hyponatremia (120 mEq/L) with undetectable vasopressin level and evidence of euvolemia (Table 1). He was initially treated with intravenous fluids (D5 0.45 normal
Figure 1. Surface expression and maturation profile of F229V-V2R. (A) Snake plot representation of the human V2R indicating the positions of residue R137 and F229. The residues composing the different transmembrane domains (denoted TMs) were determined using the method described by Abrol et al.\(^1,3\) (B) Western blot analysis performed on total cell lysates using an anti-myc antibody. The black arrowhead indicates the mature forms of the V2R, whereas the empty arrowhead shows the immature forms. (C) Densitometric ratios of mature/immature forms of the receptors obtained via Western blots. The areas used for densitometric quantification of the different bands are depicted with boxes on the WT lane in B. (D) Relative cell surface expression monitored by whole cell ELISA using an anti-myc antibody. (E) Representative confocal microscopy images of cells transfected with the indicated YFP-tagged receptors. The Western blot result shown in B is representative of six independent experiments, and the densitometric ratios in C are the mean ± SEM of three to six independent experiments. Data shown in D are the mean ± SEM of three independent experiments. *\(P<0.05\); ***\(P<0.001\).
saline), but had no improvement in his serum sodium and his BP became mildly elevated. His serum sodium and hypertension improved with fluid restriction. Despite the low level of circulating AVP, the patient was diagnosed with SIADH secondary to bronchiolitis. At 6 months, the patient had a second apneic episode, but no laboratory tests were performed. A third apneic episode occurred at 9 months of age, prompting a second hospitalization with additional laboratory evidences of euvolemic hyponatremia (plasma renin activity <20 ng/dl per hour) and again undetectable vasopressin level (Table 1). The patient was diagnosed with SIAD and the sequencing of his AVPR2 gene revealed a T to G substitution at nucleotide 1046, causing a change from phenylalanine to valine at amino acid position 229 (F229V) located near the bottom of TM5 (Figure 1A).

To determine how the F229V substitution could contribute to the pathogenesis of NSIAD, a biochemical and functional characterization of F229V-V2R was carried out in HEK293T cells and compared with the previously described R137C/L-V2R. For the three mutants, Western blot analyses showed a reduced proportion of the mature fully glycosylated (45 kD) versus the immature core-glycosylated and deglycosylated (40 and 37 kD, respectively) receptor forms compared with the WT-V2R (Figure 1, B and C). Consistent with the reduced maturation observed for the mutant receptors, surface ELISA revealed a reduced expression level for F229V (80% of WT), albeit to a lesser extent than what was observed for R137L (65% of WT) and R137C (57% of WT) (Figure 1D).

Visual assessment by confocal fluorescence microscopy (Figure 1E) revealed a similar labeling for the F229V- and WT-V2R, whereas R137C- and R137L-V2R showed an endosomal-like punctate labeling, consistent with their previously described increased constitutive endocytosis. Taken together, these results indicate that F229V-V2R has a reduced cell surface expression, resulting from an impaired maturation and, most likely, not from increased endocytosis. However, the reduced maturation efficiency and cell surface targeting of the F229V-V2R cannot account for NSIAD because decreased cell surface expression is expected to lead to a loss of function disease (nephrogenic diabetes insipidus).

The signaling activity of F229V-V2R was assessed by measuring cAMP levels in cells expressing either WT-, F229V-, R137C-, or R137L-V2R using a gene reporter assay (CRE-luciferase; Figure 2A). The F229V-V2R promoted a significantly higher level of basal cAMP level than the WT-V2R, demonstrating that the mutation increases constitutive activity. The constitutive F229V-V2R basal cAMP production was approximately 31-fold greater than WT level, compared with only 5- and 17-fold for R137C- and R137L-V2R, respectively (Figure 2A).

Previous studies have shown that the activating R137L/C substitutions lead to constitutive desensitization and endocytosis of the receptor via an increase in spontaneous recruitment of the regulatory protein β-arrestin and subsequent interaction with the clathrin adaptor protein AP-2. Using a bioluminescence resonance energy transfer (BRET)–based assay, no increased constitutive recruitment of β-arrestin2 was observed for F229V, which is in contrast to the results obtained for R137C (Figure 2B). Similar results were obtained when monitoring receptor-promoted β-arrestin2/AP-2 assembly by BRET (Figure 2C). Such increased β-arrestin and AP-2 engagement were observed despite a significantly lower expression level of R137C-V2R compared with both WT- and F229V-V2R (Figure 2, legend). Inhibition of endocytosis with a dominant-negative mutant of dynamin-2 (DynK44A) potentiated the basal BRET signal between β-arrestin2 and AP-2, emphasizing the occurrence of constitutive endocytosis. The DynK44A-promoted increased BRET signal observed for R137C-V2R was much greater than for the WT receptor, whereas no such difference was observed for F229V-V2R (Figure 2C), indicating that the F229V substitution does not increase constitutive endocytosis. Collectively, these results show that F229V-V2R does not undergo elevated constitutive desensitization, thus providing an explanation for the much higher basal cAMP level observed in cells expressing F229V-V2R compared with R137C/L-V2R (Figure 2A). The reduced constitutive β-arrestin recruitment and AP-2 engagement could not arise from an intrinsic defect of F229V-V2R to recruit β-arrestin2 because AVP stimulation increased interactions between β-arrestin2 and F229V-V2R, similar to the WT receptor (Figure 2B). Thus, the F229V substitution promotes a receptor state that possesses high constitutive activity toward adenyly cyclase while not affecting β-arrestin recruitment. Such a biased effect of a mutation is consistent with the notion that these two pathways can be controlled independently by distinct receptor conformational changes.

To provide a structural basis for constitutive activation mechanism, we used a de novo structure prediction methodology called GeNeSMBLE (GPCR Ensemble of Structures in Membrane BiLayer Environment) to predict the most stable conformations for the WT, F229V and R137C receptors. As shown in Supplemental Figure 1, the modeling predicted differences between the lowest energy structures obtained for the three receptors. Most notably, the F229V mutation resulted in a significant outward movement of TM6 away from TM3 and TM5 (compared with WT-V2R), which is consistent with the biased effect of the F229V substitution on the constitutive activity of the receptor.
classically associated with GPCR activation. Despite an uncoupling of TM6 from TM3, no such obvious movement was observed for the R137C-V2R structure. Instead an inward movement of TM2 was observed. Additional studies will be needed to assess the contribution of these structural differences to the different constitutive activation profiles of the receptor.

The V2R antagonist satavaptan acts as an inverse agonist by inhibiting basal V2R-promoted cAMP production. However, attempts to reduce the constitutive activity of the NSIAD-causing R137C/L-V2R by vaptans were unsuccessful, either in vitro or in a patient harboring the R137C substitution, strongly suggesting that these receptors were “locked” in their active state. Consistent with this notion, AVP stimulation did not promote further cAMP production in cells expressing R137C- or R137L-V2R. To determine if F229V-V2R is also locked in an active conformation, we assessed the effect of satavaptan and the recently US Food and Drug Administration–approved tolvaptan (OPC-41061). An 18-hour treatment with either compound significantly reduced the F229V-V2R–promoted constitutive cAMP production (Figure 3A) while having no effect on the cAMP levels in cells expressing the WT-, R137C-, or R137L-V2R. As shown in Figure 3B, the inhibitory effect of both satavaptan and tolvaptan occurred rapidly after drug addition, reaching their maximal effect within 5 minutes. A concentration-response curve performed for tolvaptan (Figure 3C) revealed an IC$_{50}$ of 4.9±0.1 nM for its inverse agonist activity on the spontaneous activity of F229V-V2R, a potency most likely compatible with its used in clinical setting. These data indicate that both compounds can silence the high constitutive activity of F229V-V2R, a finding that contrasts with observations made for the two other NSIAD-causing V2R mutants. Consistent with the notion that F229V-V2R is not in a locked active conformation, AVP promoted further cAMP increase, (Figure 3B, gray triangles). This indicates that this substitution, while increasing constitutive activity, maintains a receptor conformation that is amenable to pharmacological modulation.

In addition to unraveling the molecular basis underlying NSIAD resulting from F229V-V2R, our study clearly shows that inverse agonists such as satavaptan and tolvaptan are promising candidates for the treatment of patients carrying this mutation or new constitutively activating mutations that do not maintain the receptor in a locked active state. This is particularly important when considering that 10%–20% of patients diagnosed with SIADH have undetectable serum AVP levels upon water restriction, suggesting that some of these patients may in fact have NSIAD due to an activating mutation in their AVP2 gene. Finally, our study demonstrates that a disease resulting from distinct mutations of the same gene may respond differently to a given therapy, highlighting the importance of a clear understanding of the functional consequences of the mutations that will allow appropriate personalized medicine.

**CONCISE METHODS**

Microscopy, Cell Surface, and Total Receptor Expression

Microscopy images were taken from cells expressing the different YFP-tagged constructs...
WT-V2R-YFP, F229V-V2R-YFP, R137C-YFP, or R137L-V2R-YFP (WT-V2R-YFP, F229V-V2R-YFP, generated by site-directed mutagenesis of the WT-V2R-YFP) using a Zeiss LSM 510 Laser Scanning Confocal Microscope (Jena, Germany). Cell surface receptor expression was assessed by surface ELISA detecting the transiently expressed myc-tagged V2R constructs (myc-WT-V2R, myc-R137C-V2R, myc-R137L-V2R, and myc-F229V-V2R; generated by site-directed mutagenesis of myc-WT-V2R using the Quick Chang mutation kit from Agilent Technologies, Santa Clara, CA). Functional assays (AVP binding affinity and EC50 of cAMP production) of the YFP- and myc-tagged WT-V2R were performed and results were found similar to the untagged version (Supplemental Table 1). ELISAs were performed as previously described using a mouse anti-myc antibody (9E10 clone) produced by our core facility as ascites fluids and an AP-conjugated goat anti-mouse (Bio-Rad, Hercules, CA) secondary antibody. Total receptor expression was assessed by Western blot analysis performed on total lysates of cells expressing the myc-tagged receptors using the mouse anti-myc antibody and a horseradish peroxidase–conjugated rabbit anti-myc secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) as previously described.

cAMP Measurements

Intracellular cAMP of cells expressing the different myc-tagged V2R constructs was monitored using either the CRE-luciferase reporter assay (pCRE-luciferase) purchased from Clontech (Mountain View, CA) as described or the exchange protein directly activated by cAMP protein-based bioluminescence resonance energy transfer biosensor.

Engagement of the Endocytic Machinery

The β-arrestin2-Rluc/receptor-YFP and β-arrestin2-Rluc/AP-2-YFP interactions were monitored by BRET, as previously described. For β-arrestin2-Rluc/receptor interaction, YFP-tagged constructs were cotransfected with the β-arrestin2-Rluc construct in HEK293T cells and exposed or not to 1 μM AVP for 15 minutes before BRET reading. For β-arrestin2-Rluc/AP-2-YFP interaction, HEK293T cells stably expressing the AP-2-YFP construct were co-transfected with β-arrestin2-Rluc along with the indicated myc-tagged receptor construct, with or without the dominant-negative Dynamin2 (DynK44A) construct. BRET measurements were performed 48 hours post-transfection.

Structural Modeling

A de novo structure prediction methodology called GEnSeMBLE was used to predict the most stable conformations for WT-, F229V-, and R137C-V2R. The methodology is summarized in the Supplemental Material.

Statistical Analyses

Data are presented as mean ± SEM, and statistical significance of the differences were assessed by ANOVA. Pair-wise comparisons were made by the post hoc Bonferroni multiple comparison test. Differences with P < 0.05 were considered significant.

ACKNOWLEDGMENTS

We are grateful to Dr. Serradeil-Le-Gal from Sanofi-Aventis for kindly providing satavaptan
The constitutively active V2 receptor mutants conferring NSIAD are weakly sensitive to agonist and antagonist regulation. PLoS ONE 4: e8383, 2009

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.20120100777/DCSupplemental.
**Supplemental Materials and Methods:**

Chemicals were obtained from Sigma-Aldrich, unless specified otherwise. Satavaptan was kindly provided by Sanofi-Aventis (Toulouse, France) while tolvaptan was a gift from Ostuka Pharmaceutical (Tokyo, Japan).

**Clinical Data**

This is a retrospective review of the clinical phenotypes of a single individual affected with nephrogenic syndrome of inappropriate antidiuresis cared for by the authors.

**Constructs, Cell Culture and Transfections**

Expression plasmids coding for the wild-type myc-tagged V2R used in this study was previously described.¹ The myc-F229V-V2R, myc-R137C-V2R and myc-R137L-V2R were generated using the Quick Change mutation kit (Agilent Technologies, Santa-Clara, USA) using the manufacturer's protocol. YFP-tagged versions of the WT-, R137C- and R137L-receptors were previously described² while the F229V-V2R-YFP plasmid was generated from WT-V2R-YFP using the Quick Change mutation kit. The functionality of the YFP- and myc-tagged receptors was validated by radioligand binding and cAMP production assay. No difference in AVP binding affinity or potency to promote cAMP production were observed between the tagged and untagged wild-type receptors (see supplemental Table 1). The β-arrestin2-Rluc² and the dominant negative mutant of Dynamine2 (K44A)³ were also described. HEK293T or HEK293T/AP2-YFP³ cells were maintained in Dulbecco’s modified Eagles’s medium (DMEM; Wisent Bioproducts, Qc, Canada) supplemented with 10% fetal bovine serum (Wisent Bioproducts) in a 37°C humidified incubator with 5% CO₂ atmosphere. Transfections were performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer’s recommendations.
Confocal microscopy

HEK293T cells transfected with the YFP-tagged version of the different receptors studied were seeded on poly-D-lysine-coated glass bottom petri dishes (MatTek Corporation, Ashland MA) and fixed with paraformaldehyde prior to their observation under a Zeiss LSM 510 Laser Scanning Confocal Microscope (Jena, Germany) equipped with a 100x plan-Apo NA 1.4, DICIII objective and a 505 nm long pass emission filter. Excitation was performed with a 488 nm laser. Image acquisitions were performed with the LSM 510 Software, version 3.2, service pack 2.

Western blotting

Transiently transfected cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 1 mM PMSF) 48 hours post-transfection. Western blotting and SDS-PAGE were performed using standard protocols with the 9E10 anti-myc antibody (Santa-Cruz Biotechnology, CA) followed by an HRP-conjugated goat anti-mouse secondary antibody (GE healthcare, UK). Westerns were revealed using the Western Lightning™ chemiluminescence substrate (Perkin Elmer, MA) and the LAS-3000 Imaging System (Fuji, Japan). Band quantifications were done using the Multi Gauge quantification software (Fuji). The level of exposure shown was chosen to allow the visualization of the mature form of the receptor, even for the poorly mature mutant forms of the receptor. The quantification was realized using exposures that remained within the linear range of quantification of the system and the data expressed as the ratio of mature/immature forms of the receptor. For each region quantified, the luminescence signal was averaged as a function of the size of the area and the corresponding background subtracted.

Cell surface ELISA.

Cell surface receptor expression was assessed as described previously.² Briefly, HEK293T cells were seeded in 6-well plates (4x10⁵ cells/well) and transfected with the indicated receptors constructs the next day. 48h following transfection, cells were fixed with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl,
pH7.4) containing 3.7% formaldehyde for 5 min at room temperature, washed with TBS and blocked with 1% BSA-containing TBS (TBS-B). The blocking buffer was then replaced with the 9E10 mouse monoclonal anti-myc antibody diluted in TBS-B, washed again and incubated with an alkaline phosphatase-conjugated anti-mouse antibody (BIO-RAD). After washing, cell surface expression was determined by the colorimetric reaction initiated by the addition of the Alkaline phosphatase conjugate substrate kit (BIO-RAD) which was stopped by adding NaOH to the wells. The intensity of the reaction was quantified using a OASYS UVM340 microplate spectrophotometer (Montreal Biotech inc. Montreal, Canada) at 405 nm. Net surface expression was determined by subtracting the absorbance values obtained with the mock transfected wells. All the results were generated in triplicates.

**cAMP measurements**

cAMP measurements were performed using either a CRE-luciferase gene reporter assay or the EPAC-BRET biosensor as described below. For the CRE-luciferase assay, HEK293T cells were transfected with the corresponding myc-tagged receptor construct along with the pCRE-Luc vector purchased from Clontech (Mountain View, CA) and the pRL-CMV plasmid (Promega Corporation, Madison, WI). Three hours after transfection, cells were exposed to satavaptan, tolvaptan or vehicle and incubated for 16 hours at 37°C. Cells were then lysed in lysis buffer, and 10 µL of the lysates were distributed in a white opaque 96-well plate (Perkin Elmer, Waltham, MA). Luciferase activities were measured using the Veritas luminometer from Promega by injecting the Firefly luciferase substrate (D-luciferin, Biotium, Hayward, CA) or the Renilla luciferase substrate (Coelenterazin h, Prolume, Pinetop, AZ). Luminescent values obtained with the Firefly luciferase substrate (pCRE-Luc) were divided by the values obtained with the Renilla luciferase substrate (pRL-CMV) for normalization. For the EPAC-BRET biosensor, the myc-tagged WT-V2R or F229V-V2R were transfected in HEK293T cells along with the previously described biosensor construct. Following a 48-h incubation at 37°C, cells were transferred into 96-well plates at a density of 8 to 10x10⁴ cells per well in PBS. Vehicle, satavaptan (10 µM), tolvaptan (10 µM) or AVP (1 µM) were added along with the luciferase substrate DeepBlue C (Nanolight Technology, AZ, USA) and the plate was read repetitively for 20 min in a Mithras LB940 instrument (Berthold Technologies, Bad Wildbad,
Germany) using the MicroWin 2000 software (Berthold Technologies). For the dose-response curves, varying concentrations of either AVP or tolvaptan were added to the wells and the plate incubated at 37°C for 15 min prior to reading. The BRET signal is determined by calculating the ratio of the light emitted at 505-555 nm (YFP) over the light emitted at 465-505 nm (Luciferase). Experiments were performed in triplicates.

**β-arrestin2/receptor and β-arrestin2/AP2 interactions**

The interactions between β-arrestin2 and either V2R or AP2 were monitored by BRET, as previously described.\(^2\) For β-arrestin2-Rluc/receptor interaction, YFP-tagged constructs (WT-V2R-YFP, F229V-V2R-YFP and R137C-V2R-YFP) were co-transfected with the β-arrestin2-Rluc construct in HEK293T cells. After 48 h, cells were transferred into 96-well plates at a density of 8 to 10\(\times\)10\(^4\) cells per well in PBS and exposed (or not) to 1 \(\mu\)M AVP for 15 min prior to BRET reading. For β-arrestin2-Rluc/AP2-YFP interaction, HEK293T cells stably expressing the AP2-YFP construct\(^3\) were co-transfected with β-arrestin2-Rluc along with the indicated myc-tagged receptor construct, with or without the dominant negative Dynamin2 (DynK44A) construct.\(^3\) For both experiments, the Rluc substrate coelenterazin H (5 \(\mu\)M) was added 5 min prior to reading. BRET signal is determined by calculating the ratio of the light emitted at 505-555 nm (YFP) over the light emitted at 465-505 nm (Luciferase). Experiments were performed in triplicates.

**Binding experiments**

Radioligand binding experiments were performed on HEK293T or HEK293-AP2 cells transfected with plasmids encoding the specified receptor constructs. 24 h after transfection, cells were transferred into poly-D-lysine-coated 24-well culture plates. The next day, each well was first rinsed with ice-cold washing buffer (PBS containing 2% glucose and 1% BSA) prior to incubation of cells with increasing concentrations of \(^{[3]}\)Harginin vasopressin (64.2 Ci/mMol, Perkin Elmer, MA, USA) for 2 hours in binding buffer (0.1% glucose, 1% BSA, 1mM tyrosine, 1mM phenylalanine in PBS) on ice. For cell surface
receptor number determination, a saturating concentration of \([{}^3\text{H}]\)arginin vasopressin (40 \(\mu\)M) was used. After the incubation period, the attached cells were washed three times with ice-cold washing buffer to remove unbound ligand. Cells were then lysed with 0.4 M NaOH. The radioactivity was determined by scintillation counting. Specific binding was determined by subtracting the counts obtained with the mock-transfected cells. Each data point was performed in triplicates.

**Receptor modeling**

To provide a structural basis for constitutive activation in AVPR2, we used a *de novo* structure prediction methodology called GEnSeMBLE (GPCR Ensemble of Structures in Membrane BiLayer Environment)\(^5\) to predict the most stable conformations for the wild-type (WT) receptor and two mutants (F229V and R137C). The methodology samples all possible seven transmembrane (TM) helix bundle conformations in the local conformational space of helix orientations in the membrane (\(~6\) billion conformations in the current case for each of the three receptor forms) using a fast but efficient BiHelix procedure.\(^6\) This sampling procedure splits up the large 7-helix bundle conformational sampling into complete 2-helix conformational samplings involving nearest-neighbour helices, constructs the 7-helix bundle energies by adding up all the 2-helix energies appropriately, and ranks the resulting 7-helix conformations by energy. The top 2000 conformations (out of \(~6\) billion) are reranked using a more accurate energy by explicitly building the 7-helix bundles and optimizing the sidechains. The method has been used successfully to predict the structures of many GPCRs.\(^7,8\)

**References:**


receptor substitutions (R137H/C/L) leading to nephrogenic diabetes insipidus and nephrogenic syndrome of inappropriate antidiuresis: implications for treatments. *Mol Pharmacol* 77: 836-45, 2010


Table S1: Effect of YFP and myc tags addition on the functionality of the WT-V2R. AVP dose-response curve were performed on HEK293T cells transfected with either the untagged, YFP-tagged or myc-tagged version of the WT-V2R to determine the EC\textsubscript{50} of cAMP production using the EPAC BRET biosensor. \textsuperscript{3}H-AVP saturation binding experiments were also performed to determine the affinity of AVP for the different receptor species. Experimental procedures for both experiments are described in the detailed method section.

<table>
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<th>Receptor</th>
<th>Log EC\textsubscript{50} (M)</th>
<th>KD (nM)</th>
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<td>20.4 +/- 1.92</td>
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Supplementary Figure 1

Figure S1: Cytoplasmic view of the lowest energy structure for the WT, R137C and F229V-V2R. Residues 137 and 229 are shown in licorice representation; all others are shown in ball and stick representation. A) WT-V2R: The R137 (3.50) residue on TM3 forms an intra-helical hydrogen bond with T134. This arginine usually forms a salt bridge with Glu/Asp (6.30 residue) on the TM6 in many class A GPCRs, which is V266 in the AVPR2 receptor. Thus, the classical ionic lock believed to maintain GPCRs in their inactive state does not exist in the V2R. On the cytoplasmic side, TM3 is coupled to TMs 5 and 6 mainly through hydrophobic interactions. TMs 5 and 6 are linked through a hydrogen bond between T205 (TM5) and W296 (TM6). The interaction triangle on the right of Figure S1A shows the cytoplasmic facing coupling between TMs 3, 5, and 6. B) R137C: The cytoplasmic end of TM5 moves closer to TM6 (relative to WT) forming a polar interaction between H233 (TM5) and K268 (TM6). The lack of R137 leads to a cytoplasmic uncoupling of TM3-TM6 interaction as shown in the interaction triangle on the right of Figure S1B. This uncoupling is caused by an inward movement of TM2, as the salt bridge interaction between E40 on TM1 and K100 on TM2 seen in WT is lost in R137C mutant. C) F229V: The cytoplasmic end of TM6 moves away from TMs 3 and 5. The D136 residue, which forms an intra-helical interaction with R139 residue (one helix turn below D136) in the WT, R137C, and F229V structures, also forms an inter-helical salt bridge interaction with R158 (TM4). D) Superposed ribbon structures of WT-, R137C-, and F229V-V2R. The major movements that occur in the F229V involves TM6 getting uncoupled from both TM3 and TM5 whereas for R137C, the uncoupling of TM6 from TM3 is not sufficient to lead to a major movement of these helixes. However a significant inward movement of TM2 is observed compared to both WT and F229V-V2R. Both R137C and F229V mutations result in an inward movement of TM5 compared to WT-V2R. The active structures of a few GPCRs have been crystallized and suggest that the motion of TM6 plays a major role in activation.1 The predicted structures of the mutant AVPR2 receptors are consistent with this observation. In addition, the full uncoupling of TM6 from TM3 and TM5 and large outward movement of TM6 in the F229V mutant can potentially explain the high constitutive activity of the F229V mutant. The potential roles of the TM5 and TM2 movements in the constitutive activation profiles remain to be investigated, as is the role of slight outward movement of TM7 for R137C mutant in any beta-arrestin activity.

Reference: