Intracellular activation of vasopressin V2 receptor mutants in nephrogenic diabetes insipidus by nonpeptide agonists

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Binding of the peptide hormone vasopressin to its type-2 receptor (V2R) in kidney triggers a cAMP-mediated translocation of Aquaporin-2 water channels to the apical membrane, resulting in water reabsorption and thereby preventing dehydration. Mutations in the V2R gene lead to Nephrogenic Diabetes Insipidus (NDI), a disorder in which this process is disturbed, because the encoded, often intrinsically functional mutant V2R receptors are misfolded and retained in the endoplasmic reticulum (ER). Since plasma membrane expression is thought to be essential for V2R activation, cell permeable V2R antagonists have been used to induce maturation and rescue cell surface expression of V2R mutants, after which they need to be displaced by vasopressin for activation. Here, however, we show that 3 novel nonpeptide V2R agonists, but not vasopressin, activate NDI-causing V2R mutants at their intracellular location, without changing their maturation and at a sufficient level to induce the translocation of aquaporin-2 to the apical membrane. Moreover, in contrast to plasma membrane V2R, degradation of intracellular V2R mutants is not increased by their activation. Our data reveal that G protein-coupled receptors (GPCRs) normally active at the plasma membrane can be activated intracellularly and that intracellular activation does not induce their degradation; the data also indicate that nonpeptide agonists constitute highly promising therapeutics for diseases caused by misfolded GPCRs in general, and NDI in particular.

Introduction

GPCR | pharmacological chaperones | signaling | ER retention | osmoregulation

In mammals, G protein-coupled receptors (GPCRs) form the largest family of plasma membrane receptors that transduce information from hundreds of extracellular signals to intracellular messengers. Not surprisingly, mutations in GPCR genes are the cause of many genetic diseases, including retinitis pigmentosa (1), hypogonadotropic hypogonadism (2), obesity (3), hypothyroidism (4), and X-linked nephrogenic diabetes insipidus (NDI) (5). Disease-causing mutations in GPCR genes can be divided in 5 classes, based on their cell-biological fates (6). Class II comprises more than 40% of gene mutations and causes so-called “conformational diseases,” which lead to misfolded receptors that are recognized and retained by the quality control system of the endoplasmic reticulum (ER). Often, these proteins are targeted for degradation by the proteasome (7).

The vasopressin type-2 receptor (V2R) is a prototype GPCR in such studies. In states of hypernatremia or hypovolemia, arginine-vasopressin (AVP) binding to the V2R of renal collecting duct principal cells induces a cAMP-signaling cascade, resulting in translocation of aquaporin-2 (AQP2) water channels to the apical membrane and water reabsorption from pro-urine (antiuresis). Patients with V2R gene mutations suffer from X-linked congenital NDI, a disease in which the antidiuretic response to AVP is lacking, leading to polyuria and polydipsia, with the risk of severe dehydration, especially in infancy. In cell-culture studies, nearly all missense V2R mutants in NDI appear misfolded, ER-retained, and unstable. However, many of these V2R mutants appear intrinsically-functional, because they exert a vasopressin-induced cAMP response when, due to over expression, some V2R mutants are expressed in the plasma membrane (8). Therefore, the inability of intracellularly-retained V2R mutants to escape the ER quality control and traffic to the basolateral membrane is fundamental to the disease.

Like many other GPCRs, V2R is thought to function only when expressed in the plasma membrane. Therefore, to work toward a putative therapeutic treatment of NDI, nonpeptide V1R and V2R antagonists have been used, which can interact with and stabilize ER-retained V2R mutants and to rescue their cell surface expression. There, they could be activated following incubation with and displacement by high concentrations of dDAVP (9–12).

Interestingly, however, the pharmaceutical industry has recently developed nonpeptide V2R-specific agonists because these drugs can be orally administered to treat incontinence and bed wetting. Since with oral administration, these drugs have to pass the intestinal cell layer to be taken up in our vascular system, we reasoned that they must be able to pass cell membranes. If so, then they may also facilitate the transport of V2R mutants in NDI to the cell surface, where they can directly activate the mutant receptors instead of having the need for displacement by vasopressin as with antagonists.

Here, we tested how one recently developed and 2 novel V2R-specific nonpeptide agonists, but not the peptide analogue of AVP (dDAVP), induce V2R mutants in NDI to generate cAMP at their intracellular site and thereby restore the normal physiological response, i.e., the translocation of AQP2 to the apical membrane. As such, our data reveal that nonpeptide agonists are highly promising novel therapeutics to treat diseases due to misfolded GPCRs in general, and NDI due to V2R mutations in particular.

Results

Activation of the V2R Pathway by NonPeptide Agonists. The chemical structures of the recently developed nonpeptide agonist


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Many V2R Mutants in NDI Are Intrinsically Functional. With transient over-expression of V2R mutants in cells, a fraction of the ER-retained V2R mutants localizes to the plasma membrane, which can then be used to determine the intrinsic functionality (i.e., ability to bind agonist and induce a cAMP response) of ER-retained V2R mutants. To test this for V2R mutants in NDI, GFP-tagged forms of WT-V2R, 6 V2R mutants associated with NDI (L44P, Y128S, I130F, S167T, Y280C, P322S), and the nonfunctional mutant V2R-S167L (8) were over-expressed in COS cells and stimulated with 1 μM dDAVP or 1 μM VA88, VA98, or OPC51. With all compounds, cAMP levels were significantly increased for WT-V2R (5–6-fold; P < 0.01) and all V2R mutants (3–4-fold; P < 0.01), except V2R-S167L (shown for dDAVP [Fig. 1A] and VA88 [Fig. 1B]). The variations in response between V2R mutants are likely intrinsic to the mutant proteins, because immunoblotting revealed similar expression levels for WT-V2R and its mutants (Fig. 1A and B). These data show that 6 out of 7 V2R mutants in NDI are intrinsically functional.

Subcellular Localization of V2R Mutants in Polarized MDCK Cells. Next, we determined the localization of V2R mutants in NDI. In vivo, the V2R is present in the basolateral membrane of polarized collecting duct cells and expressed at low levels. As these conditions are nicely mimicked in stably transfected MDCK-V2R cells ((14) and Fig. S2A), the V2R mutants were also expressed in MDCK cells. In contrast to WT-V2R, the V2R mutants -L44P, -I130F, -S167T, -Y128S, -Y280C, and -P322S are misfolded and mainly retained in the ER, as they colocalize to a great extent with the ER-marker Protein Disulfide Isomerase (PDI; Fig. S2A).

Glycosylated membrane proteins en route to the plasma membrane change their high-mannose glycosylation, added to them in the ER lumen, for complex glycosylation as they pass the medial-trans Golgi compartment (16). While high-mannose glycosylated V2R are 60 to 62 kDa, mature receptors are 75 kDa and resistant to endoglycosidase H (endo H), while they remain sensitive to protein N-glycosidase F (PNGase F). WT-V2R in MDCK cells is expressed as a mature 75 kDa protein, because it is insensitive to endoH and reduced to its O-glycosylated form of 65 kDa with PNGase F digestion (Fig. 2A). In contrast, the V2R mutants are expressed as 62 kDa immature proteins that are quantitatively equally sensitive to digestion with EndoH and PNGase F (Fig. 2A), indicative of localization in the early secretory pathway.

To investigate further whether V2R mutants were essentially absent from the plasma membrane, cells expressing WT-V2R or its L44P and P322S mutants were subjected to cell surface biotinylation. As shown in Fig. 2B, only mature WT-V2R was detected at the cell surface, whereas no immature receptors were detected for either WT or the mutants.

To analyze the subcellular localization of the V2R mutants in more detail, EM analysis was performed on empty MDCK cells or cells expressing WT-V2R, V2R-P322S, -L44P, or -Y128S (Fig. S2B) and signals were quantified for WT-V2R and V2R-P322S (Table 1). Because the ER in MDCK cells is fragmented and therefore not easy to discern, MDCK cells expressing WT-V2R or V2R-P322S were double labeled for the ER-marker PDI and V2R (Fig. S2C). Consistent with LM studies (14), WT-V2R is expressed as 62 kDa immature proteins that are quantitatively equally sensitive to digestion with EndoH and PNGase F (Fig. 2A), indicative of localization in the early secretory pathway.

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mainly present in the plasma membrane and endosomes/lysosomes. In contrast, V2R-P322S localizes predominantly to the endoplasmic reticulum and Golgi region. However, low levels of mutant receptors were also found in endosomes and in the plasma membrane (Table 1). These data reveal that in MDCK cells, V2R mutants associated with NDI are expressed in an immature form and are mainly retained in the ER and pre-Golgi compartments, although a small fraction of V2R mutant is found in post-Golgi compartments, including the plasma membrane.

Nonpeptide Agonists, but Not dDAVP, Rescue V2R Mutants’ Functioning. Because WT-V2R and its mutants had clearly different subcellular localizations and the mutants were essentially absent from the plasma membrane, we determined the ability of the nonpeptide agonists to generate a cAMP response in our V2R mutant-expressing MDCK cell lines. Incubation with the Emax concentrations of dDAVP (100 nM) or the nonpeptide agonists (1 μM; Fig. 1B) for only 10 min induced an approx. 7–9-fold over basal cAMP response in MDCK-V2R cells, whereas no effect was observed in mock-transfected MDCK cells (Fig. 3). Administration of 100 nM dDAVP did not induce a significant cAMP response in cells expressing the V2R mutants. However, incubation with 1 μM VA88, VA89, or OPC51, significantly increased cAMP in MDCK cells expressing V2R-L44P, -Y280C, -P322S, (Fig. 3; P < 0.01), -I130F, and -S167T (data not shown). Similar cAMP data were obtained when the cells were incubated with the agonists while cell surface V2Rs were continuously blocked with a peptidic V2R antagonist (not shown). These data indicated that nonpeptide agonists, but not dDAVP, can activate intracellularly-retained V2R mutants in NDI.

Nonpeptide Agonists Do Not Affect the Localization or Maturation of V2R Mutants. Nonpeptide antagonists induce the maturation and trafficking of V2R mutants from the ER to the basolateral membrane (10–12, 17). Moreover, activation of WT-V2R by AVP induces its internalization to lysosomes (14). Because the nonpeptide agonists activate the ER-retained V2R mutants, we investigated their effects on the localization and maturation of these mutants. For this, WT and mutant V2R-expressing MDCK cells were treated for 16 h with 100 nM dDAVP, or 1 μM of the nonpeptide agonists or the CPA antagonist SR1. Subsequent CLSM analysis revealed that none of the 3 nonpeptide agonists nor dDAVP changed the ER localization of V2R-L44P or -Y280C, -P322S, or -Y128S (Fig. 4A and 4B). In contrast and as reported (11), SR1 clearly increased the basolateral membrane localization of these mutants. However, WT-V2R was internalized from the basolateral membrane by dDAVP and the nonpeptide agonists, but not by SR1 (Fig. 4A Bottom and 4B, S3). This shows that the unchanged localization of the V2R mutants with the nonpeptide agonists was not due to the absence of agonist activity of the compounds.

Because trafficking of V2R-GFP to the Golgi complex and beyond, but also of V2R mutants when incubated with SR1, increases its mass from 62 to 75 kDa (11), cells treated as above were also subjected to immunoblot analyses. Consistent with our CLSM data, none of the nonpeptide agonists induced maturation of the V2R mutants, as only the immature 62 kDa form was detected. In contrast, incubation with SR1 shifted the mass of the V2R mutants from 62 to 75 kDa (Fig. 4B; shown for V2R-L44P, -Y128S, and -P322S). For WT-V2R, treatment with VA88, VA89, or OPC51 did not significantly affect the level of maturation. In line with the agonistic activity, however, they caused an increase of a 47 kDa degradation product for V2R-GFP (Fig. 4B), which was also observed in LLC-PK1-V2R cells treated with dDAVP (18). Together, these data indicate that activation by nonpeptide agonists does not change the localization or maturation of the V2R mutants.

Nonpeptide Agonist Effects on V2R Expression and Stability. Nonpeptide antagonists stabilize the expression of intracellularly-retained V2R mutants in NDI and promote their maturation (10, 11), whereas activation of WT-V2R by agonists induces its lysosomal degradation (14, 18). As the nonpeptide agonists activate the V2R mutants, but do not induce their maturation, we investigated their effects on V2R mutant expression and stability. Semiquantification of the signals in Fig. 4B revealed that 16 h treatment with any of the 3 nonpeptide agonists did not significantly affect the expression levels of the 62 and 75 kDa forms of WT-V2R, but did increase the signal of its 47 kDa degradation product (P < 0.01). Moreover, treatment with the agonists did not significantly change the V2R-L44P or V2R-P322S expression levels (Fig. 4B), nor those of V2R-I130F, -S167T, and -Y280C. Only for V2R-Y128S, all 3 compounds significantly (P < 0.03) increased the expression of this mutant (Fig. 4B).

The unchanged expression levels observed in Fig. 4B are a balance between V2R synthesis and degradation, which may

Table 1. Electron microscopic analysis of cells expressing GFP-tagged WT-V2R or V2R-P322S

<table>
<thead>
<tr>
<th>Compartment</th>
<th>WT V2R</th>
<th>V2R-P322S</th>
</tr>
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<tbody>
<tr>
<td>ER</td>
<td>5 ± 1.2</td>
<td>36 ± 3.5</td>
</tr>
<tr>
<td>Golgi region</td>
<td>2 ± 0.3</td>
<td>21 ± 1.8</td>
</tr>
<tr>
<td>Golgi stack</td>
<td>2 ± 0.4</td>
<td>9 ± 1.4</td>
</tr>
<tr>
<td>PM</td>
<td>53 ± 1.8</td>
<td>10 ± 1.1</td>
</tr>
<tr>
<td>Endosomes</td>
<td>31 ± 2.7</td>
<td>17 ± 2.3</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>6 ± 1.8</td>
<td>2 ± 0.7</td>
</tr>
<tr>
<td>Undefined</td>
<td>1 ± 0.2</td>
<td>6 ± 0.7</td>
</tr>
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Numbers indicate the percentages of total labeling for the indicated compartments. Two different grids were scanned resulting in 3,123 gold particles for WT V2R and 2,251 for V2R-P322S. ER, endoplasmic reticulum; PM, plasma membrane.
both be increased with the agonists. As a lack of V2R mutant degradation with nonpeptide agonists activation may be clinically beneficial, we tested whether the agonists increase degradation of the ER-retained mutants. Cells were treated with the nonpeptide agonists for 6 h in the absence or presence of the protein synthesis inhibitor cycloheximide. Subsequent immunoblotting revealed that with cycloheximide, immature WT-V2R expression markedly decreased, mature V2R expression slightly decreased, and expression of the 47 kDa degradation product remained unchanged (Fig. 4C), indicating general degradation. Cotreatment with the agonists VA88, VA89, or OPC51, however, further decreased the 62 and 75 kDa signals and increased the 47 kDa signal. Together with their localization in late endosomes/lysosomes (Fig. 4A), this indicates that the nonpeptide agonists induce the lysosomal degradation of WT V2R.

Analysis of the V2R mutants showed that also their general expression was reduced by cycloheximide. Coincubation with any of the agonists, however, did not lead to a further decrease of their expression, nor did the 47 kDa signal appear (Fig. 4C). This indicated that, although the nonpeptide agonists activate the retained V2R mutants, they do not induce their degradation.

**Activation of Retained V2R Mutants by Nonpeptide Agonists Induces the Translocation of AQP2 to the Apical Membrane.** To relieve NDI in patients, the cAMP response generated from V2R mutants upon activation by nonpeptide agonists should be sufficient to activate PKA and allow re-distribution of AQP2 to the plasma membrane. To test this in vitro, MDCK-V2R-L44P cells were infected with recombinant AQP2 lentiviruses, treated with agonists for 2 h, and analyzed by immunocytochemistry. In line with the NDI phenotype, AQP2 localizes to intracellular vesicles in MDCK-V2R-L44P cells treated with 100 nM dDAVP (Fig. 5A).

**Upper**). In contrast, coinubcation with the adenylate cyclase activator forskolin, or 1 μM of the nonpeptide agonists VA89 (Fig. 5A), VA89, or OPC51 (Fig. S4) induced redistribution of AQP2 from vesicles to the apical and, to some extent, basolateral region of the cells. Note the absence of AQP2 redistribution in VA89-treated cells lacking V2R-L44P (indicated by arrows). To determine whether V2R-L44P activation actually resulted in AQP2 redistribution into the apical membrane, MDCK-V2R-L44P and MDCK-V2R control cells were treated as above and subjected to apical cell surface biotinylation. Consistent with the immunocytochemical data, AQP2 immunoblotting of the apical cell surface proteins from MDCK-V2R-L44P cells revealed a significant increase in apical membrane AQP2 following treatment with forskolin or any of the nonpeptide agonists, but not with dDAVP (Fig. 5B and C). In contrast, in MDCK-V2R cells, a significantly increased apical membrane expression of AQP2 was observed with all agonists, including dDAVP. These differences were not due to variations in AQP2 expression levels, because these were similar (Fig. 5B, lysates). These data indicate that cAMP generated by the intracellular activation of V2R-L44P is sufficient to induce redistribution of AQP2 from vesicles to the apical membrane.
Discussion

Nonpeptide Agonists Activate Intracellularly-Retained Mutant V2R Proteins in NDI. One known and 2 novel nonpeptide V2R agonists showed all of the characteristics of V2R agonists, because they activated V2R to generate cAMP, caused internalization of V2R-GFP following binding, and induced AQP2 synthesis and redistribution from vesicles to the apical membrane (Fig. S1, Fig. 4 and 5).

The general concept that activation of GPCRs activated by cell-permeable ligands needs to occur at the plasma membrane is challenged by our data with these nonpeptide V2R agonists: While 6 out of 7 V2R mutants tested were intrinsically functional receptors (Fig. 1 A and B), MDCK cells stably expressing these 6 functional V2R mutants were unresponsive to the cell-impermeable dDAVP (Fig. 3). Moreover, all V2R mutants tested mainly localize to the ER-Golgi complex (Fig. S2; Table 1). Apparently, the low levels of V2R mutant detected in the plasma membrane (Table 1) are too low to induce a detectable response. In contrast to dDAVP, however, all 3 nonpeptide agonists induced a significant cAMP response with MDCK cells expressing the 6 functional V2R mutants, (Fig. 3), even when blocking the plasma membrane V2R. Because dDAVP and nonpeptide agonists concentrations were used at equimolar concentrations, the difference in effect is not due to higher activity of the nonpeptide agonists, but most likely due to a higher propensity of the nonpeptide agonists to penetrate the cell membrane. As such, our data reveal that a GPCR that normally functions at the cell membrane can generate its corresponding signaling cascade from an intracellular location. Moreover, nonpeptide agonists can activate intrinsically-functional but disease-causing V2R mutants from these intracellular sites.

While the functional data above are clear, the lack of signaling from plasma membrane V2R-P322S is surprising. The most likely explanation is the low number of mutant receptors in the plasma membrane. Indeed, while 10% localizes in the plasma membrane, the total abundance of V2R-P322S in our EM analysis was considerably less than of WT-V2R (Table 1), which can be attributed to their reduced stability (8). Alternatively, plasma membrane V2R-P322S may signal less efficient than WT-V2R.

Recent observations that the β2-adrenergic receptor assembles with Gαs and adenylate cyclase in the ER (19, 20) and that the naturally cell permeable agonist estrogen induces signaling from the ER-localized GPR30 (21, 22) indicate that nonpeptide agonists may activate the V2R mutants to generate cAMP while located in the ER. However, considering the expression of some V2R mutants beyond the ER-Golgi complex, this remains to be established.

Intracellular Activation of V2R Mutants in NDI Does Not Change Their Localization or Stability. Even after prolonged incubation, none of the nonpeptide agonists induce any appreciable shift in localization of the V2R mutants, nor change their state of maturation (Fig. 4). In contrast, the nonpeptide V2R antagonist SR1 clearly increased V2R mutant expression at the cell surface and induced maturation (Fig. 4)(10–12, 17). The absence of translocation of V2R mutants to the cell surface is further supported by the fact that cAMP responses are obtained within 10 min following agonists addition, whereas it takes V2R antagonists 4–8 h to insert V2R mutants in the plasma membrane (11, 23). Our data are different from those of Petaja-Repo et al. (24), who found that nonpeptide agonists were able to stabilize the expression and maturation of ER-retained wild-type δ-opioid receptor (DOR). This indicates that nonpeptide agonist-induced maturation and rescue of cell surface expression is receptor-, mutation- and/or agonist-specific. Since in our study all V2R mutants tested remained unaffected in their localization and maturation, a difference in receptor is most the most likely explanation.

At present, it is unknown whether intracellular receptors are desensitized similar to their cell surface counterparts upon activation. While agonist-induced lysosomal targeting and degradation reveals that WT-V2R is down-regulated in MDCK cells, nonpeptide agonists did not increase degradation of the intracellularly-retained V2R mutants in NDI (Fig. 4). It remains to be established which step of the normal desensitization steps is abrogated with the mutant V2R proteins and whether this is generally observed for intracellular GPCRs.

High Mannose-Glycosylated V2R Mutants Are Retained in, but Not Confined to, the ER-Early-Golgi Compartment. A paradigm is that integral membrane proteins on their way to the plasma membrane exchange their high-mannose (ER) glycosylation for complex-glycosylated moieties when passing through the Golgi complex. However, although all intracellularly-retained V2R mutants in NDI localize to a great extent in the early secretory pathway (Table 1), are only high-mannose glycosylated, and are equally sensitive to endoH and PNGaseF digestion (Fig. 2A), our EM analysis revealed that a considerable pool of V2R-P322S localized to organelles beyond this stage. In transient expression studies (e.g., Fig. 1), non- or high-mannose glycosylated membrane proteins beyond the Golgi complex are commonly found, which is attributed to the inability of the ER quality control system to retain the high number of membrane proteins synthesized. This, however, does not explain the observation in our stably-transfected MDCK cells, because WT/V2R is fully matured (Fig. 2) and expressed at a higher level than the V2R mutants.

Interestingly, of several other membrane proteins, including the cystic fibrosis transmembrane conductance regulator (CFTR), protein phosphatase CD45, and the epithelial sodium channel (ENaC), post-Golgi expression of immature forms have been found for endogenously expressed proteins or in stably-transfected cells (25–27). Possibly, low levels of proteins bypass the Golgi and may directly be delivered to post-Golgi compartments, or all proteins traffic to the plasma membrane via the conventional pathway, but a population of these proteins are resistant to posttranslational processing (26). In line with this, misfolding of the V2R mutants may affect proper exchange of sugar moieties in the Golgi complex, or a small fraction may bypass this organelle in our transfected MDCK cells. Our data do show, however, that expression of only the immature glycosylated form of a mutant membrane protein is an indicator of retention in the ER or early Golgi compartment, but does not necessarily exclude that a small fraction localizes beyond the Golgi complex. While it remains unknown whether GPCR mutants traffic beyond the Golgi complex in humans, trafficking of immature membrane proteins beyond the Golgi complex has been observed for endogenously expressed membrane proteins and may thus also apply to V2R mutants in vivo.

Potential Implications of the Use of V2R Nonpeptide Agonists for NDI Patients. The lack of V2R activation in NDI patients with V2R mutations causes reduces AQP2 expression levels and abrogates AQP2 translocation to the apical membrane in renal collecting duct principal cells (28). Our data indicate that the nonpeptide V2R agonists constitute highly promising therapeutics for the treatment of NDI resulting from intracellularly-retained V2R mutants. First, the nonpeptide agonists are unlikely to cause major side effects, because they are highly selective for V2R (29). Nevertheless, monitoring of the V2R-specific extra-renal release of coagulation factors will be essential in patients. Secondly, these agonists induce a cAMP response mediated by ER-retained V2R mutants (Fig. 3) and are able to induce the cAMP-dependent expression of endogenous AQP2 (Fig. 1C).
Thirdly, the cAMP response generated by the intracellularly activated V2R mutants appears sufficiently strong to mediate the critical step in renal water reabsorption, the trafficking of AQP2 proteins from vesicles to the plasma membrane (Fig. 5).

While administration of the V1AR antagonist SR49059 to patients was the first in vivo proof of principle that nonpeptide antagonists can relieve NDI in patients (17), the direct activation of functional ER-retained V2R mutants observed here indicates that treatment with nonpeptide V2R agonists is likely superior over nonpeptide antagonists because rescue of cell surface expression of the V2R mutants and subsequent displacement of the antagonists by endogenous AVP is circumvented. Considering the low stability of ER-retained folding mutants (8), the lack of increased V2R mutant degradation upon activation by nonpeptide agonists provides an additional advantage for their therapeutic use. Determination of their in vivo therapeutic value for NDI, however, awaits approval for their use and testing in patients, because mice lacking functional V2Rs die soon after birth (30) and OPC51803, VA (9990)88, and VA (9990)89-like compounds are still in early phase clinical trials. However, the observed decrease in urine output with OPC51803 in Brattleboro rats, which have functional V2Rs but lack AVP, already reveals the activity of nonpeptide V2R agonists on a normal functioning renal concentrating mechanism (29) and underscores the potential of nonpeptide V2R agonists as therapeutic agents for NDI patients due to retained, but intrinsically-functional, V2R mutants.

In conclusion, our data show that a GPCR, which normally functions in the plasma membrane, can generate a signaling cascade from an intracellular location to induce the receptor’s physiological response. Moreover, the activation of intracellular V2R mutants by our nonpeptide agonists does not lead to increased degradation. As approximately 40% of GPCR mutants in diseases are misfolded and retained intracellularly, our data indicate that misfolded agonists are ideal therapeutics to treat patients suffering from one of the many “conformational diseases” due to misfolded and retained GPCRs in general, and NDI due to misfolded V2R mutants in particular.

Materials and Methods

Ligands. The nonpeptide V2R antagonists VA999088 and VA999089 were kindly provided by Dr. Haigh (Vantia Ltd., Chilworth, U.K.) for kindly supplying VA88 and VA89 and Dr. Claudine Serradeil-Le Gal (Sanofi Synthelabo Research, Toulouse, France) for kindly supplying SR1. We thank Renee Srinivaneck and Marc van Peski for preparation of the electron micrographs. J.K. and P.M.T.D. are recipients of VICI grants 918.36.611 and 865.07.002 of the Netherlands Organization for Scientific Research. We acknowledge the Dutch Kidney Foundation (PC 104, C06.2164), RUNMC and Netherlands Organization for Scientific Research (865.07.002) and Coordination Theme 1 (Health) of the European Community’s 7th Framework Program (HEALTH-F2-2007-201590, entitled EUNEFRON).

Supporting Information

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SI Materials and Methods

Ligands. The nonpeptide V2R agonists VA999088 and VA999089 (patent WO01/29005) were kindly provided by Dr. Haigh (Vantia-tia), while OPC51803 (1) was kindly provided by Dr. Komuro (Otsuka Pharmaceutical Co. Ltd.). The V2R nonpeptide antagonist SR121463B (2) was provided by Dr. Serra-del-Le Gal (Sanofi Synthélabo Recherche). All compounds were dissolved in dimethyl sulfoxide as 0.01 M stock solutions and diluted in culture medium as indicated. dDAVP and the peptidic antagonist [Adamantaneacetyl1, O-Et-D-Tyr2, Val4, and Arg8,9]-Vasopressin were from Sigma. For reading convenience, VA999088, VA999089, OPC51803, and SR121463B are abbreviated as VA88, VA89, OPC51 and SR1, respectively, which is, together with relevant characteristics, summarized in the Table S1.

Expression Constructs. Expression constructs encoding the WT V2R or the NDI causing mutants –L44P, -I130F, -S167T, or S167L fused at their C-termini to enhanced green fluorescent protein were as described (3). With site-directed mutagenesis (Stratagene), additional NDI-related mutations were introduced into the human V2R cDNA sequence, using the primers GTG-GGGCATGTAGCGTTCCTCTCATGATCTGG (Y128S), GTGCGTCGTCTGTTGCTGTGGGGCCCTCTTTGG (Y280C), and CCTCAACACGTCGAAATCTGATGATCATGCTATTGCAT-TAGC (P232S), and their complementary antisense primers on a pEGFP-N1-V2R template. After digesting correct clones with PstI/HindIII, the mutation-containing fragments were subcloned into the corresponding sites of pEGFP-N1-V2R. Sequence analysis confirmed that only the desired mutations were introduced.

Recombinant Lentivirus. To generate a recombinant lentivirus encoding AQP2, the AQP2 cDNA was cut from pBSII-KS (+)AQP2 using SpeI and XhoI, and cloned into the corresponding sites of pRRL-CMV-4 (Tronolab) to yield pRRL-CMV-AQP2. Subsequently, pRRL-CMV-AQP2 was digested with MluI, blunted, and cut with NheI. The 994 bp insert containing the entire AQP2 cDNA was cloned into the NheI, and the 5′-AQP2 in MDCK cells, 70–80% confluent cells were trypsinized and mixed with the PGK-AQP2 lentivirus at a multiplicity of infection of 2. Cells were then seeded on semipermeable filters at a density of 3 × 10⁵ cells/cm² in the presence of the lentivirus and incubated for 16 h at 37 °C. Subsequently, the medium was replaced with normal culture medium and the cells were grown for an additional 2 days, treated as indicated, and subjected to apical cell surface biotinylation or immunocytochemistry as described (6, 7).

Electron Microscopy. For immuno-electron microscopy, control and transfected MDCK cells were fixed in 4% formaldehyde and prepared for ultrathin cryosectioning as described (8). Immunogold labeling using anti-GFP antibodies (ABCAM, Cambridge, MA) was performed as described (9). Nontransfected MDCK cells did not show any significant label with the antiGFP antibody. For quantification, samples were selected for good overall morphology, after which counting was performed by randomly scanning the sample. Each encountered gold particle that was no further than 20 nm located from a membrane was attributed to the membrane to which it was associated.

Immunoblotting and Immunocytochemistry. Polyclonal antibody electrophoresis, Western blotting, immunodetection, and semi-quantification of signals were performed as described (6, 10). Immunocytochemistry, CLSM, and data quantification were performed as described (3). Rabbit-anti GFP (11) and rabbit-anti AQP2 (12) antibodies were obtained as described.

cAMP Measurements. Measurement of intracellular cAMP in transiently-transfected COS-M6 cells or MDCK cells stably-expressing V2R (mutants) was performed as described (3). Triplicate samples were measured, and experiments were performed at least in 3-fold. Data were analyzed using Graphpad Prism 4 (Graphpad Software Inc.).

Statistical Analysis. Statistical analysis was performed using student’s t-test. P-values < 0.05 were considered significant.


Fig. S1. Characterization of V2R agonists. (A) Chemical structures of (1) VA88, (2) VA89 and (3) OPC51. (B) Dose-response curves of dDAVP, VA88, and OPC51 on V2R. MDCK WT-V2R cells were treated with increasing amounts of dDAVP (squares), VA88 (triangles), or OPC51 (diamonds) for 10 min in the presence of 1 mM IBMX. Subsequently, cAMP levels were measured using a fluorometric competition immunoassay kit. (C) V2R agonist-induced expression of AQP2. MpkCCD cells were left untreated (C) or treated with 10 or 0.1 nM of the nonpeptide agonists or dDAVP for 4 days. Next, cells were lysed and immunoblotted for AQP2. Protein masses are indicated in kDa. (D) Apical membrane insertion of AQP2 by V2R agonists. MpkCCD cells were left untreated (C), or treated for 4 days with 1 nM dDAVP or the nonpeptide agonists. Next, cells were subjected to cell surface biotinylation and equalized total lysates and cell surface fractions were analyzed by immunoblotting using AQP2 antibodies. (E) In parallel, cells were treated with agonists as under (D) (control), incubated without ligand for 2 h (no ligand), followed by incubation with 100 nM dDAVP or 1 μM of the nonpeptide agonists for 1 h (1 h agonist). After each incubation point, cells were fixed, and immunocytochemically stained for AQP2. Representative cross-sections of the cells are shown.
Fig. S2. Intracellular retention of V2R mutants in NDI. (A) MDCK cells expressing the GFP-tagged WT-V2R, V2R-L44P, or V2R-Y128S were grown to confluence on filters. Subsequently, the cells were fixed, subjected to immunocytochemistry using anti-PDI antibodies to stain the endoplasmic reticulum (Left), and analyzed by CLSM. (B) MDCK cells expressing GFP-tagged WT-V2R (A), V2R-P322S (B), V2R-L44P (C), or V2R-Y128S (D) were processed for cryo-immunogold labeling using anti-GFP antibodies (10 nm gold particles). In WT-V2R expressing cells, a considerable amount of label is found in the microvilli (MV), whereas the nuclear envelope (NE) and Golgi (G) region are relatively devoid of labeling. Cells expressing V2R mutants display less label in the microvilli, whereas relatively more is seen in the NE and Golgi region. (Scale bar, 200 nm.) (C) Electron micrographs showing the occurrence of GFP-tagged WT-V2R (A) and V2R-P322S (B) in the endoplasmic reticulum (ER). Transfected MDCK cells were double-labeled for the ER marker PDI (10 nm gold particles) and GFP (15 nm gold). The label for PDI outlines the rather fragmented ER membranes in MDCK cells. Arrows point to ER cisternae containing GFP-tagged constructs of V2R-WT (A) or V2R-P322S (B). E = endosome; n = nucleus; NE = nuclear envelope; MV = microvilli. (Scale bars, 200 nm). For this labeling, mouse monoclonal antibodies against PDI (Stressgen) and rabbit anti-mouse antibody (Dakopatts) were used.
Fig. S3. Peptide and nonpeptide agonist action on the localization, maturation, and stability of V2R mutants in NDI. MDCK cells expressing GFP-tagged V2R-Y128S were grown to confluence on filters and subsequently left untreated, or treated with 100 nM of the peptide agonist dDAVP, 1 μM the nonpeptide agonists VA88, VA89, OPC51 or the nonpeptide antagonist SR1 for 16 h. Subsequently, cells were fixed and analyzed by CLSM.
Fig. S4. Agonist induced translocation of AQP2. MDCKII-V2R-L44P cells infected with a lentivirus containing an AQP2 expression cassette were incubated overnight with 50 μM indomethacin. (A) Then, cells were kept on indomethacin supplemented with 100 nM dDAVP, 10 μM forskolin (Forsk.), 1 μM VA88, or OPC51 for 2 h. Next, the cells were fixed, permeabilized and subjected to immunocytochemistry using rabbit anti-AQP2 antibodies and subjected to CLSM.
Table S1. Overview of the used compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>(Ant)agonist</th>
<th>(Non-)peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-desamino-8-D-AVP</td>
<td>dDAVP</td>
<td>Agonist</td>
<td>Peptide</td>
</tr>
<tr>
<td>VA999088</td>
<td>VA88</td>
<td>Agonist</td>
<td>Non-peptide</td>
</tr>
<tr>
<td>VA999089</td>
<td>VA89</td>
<td>Agonist</td>
<td>Non-peptide</td>
</tr>
<tr>
<td>OPCS1803</td>
<td>OPCS1</td>
<td>Agonist</td>
<td>Non-peptide</td>
</tr>
<tr>
<td>SR121463B</td>
<td>SR1</td>
<td>Antagonist</td>
<td>Non-peptide</td>
</tr>
</tbody>
</table>

The abbreviated name, ligand type, and (non-)peptidic nature of the compounds tested are summarized.