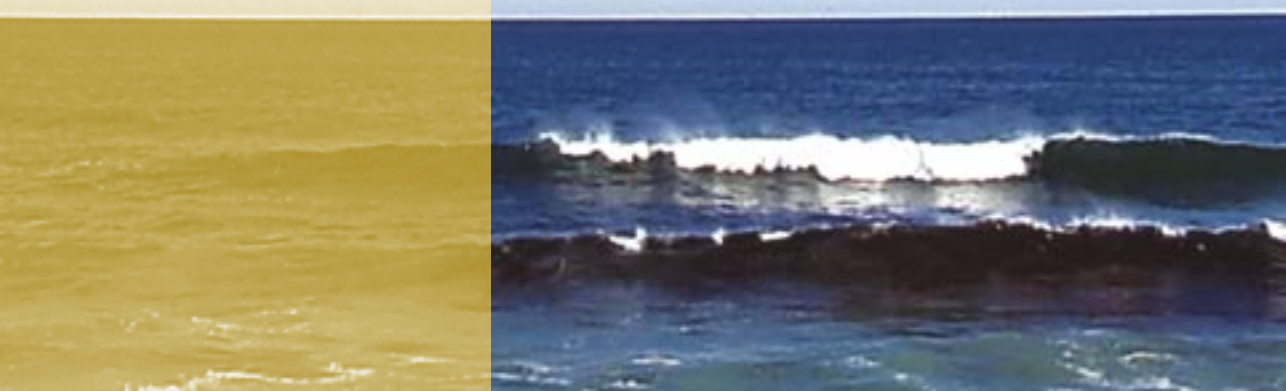
Functional rescue of mutant vasopressin V2 Receptors in Nephrogenic Diabetes Insipidus.

Proefschrift

From molecular cause to restored phenotype

Joris Hubertus Robben



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Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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Inhoudsopgave

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Chapter 1

General Introduction

Cell biological aspects of the vasopressin type-2 receptor and aquaporin-2 water channel in Nephrogenic Diabetes Insipidus

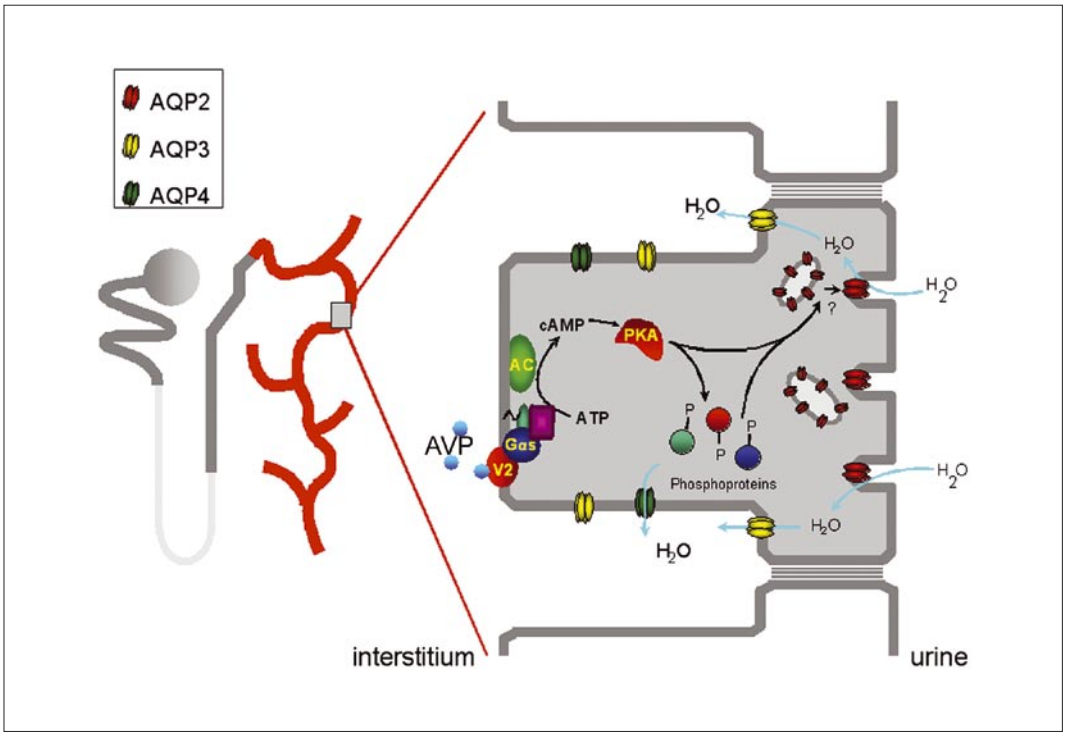

Figure 1-1 **Regulation of the Aquaporin-2 mediated water transport by vasopressin**
Nephron with magnified principal cell. In this cell, vasopressin (AVP), vasopressin V2 receptor (V2R), stimulatory GTP binding protein (Gαs), adenylate cyclise (AC), adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), and phosphorylated proteins (O-P) are indicated. For details, see text.

Introduction

Maintaining body water homeostasis is of vital importance for proper functioning of most physiological processes in the human body. Under normal conditions, the glomerular filtration rate (GFR) of the 2 kidneys amounts 180 litres/day. Of this huge volume, also called the pro-urine, approximately 90% is reabsorbed in the proximal tubule and descending limb of Henle's loop, which is a constitutive process. According to the body's demand, the remaining fluid can be reabsorbed in the collec-ting duct. This latter process is tightly regulated by an elegant system, which allows the body to adapt to periods of water load or restriction. The key hormone in this process is the antidiuretic hormone arginine-vasopressin (AVP), which is secreted by the posterior pituitary in response to states of hypernatremia or hypovolemia (48). In healthy individuals, secreted AVP will be transported by the blood to the kidney, where it can bind to vasopressin V2 receptors (V2R), which are mainly present on the basolateral (interstitial) side of the principal collecting duct cells (58). The activated V2R will induce an increase of intracellular cAMP levels via the stimulatory Gs protein and adenylate cyclase, which will

eventually lead to activation of protein kinase A and to phosphorylation of aquaporin-2 (AQP2) water channels (26; 37). Phosphorylation of at least three out of four monomers of an AQP2 tetramer is then sufficient to redistribute AQP2 homotetramers from storage vesicles to the apical membrane, rendering this plasma membrane permeable to water (35; 88). Following an osmotic gradient of sodium and urea, water will then pass the apical membrane via AQP2, and will leave the cells on the basolateral side via AQP3 and AQP4 (32; 38), thereby compensating the hypovolemic or hypernatremic state of the body. This process is summarized in figure 1. When sufficient water is reabsorbed to restore homeostasis, plasma vasopressin levels will decrease (48), and AQP2 will be internalized from the apical membrane, resulting in decreased water reabsorption. In addition to its direct regulation of water transport, AVP also increases sodium transport in the collecting duct via the epithelial sodium channel ENaC (6; 23) and urea transport via de UT-A1 transporter (78).

Malfunctioning of water reabsorption can lead to a variety of disorders, which can be of central or renal origin. Centrally, in the Syndrome of Inappropriate Secretion of Antidiuretic Hormone (SIADH), AVP levels are abnormally elevated, leading to excessive renal water reabsorption, which might result in

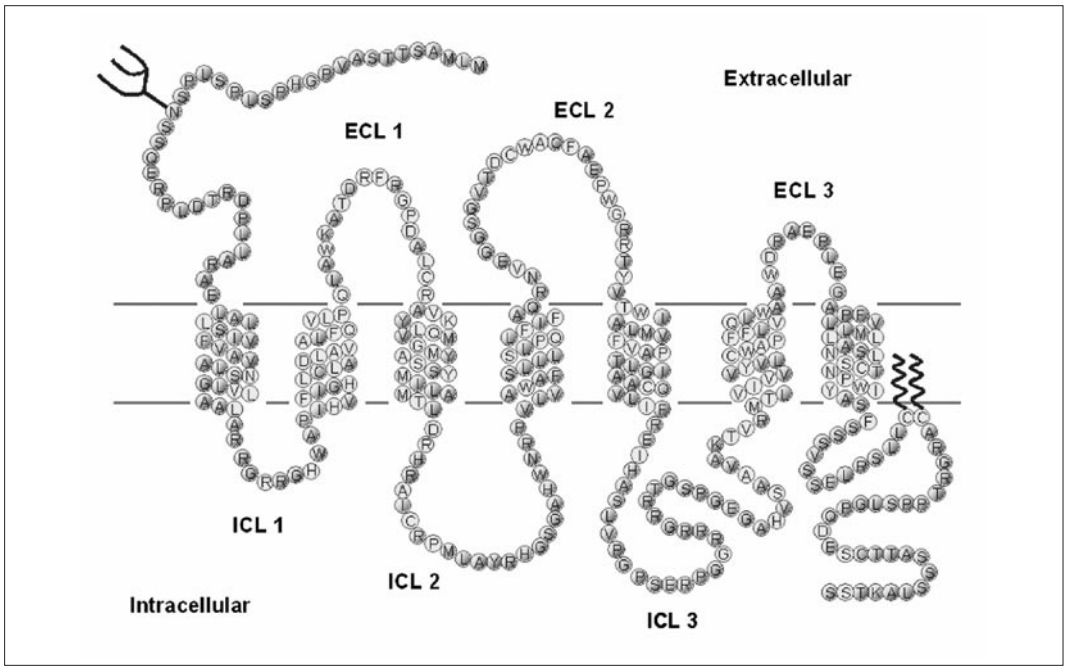

Figure 1-2 **Proposed topology of the human vasopressin V2 receptor**
The V2R has seven transmembrane domains with its N-terminus extracellular and its C-terminus intracellular. The transmembrane domains are connected by three extracellular (ECL1-3) and intracellular (ICL1-3) loops. The N-glycosylation site (at N22) in its N-terminus and the palmitoylated cysteines (C341-342) in its C-terminus are indicted. Bright amino acids are conserved between vasopressin V1, V2 and V3 receptors.

life-threatening hyponatremia (62). The most common causes of SIADH are neoplasia, like non-malignant lung carcinoma, neurological disorders, congestive heart failure, liver cirrhosis, pre-eclampsia, and drugs like thiazide diuretics or selective serotonin reuptake inhibitor antidepressants. These effects have recently been reviewed in detail and will not be discussed here (9; 57).

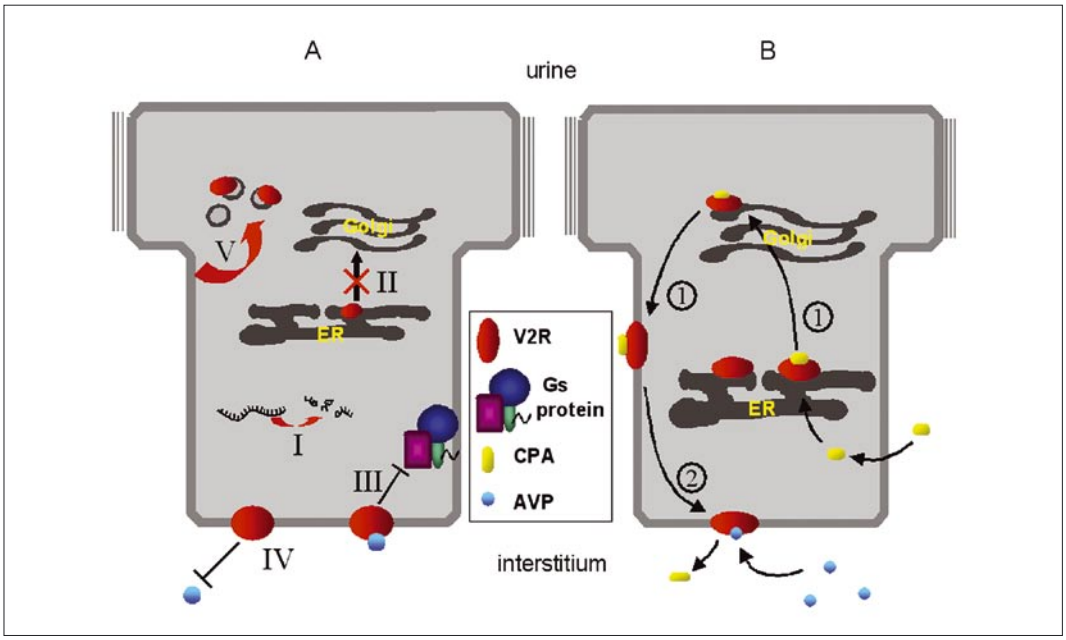

Figure 1-3

Cellular fate of vasopressin V2 receptor mutants in NDI and their rescue
A. V2R mutation classes in NDI.
Schematic view of a collecting duct cell. The different mutation classes are indicated in roman numbers. Mutation can lead to (I) mRNA instability, splicing errors, nonsense mutations; (II) ER retention; (III) impaired Gs coupling; (IV) impaired AVP binding; (V) mislocation in the cell. See text for details.

B. Pharmacological chaperones rescue class II V2R mutants
Class II V2R mutants are retained in the ER, but upon binding of cell permeable antagonists (CPA), stabilisation of their structure allows them (1) to exit the ER, mature in the Golgi and subsequent translocate to the plasma membrane. At the membrane, (2) displacement of the CPA by AVP will allow restoration of the cAMP cascade.

At the other extreme is Diabetes Insipidus (DI), in which patients are unable to concentrate their urine, resulting in polyuria and, consequently, polydipsia. In central DI (CDI), patients lack the ability to produce functional AVP, and therefore to actively concentrate their urine. Among the causes of CDI are brain carcinoma, local inflammatory, autoimmune or vascular diseases, trauma from surgery or accident and AVP gene mutations (2; 5; 25; 27; 68). Administration of the synthetic AVP homologue

1-desamino-8-D-arginine vasopressin (dDAVP), however, is usually able to drastically decrease urine output in these patients. A well-studied disturbance of water homeostasis is Nephrogenic Diabetes Insipidus (NDI). In this disorder, patients are unable to concentrate their urine, despite normal or increased serum AVP levels.

Congenital NDI

Autosomal recessive NDI. Congenital NDI can be divided in X-linked and autosomal recessive and dominant NDI. Approximately 10% of the patients diagnosed with NDI have mutations in the AQP2 gene, which is mapped to chromosome 12q13 (21; 77). Of these, >90% suffers from recessive NDI. Most, but not all (17; 21), of these patients are of consanguineous lineage and have inherited two identical mutant AQP2 alleles. In total, 34 AQP2 mutations have been described, of which 27 are involved in recessive NDI. Of the latter group, 78% comprises missense mutations. Upon expression of the encoded mutants in cell systems, nearly all mutants were misfolded and trapped in the endoplasmic reticulum (ER), followed by rapid proteasomal degradation (19; 20; 43; 46; 47; 55; 85). These AQP2 mutants are unable to form heterotetramers with wild-type AQP2 (wt-AQP2), leaving only the formation of wt-AQP2 homotetramers, which explains the healthy phenotype of the parents, who are heterozygous for the AQP2 mutation (36).

Autosomal dominant NDI. Being diagnosed in only seven families, autosomal dominant inheritance is the rarest form of NDI. By definition, the mutations in this form of NDI are only found in one AQP2 allele. As they are able to form heterotetramers with wt-AQP2 and alter its trafficking, mutants in dominant NDI interfere with the functioning of wt-AQP2. Indeed, upon expression in oocytes, these AQP2 mutants were not retained in the ER and were able to form heterotetramers with wt-AQP2 (36). Due to the mutation, however, these molecularly-functional AQP2 mutants were missorted and, because of the formation of heterotetrameric complexes with wt-AQP2, also targeted wt-AQP2 to other subcellular destinations. Extrapolated to the principal cells of the collecting duct of patients, this would lead to severely decreased amounts of AQP2 in the apical membrane, explaining the dominant mode of inheritance of NDI in their families (36).

X-linked NDI. The X-linked and most frequently occurring form of congenital NDI, and the main subject of this thesis, is caused by loss of function mutations in the AVPR2 gene, encoding the V2R (15; 44), which is a member of the family of G protein-coupled receptors (Figure 2). Over 180 AVPR2 gene mutations have been described (http://www.medicine.mcgill.ca/nephros), of which many result in severe interference with receptor signalling, thus making the principal cells of the collecting duct insensitive to AVP. The molecular mechanism underlying this insensitivity, however, differs between mutants. As upcoming pharmacological treatments for NDI likely depend on the underlying mechanism, we recently divided GPCR mutations in general and V2R mutations in particular in five different classes according to their cellular fate (18; 69) (Figure 3A and table 1).

Class I comprises all mutations that lead to improperly processed or unstable mRNA, like promoter alterations, or mutations that result in exon skipping or aberrant splicing. This class also holds frame shift and non-sense mutations, which result in truncated proteins like V2R-Q119X, -W293X and -R337X and –C358X (chapters 8 and 9).

Class II mutations are missense or in-frame insertions/deletions resulting in fully translated proteins. Due to the mutation, however, mutant receptors are misfolded and retained in the ER, as the ER is the organelle that has the cellular quality control over proper folding and maturation of synthesized proteins. Misfolded proteins are subsequently mostly targeted for proteasomal degradation (24). Intracellular entrapment of missense V2R mutants and their rapid degradation likely represents the most important cause of X-linked NDI, as more than 50% of the mutations in V2R are missense mutations and cellular expression revealed that most of these result in ER-retained proteins. The extent of ER retention, however, may differ between mutants, and may represent differences in their folding state. Hermosilla et al. recently reported that of eight V2R mutants that are retained, only three were strictly kept in the ER, whereas the five other mutants were transported to the ER-Golgi intermediate compartment, followed by retrograde transport to the ER (28).

Class III comprises similar mutations as those in class II, but the resulting mutants are not considered misfolded by the ER and can continue their trafficking to the plasma membrane. However, these mutations disturb binding of the stimulatory Gs protein, leading to a reduced activation of adenylate cyclase and thus formation of cAMP.

Class IV mutations also result in full-length receptors expressed at the cell surface, but here the mutation interferes with, or reduces, AVP binding. These mutations especially involve residues thought to be in or close to the AVP binding pocket; V2R-ΔR202 is a clear example (1).

Finally, class V mutations allow normal protein synthesis and maturation, but they cause misrouting to different organelles in the cell. The NDI R137H mutation, located in the well conserved DRY/H motif of GPCRs, is a member of this class, as V2R-R137H is constitutively internalized from the plasma membrane, and therefore only briefly available to bind AVP (8; 11).

Sometimes, mutants do not exert a full phenotype of a particular class and then often also show features of another class. For example, some V2R missense mutants are partially ER-retained (class II), but are also partially expressed in the plasma membrane (chapter 4), where they might show a reduced G protein coupling (class III) or AVP binding (class IV). As such, it provides an explanation for the observed small anti-diuretic response to high doses of dDAVP in NDI patients harbouring such mutations (66) (Table 1). As water transport is driven by an osmotic gradient, blood osmolalities and sodium levels are also important for a full interpretation of these increased urine osmolalities with dDAVP, but these have only been reported for a few patients (Table 1).

Nucleotide	Amino acid	Referred/analysis	Func.	Cons.(loc.)	Class	Diagnosis	Treatment
492T>C	L44P	(34; 65-69)	F	Y (tmd1)	II	1 wk	dD :NR
498T>A	I46K	(70)	F	N (tmd1)	II	5 yrs	ndD :NR
546T>C	L62P	(18; 68)	?	Y (tmd1)	II ?	21,53 mo	dD:NR
545-553del	Δ62-64	(34; 71)	G	N (tmd1)	II	?	?
574G>A	W71X	(72)	-	N (ICL1)	I	?	dD:NR
612C>A	A84D	(19)	A	N (tmd2)	II	?	?
614G>A	D85N	(70; 73; 74)	F	Y (tmd2)	III	32 mo	nD,dD-deh.:>400
623G>A	V88M	(68; 75; 76)	?	Y (tmd2)	II	16 mo	ndD:NR; deh.:>500, PS 158
692T>C	W99R	(19)	A	Y (ECL1)	II, IV	?	?
671C>T	R104C	(77; 78)	F	Y (ECL1)	II	8 d	ndD:NR; dD:>300; thiaz., dDAVP
674T>G	F105V	(70)	A	Y (ECL1)	IV	?	?
698C>T	R113W	(68; 69)	F	Y (tmd3)	II, IV	?	dD-deh.:NR
749A>T	I130F	(34; 70)	F	N (tmd3)	II	1 mo	ndD:NR
771G>A	R137H	(72; 73; 80-86)	G	Y (ICL2)	II, III, V	?	dD-deh.:NR (292); dD:>400 PO(282)
860T>A	S167T	(34; 51; 65; 66)	F	N (tmd4)	II	1 mo	dD:NR
861C>T	S167L	(34; 51; 52; 65; 66; 68)	A	N (tmd4)	II	2 wk, 8 mo	ndD,dD:NR
902C>T	R181C	(68; 71; 86-88)	A	N (tmd4)	IV	6 mo	ndD:NR
914G>T	G185C	(75; 89; 90)	A	N (ECL3)	IV	7,9,108,128,204 mo	dD-deh.: >300
963G>A	G201D	(34; 79)	F	Y (ECL3)	II, IV	?	dD-deh.:>400
965C>T	R202C	(18; 19; 89; 91; 92)	A	N (ECL3)	IV	18 mo	?
965-967del	ΔR202	(37)	A	N (ECL3)	IV	?	dD:NR
972C>A	T204N	(34; 40; 68)	A	N (ECL3)	II	15 mo	dD:NR; dD':>200
975A>G	Y205C	(36; 40; 75; 76; 90)	A	Y (ECL3)	II	2 wk, 2.5 mo	dD:NR
978T>A	V206D	(34; 40; 68)	A	N (tmd5)	II	?	dD:NR
1431C>T	P322S	(37; 76)	F	Y (tmd7)	III, IV	10 mo,9 yrs	dD-deh.:>400
1476C>T	R337X	(36; 55; 68; 71; 92-96)	A	N (C-tail)	I	4 mo	dD:NR, deh>300, PS 169

Table 1-1 **V2R mutations involved in NDI**

The mutants in this table were selected based on the combined availability of published cell-biological and patient data. Abbreviations: Func., Functionality; Cons., conserved; Loc., location; F, functional; A, disturbed AVP binding; G; disturbed Gs protein binding; conserved (Y) or not (N) between vasopressin receptor; tmd, transmembrane domain; ECL, extracellular loop; ICL, intracellular loop; wk, week; mo, month; yrs, years; dD, infusion with dDAVP; ndD, nasal dDAVP; deh, dehydration; NR, non-responsive; >400, urine more than 400 mOsm/kg; PO plasma osmolality in mOsm/kg; PS, plasma sodium (mOsm/kg).

Therapies in congenital NDI

Conventional therapies. The most important component of treatment for NDI is replacement of urinary water losses by adequate supply of fluid, in combination with a decreased solute diet to decrease obligatory water excretion. Diuretics such as hydrochlorothiazide and amiloride have been shown to effectively lower urine volume in NDI, which is, at least for hydrochlorothiazide, more pronounced in combination with a low salt diet (22; 39). The combined administration of hydrochlorothiazide with either a prostaglandin synthesis (or cyclooxygenase [COX]) inhibitor such as indomethacin (2 mg/kg/day), or the potassium-sparing diuretic amiloride, was shown to be much more effective in reducing urine volume than the thiazide-diuretic alone (3; 33; 41; 42; 51; 67). Long-term use of prostaglandin-synthesis inhibitors, however, is often complicated by gastrointestinal and haematopoietic side effects. In addition, renal dysfunction has been described during indomethacin therapy, most often consisting of a reduction in GFR. Because of the known gastrointestinal safety of selective COX-2 inhibitors as compared to nonselective COX-inhibitors, a potential role for these drugs in the treatment of NDI has been put forward. In one male NDI infant, the effectiveness of a specific COX-2 inhibitor (rofecoxib) in decreasing urinary free water losses was indeed

demonstrated (65). Nevertheless, in view of the recent discovery that prolonged use of that particular COX-2 inhibitor can cause severe cardiac side-effects (84), we suggest that COX-2 inhibitors should not be used in the treatment of NDI until it has been strictly determined which of these specific inhibitors are completely safe. Altoghether, while decreasing urine volume to a great extent, these conventional treatments do not completely overcome the excess of water excretion, as adult patients treated with these drugs void 4-8 litres per day. Therefore, efforts to develop tailored therapies have been initiated, and are discussed in chapters 5-8 of this thesis.

Chemical and pharmacological chaperones to rescue V2R mutants.

Promising are the approaches recently developed for class II V2R mutants. While their in vivo expression is likely low, transient overexpression of class II mutant proteins in HEK293/COS cells, of which a small fraction is often expressed in the plasma membrane, allows the determination of the molecular functionality of such mutants (chapters 4 and 7) (66; 93; 94). In line with these findings, some patients harbouring functional and partially ER retained V2R mutants showed an increased urine concentration in response to high doses of AVP (49) (Table 1).

An important step forward in a putative treatment of these patients was the discovery that stabilization of mutant receptors by cell permeable V2R antagonists, like SR121463B, aid in the mutant's folding, thereby facilitating their translocation to the plasma membrane (53; 86)(Figure 3B). Because of their assistance in receptor folding, such cell permeable ligands are termed 'pharmacological chaperones' (10; 52). Although V2Rs with mutations at residues of major structural importance could not be rescued (e.g. H80R, W164R, S167L), the plasma membrane expression of most class II V2R mutants was restored (94). To be of therapeutic use, the rescuing antagonist has to be displaced by AVP after translocation of the V2R mutant to the basolateral membrane. As the actual functional rescue of the V2R mutant is determined by the competition between AVP and the rescuing ligand at the plasma membrane, low affinity V2R ligands, like the V1R inverse agonists SR49059, are thought to be clinically more successful than high-affinity compounds such as SR121463B (11). Indeed, Bernier et al. recently found that SR49059 showed a small, but significant, reduction in water intake and urine output in 5 NDI patients with missense V2R mutations, thus demonstrating the proof-of-principle that pharmacological chaperones can also rescue mutant V2R in vivo (12). Of great importance for the treatment, the relatively high blood levels of SR49059 came with a minimum of side effects (at least on the short term), which is due to their high specificity for the V2R. As several other V1R and V2R antagonists and agonists have been developed and have been FDA approved, or are close to FDA approval, we tested to what extent they are able to rescue the function of class II V2R mutants in NDI (chapters 5-7).

Antibiotics to bypass stopcodons. Antibiotics to bypass stop-codons.

A future therapy for patients harbouring premature stopcodon mutations (class I) is based on the ability of aminoglycoside antibiotics to cause translational read-through. Premature stop mutations in the dystrophin gene that are involved in Duchenne muscle dystrophy are the prototypic mutations that have been used to investigate the read-through effect of aminoglycosides (29). In addition, a gentamycine analogue caused read-through of nonsense V2R mutants in vitro and in vivo (79; 83). However, the efficiency by which aminoglycosides confer read-through depends strongly

on the nucleotides flanking the premature stop codon, and on the compound used (45; 56). In chapter 8, we tested the effect of several aminoglycosides on several nonsense V2R mutants in NDI.

Aim of the thesis

The aim of this thesis was to describe the cellular defects underlying congenital NDI and, based on the type of cellular defect caused by a mutation and to identify means to overcome this defect. The identification of compounds that can restore or improve the function of mutant proteins and an increased understanding of the molecular and cellular mechanisms of functional rescue will allow the development of novel therapies for NDI and other conformational disorders.

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Chapter 2

Polarization, key to good localization

Summary

Polarisation of cells is crucial for vectorial transport of ions and solutes. In literature, however, proteins specifically targeted to the apical or basolateral membrane are often studied in non-polarised cells. To investigate whether these data can be extrapolated to expression in polarised cells, we studied several membrane-specific proteins. In polarised MDCK cells, the Aquaporin-2 water channel resides in intracellular vesicles and apical membrane, while the vasopressin-type 2 receptor, anion-exchanger 1 (AE1) protein and E-Cadherin mainly localise to the basolateral membrane. In non-polarised MDCK cells, however, Aquaporin-2 localises, besides plasma membrane, mainly in the Golgi complex, while the others show a dispersed staining throughout the cell. Moreover, while AQP2 mutants in dominant nephrogenic diabetes insipidus are missorted to different organelles in polarised cells, they all predominantly localise to the Golgi complex in non-polarised MDCK cells. Additionally, the maturation of V2R, and likely its missorting, is affected in transiently-transfected compared to stably-transfected cells. In conclusion, we show that the use of stably-transfected polarised cells is crucial in interpreting the processing and the localisation of membrane targeted proteins.

Introduction

In the body, epithelial layers form the boundary between different compartments, separating the interstitium from the outside world, and are of vital importance in maintaining ionic homeostasis by allowing regulated vectorial transport of ions and solutes. Crucial in this process is the establishment of an asymmetric cell surface distribution, dividing the plasma membrane in two structurally and functionally different domains, known as the apical and the basolateral membrane. The process of cell polarisation is guided by different polarisation cues, induced by cell-cell and cell-extracellular matrix (ECM) contacts, resulting in the formation of tight junctions and a reorganisation of the cytoskeleton (reviewed in (42) and (36)). Typical is the reorganisation of the microtubule network to an apico-basolateral array, which allows the vectorial transport to and from the apical and basolateral domains, which is important for maintaining asymmetry, as well as for transepithelial transport (42).

Two of such proteins for which proper polarised expression is important are the water channel Aquaporin-2 (AQP2) and its upstream vasopressin type-2 receptor (V2R) (22), which are essential in the process of vasopressin-regulated concentration of urine. After synthesis and homotetramerisation in the endoplasmic reticulum and transport through the Golgi, AQP2 is stored in so-called storage vesicles localised close to the apical membrane (12; 29; 38). Vasopressin binding by basolaterally-localised V2R leads to a transient rise in intracellular cAMP levels resulting in protein kinase A (PKA) activation and subsequent AQP2 phosphorylation at serine 256 (S256) (20). Phosphorylation of minimally three monomers of the AQP2 tetramer results in an apical translocation of the protein, enabling reabsorption of pro-urinary water from the apical side (16). The constitutively expressed basolateral water channels AQP3 and AQP4 enable the basolateral exit of reabsorbed water to the interstitium.

Recently we generated polarised cell models for the regulation of V2R and AQP2. In MDCK cells, GFP-tagged V2R and untagged AQP2 were stably transfected.

The resulting GFP-V2R was shown to be (mature) complex-glycosylated, localised to the basolateral membrane of polarised cells and was stimulated and internalised upon vasopressin stimulation, thereby closely resembling the in vivo situation (34). Polarised MDCK-AQP2 cells show a subapical localisation of AQP2, which redistributes to the apical membrane after an increase in cellular cAMP after AVP stimulation or by forskolin (9). AQP2 mutations found in nephrogenic diabetes insipidus (NDI), a disorder in which the kidney is unable to concentrate urine, often interfere with this routing, resulting in a different localisation of AQP2 (14; 17; 23; 26; 28). Other often used mutations, though not NDI derived, are the S256A and S256D mutants, which represent constitutively non-phosphorylated and phosphorylated AQP2, respectively. These two mutations have been crucial in understanding the routing of the AQP2 molecule after vasopressin signalling (16; 20; 41). In steady state and independent of forskolin stimulation, AQP2-S256A resides in intracellular vesicles. In contrast AQP2-S256D is constitutively localised in the apical membrane.

The collecting ducts of the distal nephron, roughly consists of two major cell types: the principal cells, which express AQP2 and V2R, and the a-intercalated cells. The polytopic chloride-bicarbonate exchanger AE1 is expressed in the basolateral membrane of the latter cell type and is involved in the regulation of our pH homeostasis (1). Mutations in the gene for AE1 cause autosomal dominant distal renal tubular acidosis (ddRTA) and are characterised by defective trafficking (11; 18; 19).

Madin-Darby canine kidney (MDCK) cells provided a polarised cell model in which the routing of these specific apical and basolateral proteins could be studied in resembling organ physiology. However, in literature, several proteins, known to target specifically to either the apical or the basolateral membrane, are investigated in non-polarised cells models ranging from cells unable to polarise, like HEK293 or HeLa, to cells that can polarise, but are not grown as such (e.g. MDCK or LLCPK-1). In these studies localisation to the plasma membrane is often extrapolated as an apical or basolateral membrane localisation in polarised cells. To assess whether this extrapolation can be made, we investigated the consequence of using a non-polarised versus a polarised cell model on the localisation of the above-mentioned membrane proteins. In addition, we analysed whether maturation of these proteins differ between stably-transfected versus transiently-transfected cells.

Materials and methods

Cells

The stably transfected MDCK cell lines MDCK-AQP2 (9); MDCK-AQP2-S256A (41); MDCK-AQP2-S256D (41); MDCK-AQP2-P262L (5); MDCK-AQP2-R254L (6); MDCK-V2R-GFP (34); MDCK-HA-AE1 (37) were cultured as described (8). Stable MDCK cells containing AQP2-E258K were constructed as described (8). COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Biowittaker, Verviers, Belgium) supplemented with 10% foetal calf serum (PAA Laboratories, Karlsruhe, Germany). As the original American Type Cell culture MDCK cell line is known to be multiclonal, several groups have selected clonal MDCK cell lines. Here, MDCK high resistance (HRS) or type I cells (33) have been used throughout. AQP2 constructs have been transfected to MDCK-HRS cells with passage

numbers between 90 and 120. In case a transfection had been repeated with a same construct in MDCK cells of a late passage number, localization and variation thereof (see below) were similar. All AQP2 stable cell lines were derived from the same standard stock of cells. Stably-transfected cell lines were first tested for the expression of the respective gene of interest by immunoblotting. Then, at least four clones were analyzed by immunocytochemistry to select a consensus localization of the protein of interest. Selected clones were used for a limited number of passages (<20) as some of the clones lost expression when grown at higher passage numbers.

Immunocytochemistry and immunoblotting

Cells were seeded on polycarbonate filters (Costar, Cambridge, MA, USA) at a density of either $1.5*10^5$ cells/cm^2 for polarised cells or $9.4*10^3$ cells/cm^2 for non-polarised cells. Immunocytochemistry and confocal laser-scanning microscopy (CLSM) were performed as described (10). As primary antibodies 1:50-diluted mouse anti-early endosomal antigen-1 (EEA1; BD Transduction Laboratories, Lexington, KY, USA), 1:50-diluted mouse anti-Golgi marker 58K (Sigma Aldrich, St. Louis, MO), 1:200-diluted rat anti-E-Cadherin (Sigma Aldrich, St. Louis, MO), 1:50-diluted mouse anti-HA (Sigma Aldrich, St. Louis, MO) and 1:50-diluted rabbit anti-AQP2 (7) were used. Anti-rabbit and anti-mouse secondary antibodies coupled to Alexa 488 or 594 (Molecular Probes, Leiden, The Netherlands) were used in a 1:100 dilution. For staining surface FLAG-tagged GFP-V2R, a slightly modified protocol was used: COS cells were washed and incubated with 1:100-diluted mouse anti-FLAG antibody (Sigma Aldrich, St. Louis, MO) at 4°. Subsequently, cells were fixed and immunocytochemistry and CLSM were continued as described above. The glycosylation pattern of the transfected GFP-V2R was determined by immunoblotting as described (34).

Results

Localisation of wild-type Aquaporin-2 in polarised and non-polarised cells.

To establish whether localisation in non-polarised cells can be extrapolated to a polarised situation as exists in vivo, we first studied the localisation of AQP2. For this, we used stably-transfected MDCK-AQP2 cells, seeded at low and high density. Seeded cells were allowed to grow for three days, resulting in a polarised monolayer for the higher cell density only. Before fixation, the cells were treated with the adenylate cyclase activator, forskolin, to induce apical membrane localisation of AQP2. Without stimulation, AQP2 has been reported to be localised to the Golgi complex or to vesicles different from the Golgi (4; 9; 32; 40). Therefore, we performed a co-localisation experiment with the Golgi complex marker protein 58K (3). Following immunocytochemistry, we saw a clear difference in AQP2 localisation between the two cell conditions. In the polarised cells, AQP2 mainly resided in the apical membrane. However, also some vesicular staining of AQP2 was observed, indicating that not all AQP2 was translocated to the apical membrane (Figure 1, upper panel). These intracellular vesicles, however, were different from the Golgi complex, as no co-staining was observed with 58K. In non-polarised cells, however, AQP2 partially localises to the plasma membrane, but also appeared to reside in structures close to the nuclear membrane, which showed a clear co-localisation with 58K (Figure 1, lower panels). This indicated that in non-polarised MDCK cells AQP2 partially resides in the Golgi-complex. Notably, the xz scans in

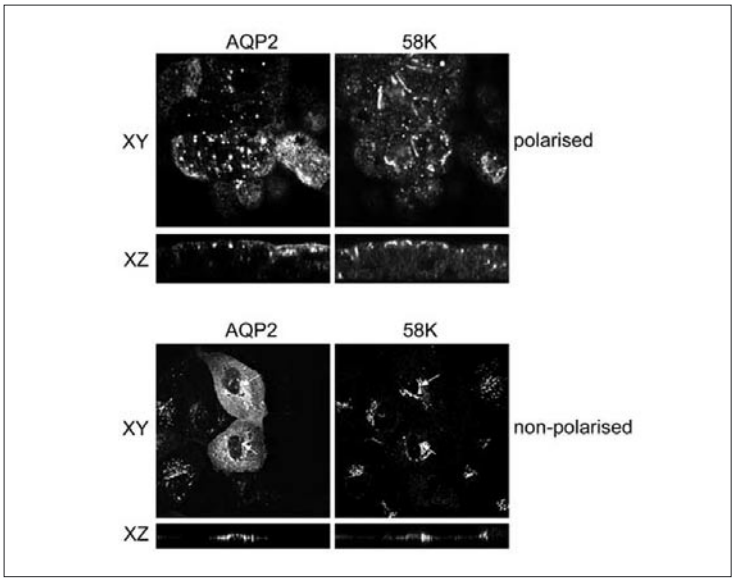

Figure 2-1 **Localisation of Aquaporin-2 in polarised and non-polarised MDCK cells.** MDCK cells, stably transfected with wild type AQP2, were grown to full confluency (top panels) to obtain a polarised cell layer or sparsely seeded (lower panels) to obtain non-polarised cells. The left panels show AQP2 staining, while the right panels represents staining of the Golgi complex marker protein 58K. Arrows indicate co-staining. The xz view is taken at approximately midway of the xy panel. Please note the difference in height between the top and bottom xz panels, indicative of the difference in polarisation.

Figure 1 also show a clear difference in height between the two conditions confirming the difference in polarisation status.

Localisation of AQP2 mutants in polarised cells versus non-polarised cells

To investigate whether polarisation is also crucial in determining the implication of AQP2 mutants, often impaired in their routing, we investigated the localisation of AQP2-S256A and AQP2-S256D, as these mutants respectively represent strictly intracellular or apical localisation. Stably transfected MDCK cells expressing these proteins were seeded in low and high density and immunocytochemically analysed in combination with subcellular marker proteins. Indeed, in polarised cells, AQP2-S256D staining expectedly showed a clear apical localisation, while the basolateral marker protein E-Cadherin revealed the basolateral membrane (Figure 2A, upper left panels). In non-polarised MDCK-AQP2-S256D cells, however, AQP2-S256D did not localise to the plasma membrane, but was localised intracellularly. Similar to wt-AQP2, AQP2-S256D showed a considerable co-localisation with the 58K Golgi marker protein in non-polarised cells, while it is also present in other intracellular, but unknown, structures (Figure 2A, lower left panels).

Examining AQP2-S256A, we found that in polarised cells this protein is localised in vesicles in the cytosol, which co-stained to a low extent with the early endosome marker EEA1 (Figure 2A, upper right panels). Non-polarised cells showed a similar intracellular distribution for AQP2-S256A without any co-localisation with the Golgi complex marker protein 58K (Figure 2A, lower right panels). This may indicate that the subcellular localisation of AQP2-S256A was not affected by the difference in cellular polarisation.

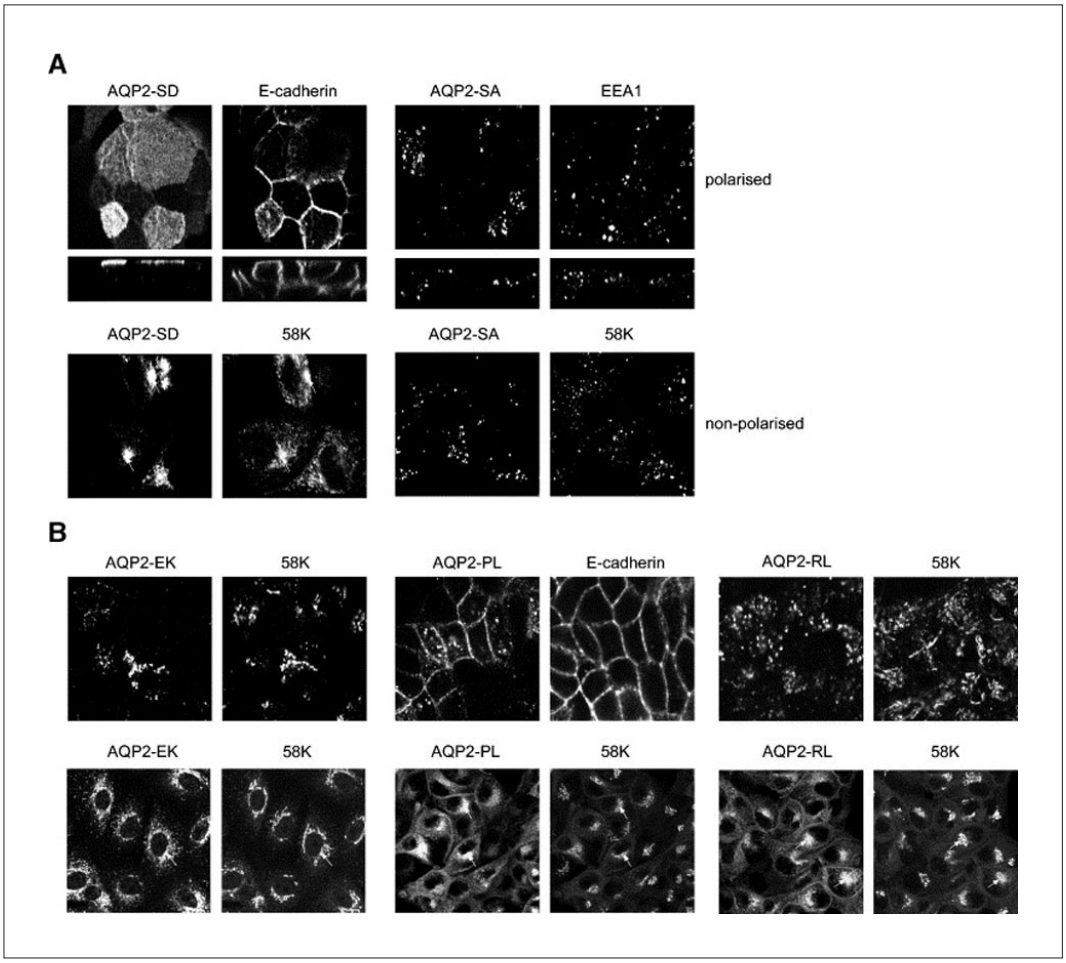

Figure 2-2 **wLocalisation of mutant AQP2 is dependent on polarisation.**
MDCK cells stably transfected with mutant-AQP2 (as indicated) were grown as described either to polarity or as non-polarised cells. The top panels represent the polarised cells; the lower panels represent non-polarised cells. The marking above the panels indicate for which AQP2 mutant was stained or for which subcellular marker protein. Arrows indicate co-staining. AQP2-SD, AQP2-S256D; AQP2-SA, AQP2-S256A; AQP2-EK, AQP2-E258K; AQP2-PL, AQP2-P262L; AQP2-RL, AQP2-R254L.

As missorting of naturally-occuring AQP2 mutants is of fundamental importance for our understanding of dominant NDI, we also investigated the localisation of NDI-causing AQP2 mutants. AQP2-E258K has been reported to be retained in the Golgi complex in oocytes, but to late endosomes/lysosomes in polarised liver cells (13; 27). Upon analysis in polarised MDCK cells, stably-expressed AQP2-E258K indeed localised to an intracellular compartment, which was distinct from the Golgi complex, indicated by the lack of co-localisation with 58K (Figure 2B, upper left). In non-polarised MDCK cells, however, AQP2-E258K co-localised with 58K to a great extent (Figure 2B, lower left). Recently, we also reported that in polarised MDCK cells, AQP2-P262L resides in the basolateral membrane and intracellular vesicles, distinct from the ER and Golgi (5), and that AQP2-R254L localises to intracellular vesicles (6). Co-localisation studies in polarised cells confirmed this localisation for AQP2-P262L (Figure 2B, upper middle panel), while co-staining with 58K revealed that AQP2-R254L did not localise to the Golgi complex (Figure 2B, upper right). In non-polarised cells, however, both AQP2-P262L and AQP2-R254L showed considerable co-localisation with 58K (Figure 2B, lower middle and right), indicating that in non-polarised cells, these proteins localise to a great extent to the Golgi complex.

Polarised versus non-polarised: are the differences in sorting found for other directed membrane proteins?

As these results could be a unique feature of AQP2, we next investigated whether the localisation of other membrane proteins was also depending on the extent of polarisation. Therefore, we seeded MDCK-V2R-GFP cells and examined the V2R localisation one and three days after seeding in conjunction with the basolateral marker protein E-Cadherin. At day 1, at which the cells are not confluent (note the flatness of the cells in the xz plane), E-Cadherin was diffusely expressed throughout the cell, and showed no plasma membrane staining (Figure 3A, upper panel). In these cells, some plasma membrane localisation for V2R-GFP was found, but the majority of the staining was scattered throughout the cytosol. At day 3, however, full confluency and therefore polarisation was reached, as is shown by the height of the cells in the xz plane. Analysis of the localisation now revealed a strong basolateral staining for E-Cadherin (Figure 3A, lower panel). Additionally, besides some late endosome localisation (34), V2R-GFP was also mainly localised to the basolateral membrane, in a clear overlap with E-Cadherin (Figure 3A, lower panel).

Proteins of non-principal cell origin also require polarity

Besides V2R, we also analysed another integral membrane protein, being the chloride-bicarbonate exchanger (AE1), normally expressed in basolateral membrane of a-intercalating cells (11). As confirmed in Figure 3B (lower panel), AE1 indeed localises to the basolateral membrane of stably-transfected polarised MDCK cells (11). Grown non-polarised, however, results in the loss of the basolateral localisation (Figure 3B, upper panel). Instead, these cells show an intracellular and apical staining for AE1. Similar results have been obtained using rat IMCD cells, endogenously expressing AE1 (data not shown).

Expression of membrane proteins in transiently-transfected cells

In the experiments described above we employed stably transfected MDCK cells, partly because these cells are not efficiently transfected in a transient fashion. For this reason, transfection of COS and HEK293 cells is popular, as these cells can be

transfected transiently to a high extent. Concurrently, however, these cells are unable to polarise. To determine whether transient expression can also be of influence on the localisation of membrane proteins, we decided to study V2R in transiently-transfected COS cells. In its itinerary to the plasma membrane, V2R-GFP is high mannose gly-

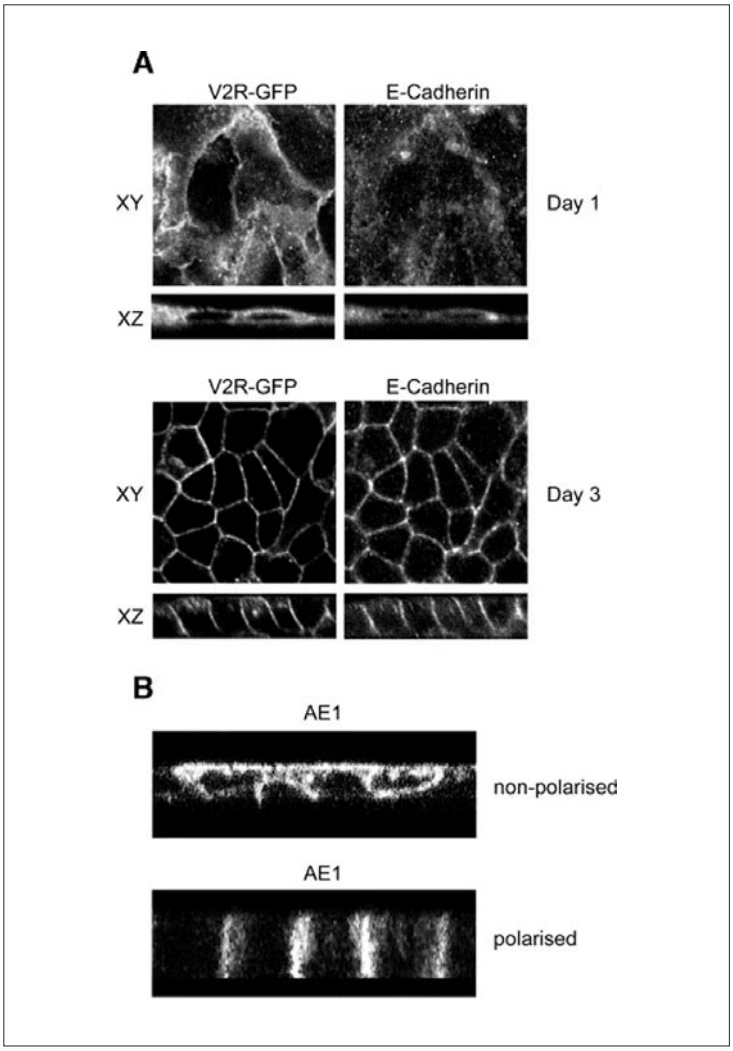

Figure 2-3

Figure 3. Polarisation leads to correct basolateral localisation of V2R.
A) MDCK-V2R-GFP cells at day 1 (top panels) and day 3 (lower panels) after seeding, representing non-polarised and polarised cells, respectively. Left panels show the GFP signal, right panels show E-Cadherin. The xz view is taken at approximately midway of the xy panel. Note the difference in height between the cells on day 1 and day 3 in the xz panels. B) xz planes of MDCK-AE1 cells grown to polarity (top panel) or as non-polarised cells (lower panel), stained for AE1.

cosylated in the endoplasmic reticulum, resulting in a protein of 60-62 kDa. In the Golgi complex, this glycosylation is changed for complex-glycosylation, resulting in a mature protein of 75 kDa, which is not changed upon its further route to the plasma membrane (35). Immunoblot analysis of the V2R-GFP, stably expressed in polarised grown-MDCK or transiently expressed in non-polarised COS cells revealed a striking difference. In transiently-transfected COS cells, the ER-glycosylated form of V2R-GFP was much more pronounced than the complex-glycosylated form, when compared to these bands in stably-transfected cells (Figure 4A). This indicates that V2R-GFP matured better in stably transfected cells compared to transiently transfected cells and that maturation depends on polarisation of the cells. To determine whether the extent of polarisation makes a difference in the level of maturation, we analysed the level of maturation of V2R-GFP in polarised versus non-polarised MDCK cells. We employed the seeding and harvesting conditions as described for Figure 3. Immunoblot analysis of equal protein amounts revealed similar maturation of V2R-GFP at days 1-3 (Figure 4B). This indicated that the reduced level of maturation in COS cells is due to the transient expression of V2R-GFP in COS cells and not due to differences in polarity. Similarly, with transient expression of AQP2 in COS, HEK293 or HeLa cells, the ER-glycosylated form is the most predominant form (data not shown). Consistent with the immunoblot data, parallel immunocytochemistry revealed a strong intracellular expression of V2R-GFP in transiently transfected COS cells (Figure 4C). As the cells were transfected with a GFP-V2R construct bearing an extracellular FLAG tag, we could discriminate surface from intracellular V2R by staining for the FLAG tag in non-permeabilised cells. Staining for surface expression, revealed that a fraction of GFP-V2R reached the plasma membrane, which is consistent with the partial maturation found on immunoblot (Figure 4A and C). These experiments indicate that, next to the lack of polarisation, transient expression also influences the localisation of membrane proteins.

Discussion

Polarisation is recognised as an important feature of epithelial cells. The establishment of asymmetry in epithelial cell layers allows regulated vectorial transport of ions and solutes, crucial for maintaining ion homeostasis. Hence, specialised epithelial layers form the boundary between different compartments in the body, and between the body and the outer environment. The kidney and more specifically nephrons, consist of a series of tubules lined with epithelium specialised in ion transport. Polarisation of these cells and the concomitant reorganisation of the cytoskeleton ensures the correct transport of ions either towards the urine for secretion or the blood for reuptake (36; 42). Here, we studied the effect of polarity and the localisation of several renal proteins.

The use of non-polarised cells hides the true localisation of AQP2

We show that in non-polarised cells, the localisation of AQP2 is not maintained to intracellular vesicles and plasma membrane, but then also localises to the Golgi complex (Figure 1). In vivo, AQP2 has been localised to multivesicular bodies and smaller vesicles, but not to the Golgi complex (29), indicating that fully polarised MDCK cells are a better physiological model than non-polarised cells to study trafficking of AQP2.

The necessity of using polarised cells to investigate the routing and function of AQP2 is demonstrated further by the experiments described in Figure 2. We show

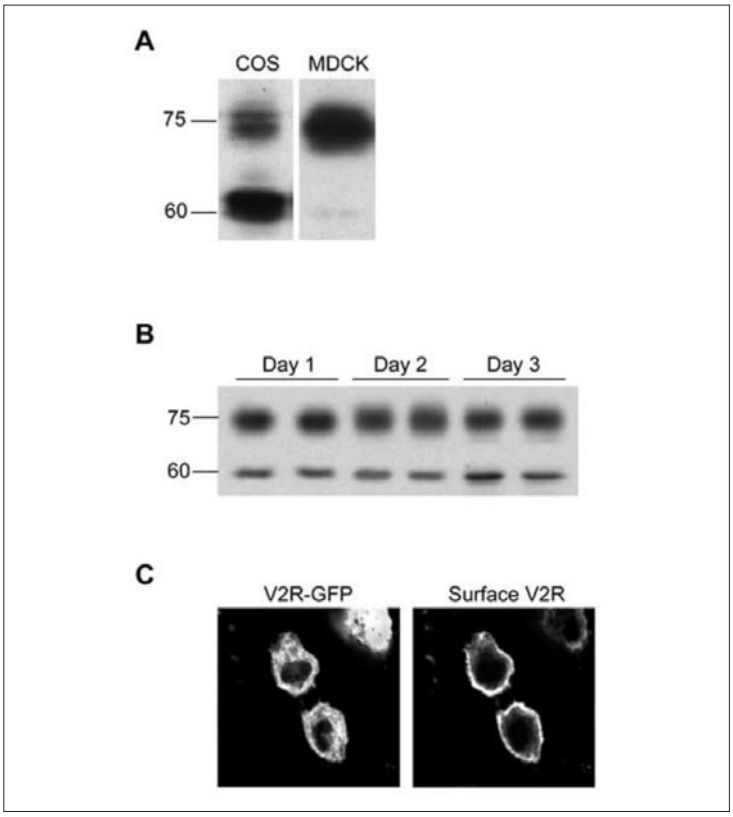

Figure 2-4 **Transient transfection leads to aberrant localisation and maturation.**
A) Immunoblot for GFP-V2R of transiently transfected COS cells (left panel) and stably transfected MDCK cells (right panel). The 60 kDa protein band represents the core protein, while the 75 kDa protein band represents the mature, complex glycosylated protein. All lanes were of equal protein loading. Please note the high amount of core protein in the left lane compared to the right lane. B) Immunoblot for V2R-GFP in MDCK-GFP-V2R lysates. Lysates were taken at different time-points after seeding, representing the change in polarisation status (see Figure 3A). The 60 kDa protein band represents the core protein, while the 75 kDa protein band represents the mature, complex glycosylated protein. All lanes were of equal protein loading. Please note the lack of difference between the different days of polarisation. C) Immunocytochemistry of non-permeabilised transiently-transfected COS cells. The left plane shows the GFP signal of total GFP-V2R. The right panel represents the FLAG-signal, which represents due to lack of permeabilisation, surface GFP-V2R only.

that the constitutively active mutant AQP2-S256D does not localise to the plasma membrane in non-polarised cells, while in polarised it is found in the apical membrane. The routing of the AQP2-S256A mutant, on the contrary, does not seem to be different between the two experimental conditions used (Figure 2). As the vesicular staining only partially co-localises to the endosomal marker EEA1, AQP2-S256A is probably

retained in storage vesicles. Translocation to the apical membrane of AQP2 is induced by phosphorylation of the S256 residue (20; 29). As the phosphorylation is impaired in the AQP2-S256A mutant, no translocation can take place, either in polarised or in non-polarised cells. The apparent lack of difference for AQP2-S256A localisation between polarised and non-polarised cells could indicate that its localisation in storage vesicles is not depending on the polarisation status of the cells. This leads to the speculation that in non-polarised cells, translocation from storage vesicles after cAMP signalling is directed towards the Golgi complex. Indeed the Golgi complex has been recognised as a critical cellular organ involved in protein trafficking (36). Low et al (24), have also shown that upon losing polarity, a shift in membrane protein trafficking towards intracellular compartments could be seen. This could also explain the difference between the storage vesicle localisation of AQP2-S256A and the Golgi-like staining of wild type AQP2 in non-polarised cells. It needs to be noted, however, that even in fully-polarised LLC-PK1 cells we found that stably-expressed AQP2 is also partially expressed in the Golgi complex (not shown). This is consistent with data from Brown and co-workers, who also (partially) localized AQP2 to the Golgi complex in stably-transfected unstimulated LLC- PK1 cells (25). At present, it is unclear whether this difference is due to the tubular origin of the cells (LLC- PK1 from proximal tubules; MDCK from collecting duct) or is cell-type dependent. Clearly, however, our data reveal that for studying protein trafficking in a physiological relevant setting, it is important to search for a cell model that mimics the in vivo localisation and regulation mostly closely.

The molecular cause of dominant NDI: polarity makes the difference

In Figure 2B we investigated the mutants AQP2-E258K, -P262L and -R254L, all of which cause NDI by defective routing to different organelles (5; 6; 14; 27). In polarised cells, AQP2-E258K is missorted to the Golgi complex and late endosomes/lysosomes, AQP2-P262L resides in intracellular vesicles of unknown identity, but which are different from the Golgi complex or early endosomes, while AQP2-R254L co-localises partly with early endosomes and, due to its resemblance in localisation with AQP2-S256A and its inability to be phosphorylated after forskolin stimulation, likely resides in genuine AQP2 storage vesicles(5; 16; 20; 41). In non-polarised cells, however, all these mutants mainly localise to the Golgi complex, a location indiscernible from that of wild type AQP2 (Figure 1). These data indicate that analysis of the sorting of these mutants in polarised cells was essential to identify the cellular cause underlying dominant NDI.

Missorting due to a lack of polarisation is a general phenomenon

The experiments described in Figure 3 suggest that the mislocalisation in non-polarised cells is a common feature for proteins that have a regulated basolateral or apical transport. V2R is a predominantly basolaterally localised protein, which is largely retained intracellular in non-polarised cells. As shown in Figure 3B, AE1 shows a similar sensitivity with regard to the polarisation status of the cell. Concurrently, we found that the basolateral marker protein E-Cadherin was also dispersed throughout the cell when grown non-polarised, whereas it was localised in the basolateral membrane in the confluent state (Figure 3A).

Transient expression affects proper maturation.

Compared to stably-transfected MDCK cells (Figure 4B), transiently-transfected COS cells showed a defective maturation of V2R-GFP, while the extent of

maturation of V2R-GFP was not changed by the extent of polarity of MDCK-V2R-GFP cells. Because the expression levels are usually very high in transient transfection assays , overloading of the capacity of the ER and Golgi complex to fold and assemble these proteins properly is a likely explanation for this. This hypothesis is supported by the observed correlation between the total expression of an ER-retained AQP2 mutant in Xenopus oocytes and the increased levels of unglycosylated versus high-mannose glycosylated bands (15). While the V2R-GFP is already mislocalised in stably-transfected cells grown non-polarised, the affected maturation of V2R-GFP in transiently-transfected non-polarised cells (here COS cells) likely adds to its mislocalisation. Our data, therefore, indicate that caution should be attained when using transiently-transfected cells to study membrane proteins.

In polarised cells, the co-localisation of the molecular motors dynein and dynactin with AQP2 (30) indicates that the transport of AQP2 bearing vesicles is mediated through microtubules, the organisation of which is crucial for proper transport. Additionally AQP2 was found to bind to a multiprotein motor complex. Knepper and co-workers have shown by screening AQP2 bearing vesicles for associated proteins in renal inner medullary collecting duct that several myosin family members are associated with AQP2 vesicles (2). This indicates that the transport of AQP2 vesicles is also mediated by actin-based motors. Indeed, AQP2 has been shown to interact with actin (31) and the organisation of the actin cytoskeleton controls the apical membrane insertion of AQP2 (21; 39). In cancer, loss of polarisation is one of the first signs of malignancy. Depolarisation leads to a change in the intracellular cytoskeleton and a rearrangement of microtubules (42). Therefore routing to and from organelles is organised differently. Furthermore, also components of the plasma membrane fusion machinery, normally localised apically, redistribute to intracellular localisations when MDCK cells lose polarity (24). In this respect, the missorting of the membrane proteins in non-polarised cells as found here might be due to a changed organisation of the microtubular and/or cytoskeletal network, which will be subject of future research.

In conclusion, our study shows that for studying the routing of specifically sorted apical or basolateral membrane proteins it is crucial to use a polarised cell model and that the use of non-polarised cell models can cloud the interpretation of mutant phenotype, as is shown here for the regulated water channel AQP2.

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Chapter 3

Regulation of the vasopressin V2 receptor by vasopressin in polarized renal collecting duct cells

Abstract

AQP2-mediated water reabsorption in the renal collecting duct is regulated by arginine-vasopressin (AVP), which acts via the vasopressin V2 receptor (V2R). We generated a polarized cell model for renal collecting duct cells to study localization of the V2R and receptor translocation after treatment with the synthetic AVP analogue dDAVP. Receptor proteins fused to the green fluorescent protein were visible on western blot as multiple bands of approximately 75 kD, representing the complex glycosylated form of the receptor. In the absence of agonist, the majority of V2R (75%) was located in the lateral plasma membrane, while the remaining part was located in late endosomes and lysosomes. A dose-dependent internalization of receptor proteins was observed upon agonist treatment, which was maximal when 100 nM dDAVP was administered. Internalization could be prevented by administration of a V2R antagonist. Using confocal laser scanning microscopy, we found V2R transiently localized in early endosomes 15 and 30 minutes after stimulation with agonist, whearas the localization changed to late endosomes and lysosomes beyond these timepoints. V2R is subsequently degraded in the lysosomes, hereby decreasing the halflife of the receptor from >8 hours in the non-stimulated situation, to 2.77 hours. We therefore conclude that after stimulation with dDAVP, V2R is transiently present in early endosomes, from where it is translocated to the late endosomes and lysosomes, where it is degraded.

Introduction

The vasopressin V2 receptor (V2R) is a member of the 7 transmembrane (TM) family of G protein-coupled receptors (GPCRs), which is expressed in the basolateral membrane of epithelial cells lining the distal tubule, connecting tubule and collecting ducts. Through these cells, its major role is the regulation of the body water homeostasis, by determining the level of reabsorption of water from pro-urine through Aquaporin-2 (AQP2) water channels. Upon binding of the antidiuretic hormone arginine-vasopressin (AVP), it activates adenylate cyclase via a stimulatory G (Gs) protein. The subsequent increase of intracellular cAMP induces protein kinase A (PKA) to phosphorylate, amongst other proteins, AQP2, which subsequently is redistributed from intracellular vesicles to the apical membrane, resulting in urine concentration. Removal of AVP reverses this process, restoring the water-impermeable state of the apical membrane.

The V2R is involved in several pathophysiological conditions. Mutations in the human V2R result in X-linked Nephrogenic Diabetes Insipidus (NDI), a disorder in which patients are unable to concentrate their urine in response to AVP, resulting in the excretion of large volumes of diluted urine (1; 2; 11). Paradoxically, the V2R is also involved in states of excessive reabsorption of renal water, which is commonly found in patients suffering from congestive heart failure, liver cirrhosis, pre-eclampsia and the syndrome of inappropriate release of AVP (SIADH) (14; 19). Of these, the first three are due to an increased pituitary release of AVP induced by a sensed underfilling of the blood system, which can lead to life-threatening hyponatremia.

Considering the importance of the V2R in health and disease and the difficulty to study GPCRs in vivo, several studies have focused on the regulation of the trafficking of the V2R and its mutants in NDI in cell models. However, nearly all the

V2R expression studies have been performed in non-polarized cells and it has been reported that the regulation of proteins may differ between polarized and non-polarized cell types (23). Indeed, in transiently transfected cells, the observed high levels of immature expression and extensive intracellular localization of the V2R {28, 79}, while in vivo, the V2R is mainly localized in the basolateral membrane {282}, make these cell models less suitable to study the regulation of the V2R. Therefore, we set out to generate a polarized renal cell line that would constitute a good model for V2R regulation in vivo. Using this cell line, we subsequently analyzed the V2R localization, changes therein upon treatment with the synthetic AVP analogue dDAVP, and whether the V2R recycles to the plasma membrane or not.

Materials and Methods

Materials

MG-132 was from Calbiochem (La Jolla, CA); chloroquine diphosphate, cycloheximide, dDAVP, [Adamantaneacetyl^1, O-Et-D-Tyr^2, Val^4, Aminobutyryl^6, Arg^{8,9}]- vasopressin (a V2R antagonist) were from Sigma Aldrich (St. Louis, MO). The expression construct encoding wt-V2R, C-terminally-tagged with Green Fluorescent Protein (V2R-GFP (20)) was kindly provided by Dr. Alexander Oksche (FMP, Berlin)

Culture of MDCK cells

MDCK type I and II cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Biowitthaker) supplemented with 5% FCS (PAA laboratories), gentamycin, L-glutamin, sodium carbonate, and 1% non-essential amino acids. Transfection of these cells with 25 mg of the V2R-GFP expression construct was performed using the calcium phosphate method as described. (6)

Immunocytochemistry

For immunocytochemistry, the cells were seeded on Costar filters at a density of $3x10^5$ cells/cm^2 and grown for three days. Immunocytochemistry and confocal laser scanning microscopy (CLSM) was performed as described (6). As primary antibodies, 1:100-diluted rat anti E-Cadherin (Sigma, St. Louis, MO), 1:100-diluted mouse anti early endosomal antigen 1 (EEA-1; BD Translab, San Diego, CA) or 1:200-diluted mouse anti lysosome associated membrane protein 2 (LAMP-2) antibodies (13); a kind gift of Dr. Le Bivic, Marseille, France) were used. As secondary antibodies, 1:100-diluted goat anti-rat IgG and affinity-purified goat anti-mouse IgG, both coupled to Alexa-594, were used (Molecular Probes, Leiden, The Netherlands). For determination of the level of co-localization, individual pictures of 15-20 cells were contrast-stretched for the green and the red signal, after which the percentage of co-localization was calculated using Metamorph software (Universal Imaging Corporation, Downingtown, PA). Averaged data obtained from three independent pictures was used to determine the rate of co-localization.

Immunoblotting

For immunoblotting, total cell lysates were obtained by dissolving cells in Laemmli buffer containing 0.1 M DTT. Removal of sugar moieties from proteins of cell lysates with endoglycosidase H (Endo H) or protein N-glycosidase F (PNGase F; both from New England Biolabs, Beverly, MA) was done according to the manufacturer's

protocol. Protein samples were analyzed on a 10% PAAG and subsequently blotted onto PVDF membranes (Millipore Corporation, Bedford, MA) as described (6). For detection of V2R-GFP, 1:5000 diluted rabbit anti-GFP antiserum (4) was used (kindly provided by Dr. B. Wieringa (UMC Nijmegen)). As secondary antibodies, goat anti-rabbit IgGs (Sigma, St. Louis, MO, USA) were used at a 1:5000 dilution.

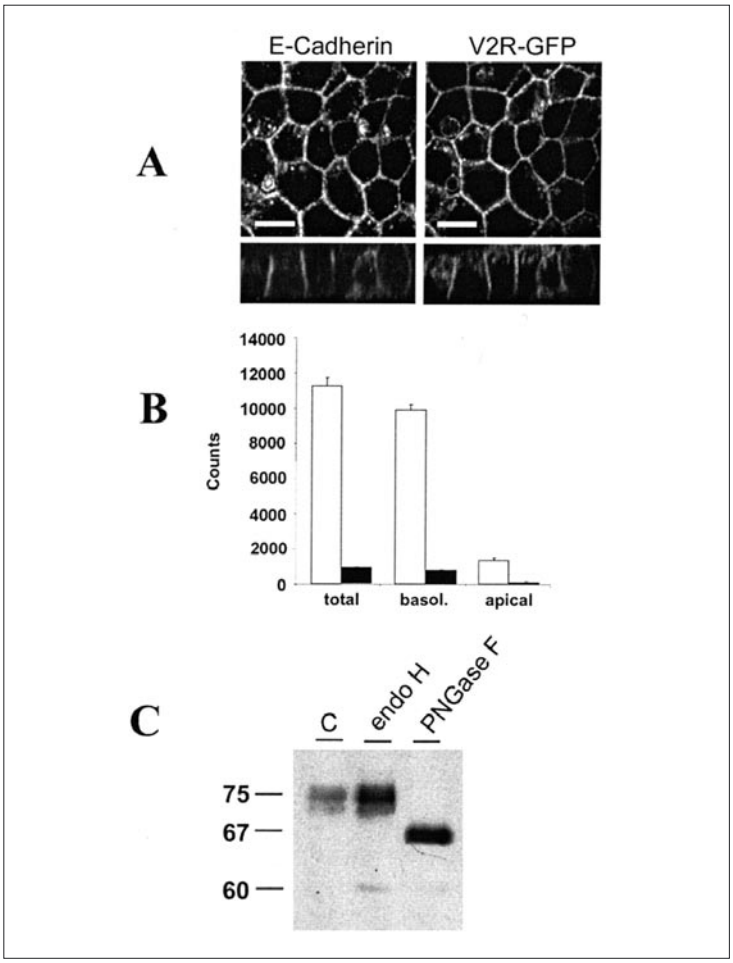

Figure 3-1 **Localization and glycosylation of V2R-GFP in polarized MDCK cells**
(A) MDCK type I cells stably-transfected with a V2R-GFP expression construct were grown to confluence, fixed and subjected to immunocytochemistry with rat anti-E-Cadherin antibodies and Alexa 594-conjugated goat anti-rat antibodies. CLSM analysis revealed a large portion of V2R-GFP co-localized with the E-cadherin. (B) Confluent MDCK-V2R-GFP cells were lysed in Laemmli buffer, and directly loaded (control: c) on a 10% SDS-PAAG or pre-treated with either endo H or PNGase F (indicated). Following immunoblotting with rabbit anti-GFP antibodies, core V2R-GFP, O-glycosylated V2R-GFP and complex-glycosylated V2R-GFP were detected. The mass of these proteins, as deduced from those of marker proteins, is indicated in kDa.

Results

Localization of the V2R stably-expressed in polarized MDCK cells

Madin-Darby Canine Kidney (MDCK) cells, which are derived from the renal collecting duct, have shown to be a good cell model for the regulation of the AQP2 water channel (6). Therefore, to set up a proper collecting duct cell model for studying routing of V2R, an expression construct encoding human V2R C-terminally-tagged with GFP was transfected into MDCK type I and type II cells. Following selection for G418 resistance, clones were analyzed for protein expression using immunoblotting. Numerous positive clones were isolated from both cell lines. Confocal laser scanning microscopy (CLSM) of six of these clones demonstrated that in both cell types V2R-GFP was predominantly expressed in the lateral membrane, where it co-localized with the basolateral marker E-cadherin. (Fig. 1A). Besides, V2R-GFP was detected intracellularly (see below). As the lateral staining was more distinct in MDCK type I cells, a representative clone from these cells was selected for our further studies.

Glycosylation of the V2R in polarized MDCK cells

Based on studies in non-polarized cells, high-mannose sugar moieties are added to N22 of the V2R in the endoplasmic reticulum(9), which can be specifically cleaved off by endoglycosidase H (Endo H). On its further transport to the membrane, these sugar groups will be exchanged for complex sugar groups in the Golgi complex, which can be biochemically cleaved off by PNGase F, but not by Endo H. In addition, in COS cells, V2R has been reported to be O-glycosylated (18), a process that occurs in the Golgi complex. To test the level of maturation of the V2R in MDCK cells, we therefore investigated the glycosylation state of V2R-GFP. Total cell lysates were untreated or treated with Endo H or with PNGase F and analyzed by immunoblotting using anti-GFP antibodies (Fig. 1B). For control cells, V2R-GFP was detected as a set of bands between 70-80 kD. Upon treatment with Endo H, these 70-80 kD bands remained, but a weak band of approximately 60 kD appeared. The size of this band is consistent with that of non-glycosylated V2R-GFP. Treatment with PNGase F resulted in a shift of the 70-80 kD bands to a band of approximately 67 kD. This shift with PNGase F, but not with Endo H, indicated that most of V2R-GFP in MDCK cells is complex glycosylated. The band at 67 kD likely represents O-glycosylated V2R-GFP, as this form of glycosylation is insensitive to Endo H and PNGase F (18). Together, these data reveal that in MDCK cells the majority of V2R-GFP is expressed in a mature form.

dDAVP-induced internalization of the V2R is dose dependent

One of the features of G protein-coupled receptors is that they are internalized from the plasma membrane upon stimulation with their selective agonists, which leads to a desensitization of the tissue for the hormone. These agonist-bound GPCRs are then usually transported via endosomal compartments to late endosomes, where the acidic environment leads to a dissociation of receptor and agonist. Subsequently, GPCRs are then recycled to the cell surface or are targeted for degradation in lysosomes (24).

To investigate whether the V2R in our cells is internalized with its agonist and whether the level of internalization is dose-dependent, polarized MDCK-V2R cells were treated with 1, 10 or 100 nM of the synthetic vasopressin analogue dDAVP for 1 hour. These specimen were then subjected to immunocytochemistry for the basolateral marker protein E-cadherin after which its level of co-localization with V2R-GFP

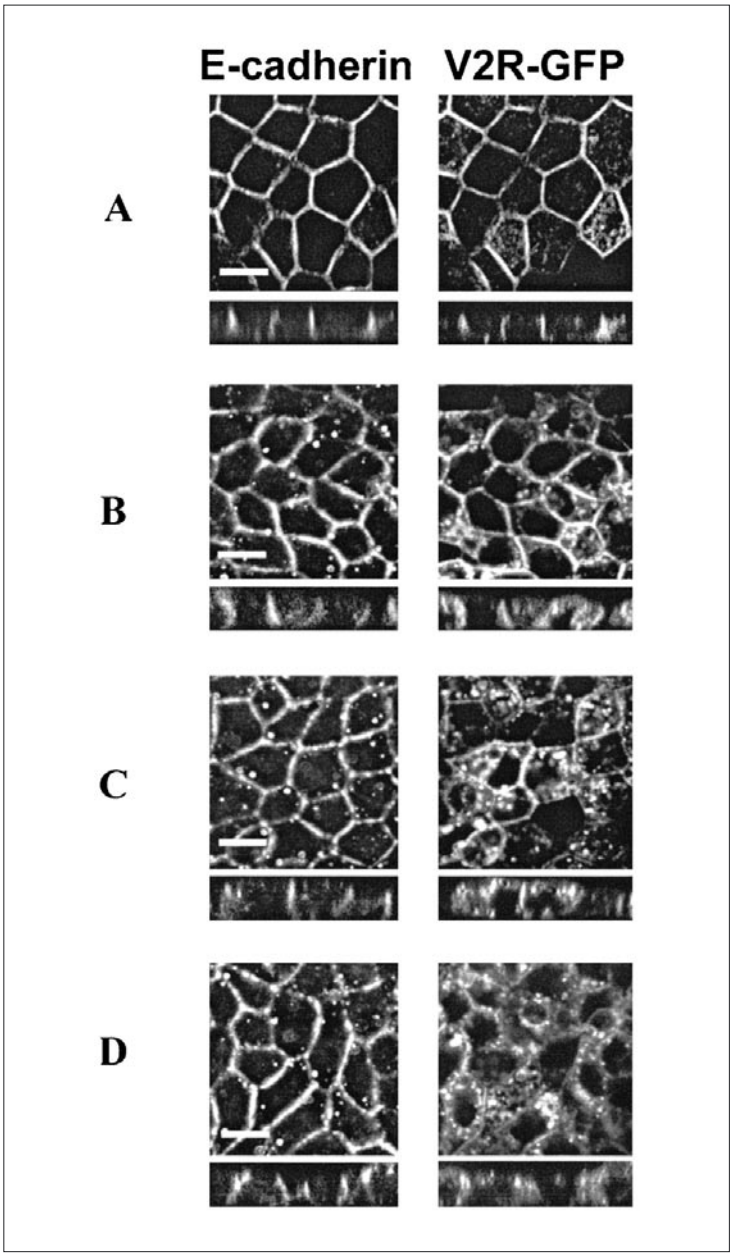

Figure 3-2 **Dose-dependent internalization of V2R-GFP with dDAVP**
Confluent MDCK-V2R-GFP cells were incubated at the basolateral side with normal medium (A), or medium containing 1 (B), 10 (C) or 100 nM (D) dDAVP for one hour. Then, the cells were fixed and subjected to immunocytochemistry as described in the legend of figure 1. The staining of E-Cadherin (left) and V2R-GFP (right) are shown. Bar, 10 µM

was determined by CLSM analysis (Fig. 2A-D). Subsequent semi-quantification revealed that in control cells 74.7 +/- 4.5% of V2R-GFP colocalized with E-cadherin, while 1, 10, 100 nM dDAVP treatment resulted in 41.5 +/- 3.3%, 25.4 +/- 5.4% and 16.6 +/- 4.3% co-localization, respectively. These data showed that the level of internalization was dependent on the dose of the agonist.

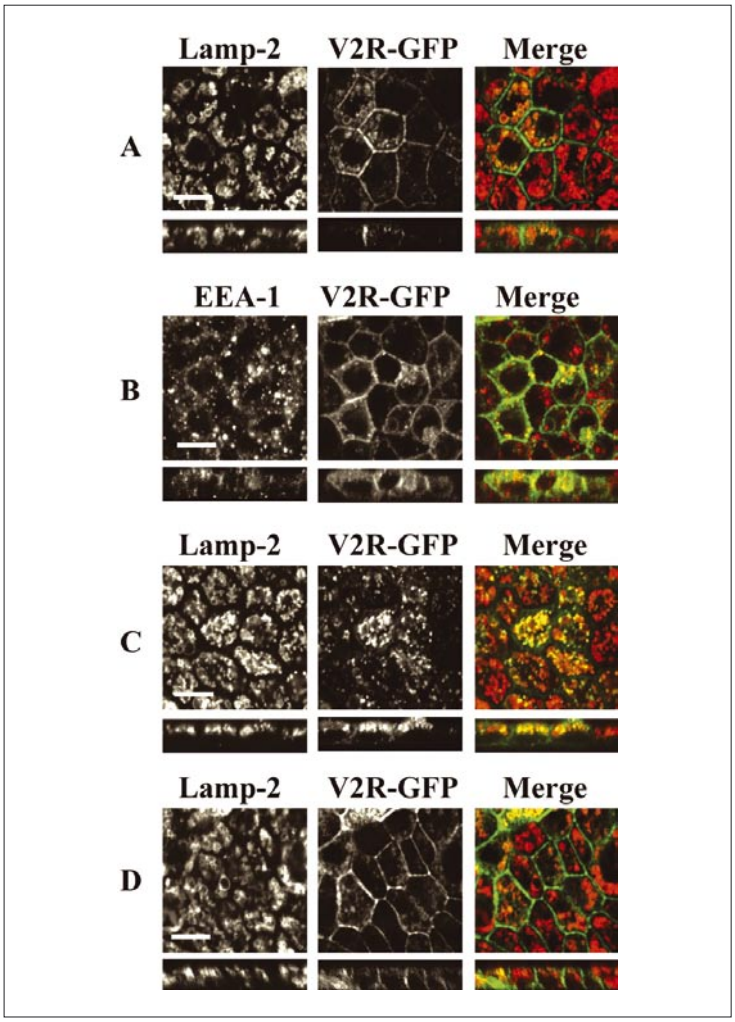

Figure 3-3 **Time-dependent internalization of V2R-GFP with dDAVP**
Confluent MDCK-V2R-GFP monolayers were incubated at the basolateral side with normal medium (A), medium containing 100 nM dDAVP for 30 minutes (B) or 1 hour (C), or with medium containing a mixture of 100nM dDAVP and 1 mM V2R antagonist (D). Then, the cells were fixed and subjected to immunocytochemistry with antibodies raised against LAMP2 (late endosomes/lysosomes; A, C and D) or EEA1 (early endosomes; B), followed by CLSM analysis. In the merged figures, V2R-GFP is given in green, while the marker proteins are in red. Bar, 10 μM

dDAVP-induced internalization of the V2R in time

To study the level of V2R-GFP internalization in time, MDCK-V2R cells were incubated with 100 nM dDAVP for different periods of time and subjected to immunocytochemistry using E-cadherin antibodies. CLSM analysis and subsequent semi-quantification of the signals revealed that in control cells 75.9 +/- 4.5% of the V2R was localized in the lateral membrane, while the level of co-localization decreased to 31.2 +/- 6.5%, 10.0 +/- 3.5% and 9.2 +/- 4.4% at 30, 60 and 120 minutes after addition of dDAVP, respectively (data not shown). These data revealed the internalization of V2R-GFP from the basolateral membrane is time-dependent and that it has reached a plateau level at 60 minutes of dDAVP treatment.

Time-resolved localization of the V2R in early endosomes with dDAVP

Subsequently, we analyzed the further route of dDAVP-induced internalized V2R in time. Receptors internalized from the basolateral membrane are likely to pass early endosomes on their path to the late endosomes and lysosomes. Therefore, to examine the passage of the V2R through endosomes in time, MDCK-V2R cells were treated with 100 nM dDAVP for different periods of time and subjected to immunocytochemistry using early endosome antigen-1 (EEA-1) antibodies. Subsequent CLSM analysis indicated that the level of co-localization of the V2R with EEA-1 was indeed transient (Fig. 3). Semi-quantification of the level of co-localization revealed no significant level of co-localization in untreated cells (3.1 +/- 2.0%). At 15 or 30 minutes after administration of dDAVP, the level of co-localization increased to 15.0 +/- 3.0% and 20.4 +/- 2.9%, respectively, while at 60 minutes, the rate of co-localization decreased to 8.5 +/- 1.7 %.

Time-resolved localization
of the V2R in late endosomes/lysosomes with dDAVP

To determine the level of localization of V2R-GFP in late endosomes/lysosomes in time, co-localization studies were done with the a marker proteins for these organelles, the lysosomal-associated membrane protein-2 (LAMP-2). Again, following administration of 100 nM dDAVP to the basolateral side of MDCK-V2R cells for different periods of time, the cells were subjected to CLSM analysis and semi-quantification of the level of co-localization. In control cells, a considerable fraction (26.8 +/- 3.8%) of V2R-GFP already co-localized with LAMP-2. Administration of dDAVP for 30 minutes and 1 hour increased the rate of co-localization with LAMP-2 to 48,6 +/- 5.5% and 80.2 +/- 5.8%, respectively, which was not further increased for longer periods of time (not shown). Incubation of MDCK-V2R cells with 100 nM dDAVP combined with 1 mM of the V2R antagonist prevented the accumulation of V2R-GFP in late endosomes/lysosomes (28.1 +/- 6.2% colocalization; Fig. 3D), which indicated that the late endosomal/lysosomal targeting of the V2R is specifically initiated by binding of dDAVP to the V2R.

Agonist stimulation increases lysosomal degradation of V2R

From the late endosomal/lysosomal compartment, internalized receptors are degraded or recycle back to the plasma membrane (3; 7), Therefore, to determine the stability of unbound or dDAVP-bound V2R in time, we blocked protein synthesis in MDCK-V2R cells with 50 μM cycloheximide, immunoblotted for V2R cells and relatively-quantified the amount of V2R-GFP in time (Fig.4, left panel). Without dDAVP, 12.3 +/- 4.8%, 27,9 +/- 7.3% and 34.3 +/- 6.4% decreases in V2R-GFP expression were

observed for 2, 4 and 8 hours incubation, respectively. Treatment of V2R-GFP expressing cells with dDAVP (Fig. 4, right panel) induced a much faster degradation of V2R, because at 2, 4 and 8 hours dDAVP treatment, its expression was reduced 47.9 +/- 6.8%, 64.9 +/- 7.2% and 82.3 +/- 4.6%, respectively. From these data it could be deduced that 100 nM dDAVP reduced the half-life of V2R from 11.52 +/- 2.8 hours to 2.77 +/- 0.41 hours (n=3). Co-incubation with 100 μM chloroquine for 8 hours, which blocks lysosomal degradation, only resulted in 11,2 +/- 4.3% degradation, which indicated that the majority of dDAVP-induced degradation of the V2R was through the lysosomal pathway. When the proteasomal blocker MG-132 was used in addition to chloroquine , V2R-GFP levels were similar to the untreated situation, indicating that a small amount of the dDAVP induced internalized V2R may be degraded by the proteasome. To test whether the V2R is also able to recycle to the basolateral membrane after dDAVP treatment, we treated MDCK-V2R cells for 2 hours with dDAVP followed by a 4 hour incubation in culture medium lacking dDAVP to allow receptor recycling. Subsequent immunocytochemistry for the basolateral marker E-Cadherin, CLSM analysis and semi-quantification, however, showed no detectable levels of re-occurrence of the V2R on the plasma membrane (not shown).

Discussion

MDCK type I cells expressing V2R-GFP are a proper model for V2R regulation in renal collecting duct cells

In vivo, studying localization and translocation effects of the V2R is difficult, as its expression levels are very low and isolation of the specific nephron segments expressing V2R is extremely laborious. Therefore, analysis of their molecular regulation depends on data from cell culture studies. Therefore, to identify pathways involved in the molecular regulation of the human V2R, a polarized epithelial cell model that shows trafficking of the V2R as anticipated from in vivo studies is most desirable. To generate such a cell line, MDCK type I, which resemble renal collecting duct cells, and type II cells, which resemble proximal tubular cells (17) were transfected with a human V2R-GFP expression construct. As seen in vivo(15), both cell lines showed a strong expression of V2R-GFP in the basolateral membrane, while it also localized to intracellular compartments. Since more than 20% of V2R-GFP in such MDCK type I cells co-localizes with LAMP2, this intracellular localization of V2R-GFP mainly represents late endosomes or lysosomes. This may imply that, even in the absence of agonists, V2R-GFP is continuously internalized from the plasma membrane, which may be due to basal receptor activity (22).

The MDCK type I cells showed a more distinct plasma membrane staining of V2R than type II cells and were therefore selected for further studies.MDCK type I cells have a higher transcellular resistance than type II cells (16), which might be due to a more rigid basolateral membrane and might explain the observed differences. Using the same construct, Schülein et al. reported a similar localization of V2R-GFP in another MDCK cell type, which indicates that this localization is common for MDCK cells (21).

Biochemical analysis further revealed that in the selected MDCK cells, the vast majority of the receptor is expressed as complex- and O-glycosylated V2R-GFP. Since these glycosylation modifications only take place in the Golgi complex on

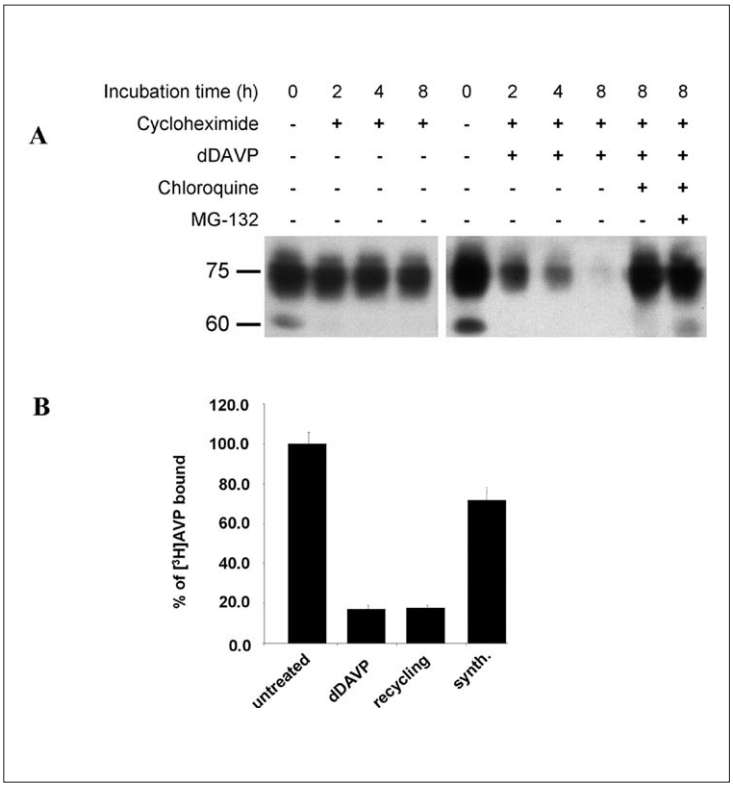

Figure 3-4 **Figure 4. Lysosomal degradation of V2R-GFP upon agonist treatment**
MDCK-V2R-GFP cells that were grown to confluence on filters were incubated for the indicated time (in hr) with or without 100 nM dDAVP, 50 μM cycloheximide, 100 μM chloroquine and/or 20 μM MG-132 (indicated). Following these treatments, the cells were lysed in laemmli buffer, loaded on a 10% PAAG and subjected to GFP immunoblotting as described in the legend of figure 1. The masses of unglycosylated (60) and complex-glycosylated (75) V2R-GFP are indicated in kDa.

properly-folded V2R, these data also indicate that the V2R folds and matures properly in MDCK type I cells. Although we can not exclude that some V2R-GFP is retained in the endoplasmic reticulum, the minor amount of high-mannose V2R-GFP detected in our cells upon endo H treatment likely represents V2R-GFP just synthesized at the endoplasmic reticulum and on its way to the Golgi complex.

In transiently transfected non-polarized cells, a substantial amount of the expressed V2R is immature and expressed in intracellular compartments, whereas in our stably transfected polarized renal cells, V2R-GFP is mainly located in the basolateral membrane and exerts a high level of maturation. Therefore, we believe we have been able to generate a proper polarized cell model for studies on the regulation of the V2R as found in renal principal cells.

Sequestration of the V2R is dose dependent

In vivo and in polarized in vitro cell cystems, basolateral stimulation of V2R with AVP or dDAVP leads to translocation of AQP2 from intracellular vesicles to the apical membrane (5; 10). However, it is unknown how this stimulation affects the localization of the V2R in polarized cells. Our study with MDCK-V2R cells demonstrates that the internalization of the V2R is dose-dependent and time-dependent. The level of internalized V2R-GFP upon incubation with 1, 10 or 100 nM concentrations of dDAVP for 1 hour revealed that in our cell model V2R-GFP sequestration already occurs at a physiological concentration of dDAVP (1 nM), which is increased at higher concentrations. The increased level of internalized V2R with increasing dDAVP concentrations most likely is a consequence of increased receptor occupation by the hormone.

dDAVP induces the sequestration of V2R via early endosomes to late endosomes/lysosomes

To determine the path and time frame of V2R internalization following binding of dDAVP, time-resolved co-localization experiments were performed between V2R-GFP and the cell organelle markers E-cadherin, EEA-1 and LAMP2. As pointed out above, under unstimulated conditions, the majority (75%) of the V2R localized to the lateral membrane, whereas the remaining V2R mainly localized to late endosomes/lysosomes. No significant localization to early endosomes was observed under this condition. Upon incubation with 100 nM dDAVP, co-localization studies with E-Cadherin revealed that half of V2R-GFP was internalized within 30 minutes, while the maximal level was obtained within 1 hour. During the first half of this hour, most of the V2R passed the EEA1-positive early endosomes, because their co-localization increased from 3 to 15% within 15 minutes following dDAVP treatment, which was sustained to at least 30 minutes, and subsequently reduced again to 8% at 1 hour after the start of the dDAVP treatment. At present it is unclear whether all sequestered V2R passes EEA1-positive early endosomes or whether a particular fraction trafficks via EEA1-negative endosomes. However, the colocalization of V2R-GFP of about 30, 20 and 50% with E-Cadherin, EEA-1 and LAMP-2, respectively, suggests that most, if not all, V2R-GFP trafficks along this pathway. Following its passage through early endosomes, V2R-GFP accumulates in late endosomes/lysosomes as the level of co-localization of the V2R with LAMP2 increased from 27% at t=0 minutes to 58%, 80% and 83% at t=30, 60 and 120 minutes, respectively, after the start of the dDAVP treatment, respectively. Since the level of V2R internalization is dose dependent, it is anticipated that the time frame of V2R-GFP sequestration is increased with lower doses of dDAVP.

Innamorati et al. reported that in transiently transfected HEK293 cells, dDAVP treatment resulted in the accumulation of HA-tagged V2 receptors in rab11-positive perinuclear recycling compartment (8). They did not observe any localization of HA-tagged V2R in late endosomes/lysosomes. We did not find any co-localization of the fully-internalized receptor with rab11 (not shown). These discrepancies may underscore the cell biological differences in protein localization in polarized versus of non-polarized cells.

dDAVP-induced sequestration of V2R is targeted for lysosomal degradation

From the early endosomes, the internalized receptor can either be recycled to the plasma membrane or degraded in proteasomes or lysosomes after being released

from its agonist in late endosomes(24). In our cell model, V2R-GFP accumulation in late endosomes/lysosomes was observed when cells were stimulated with 100 nM dDAVP for either 30 minutes or 1 or 2 hours. The majority of these receptors is indeed targeted to lysosomes for degradation, as its dDAVP-induced degradation could be prevented to a large extent by blocking of the lysosomal degradation pathway with chloroquine. Consistent with the lysosomal degradation of agonist-bound V2R, dDAVP induced internalization and subsequent degradation reduced the half-life drastically (from 11.5 to 2.8 hours). This is in line with the results of Martin et al., who also observed this effect in transiently-transfected COS cells (12). However, the half-lives they observed for both the unstimulated as well as the AVP stimulated receptor were considerably lower than the half-lives we found. These differences in stability may be due to the cell type used, transient versus stable expression systems, differences in the level of V2R maturation between the different cells and/or due to the usual high expression of V2R in transiently-transfected cells.

In conclusion, we have set up and characterized a polarized renal cell model that shows a V2R maturation, localization and dDAVP-induced internalization and degradation as can be expected for the V2R in vivo. This cell line is therefore a suitable cell model to study the molecular determinants and pathways involved in the regulation of V2R trafficking and to determine the effects of molecular and pharmacological chaperones on the trafficking of ER-retained V2R mutants identified in Nephrogenic diabetes Insipidus.

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Chapter 4

Characterization of vasopressin V2 receptor mutants in Nephrogenic Diabetes Insipidus in a polarized cell model

Abstract

X-linked Nephrogenic Diabetes Insipidus (NDI) is caused by mutations in the gene encoding the vasopressin V2 receptor (V2R). For the development of a tailored therapy for NDI, knowledge of the cellular fate of V2R mutants is needed. It would be useful when this fate could be predicted from the location and type of mutation. To identify similarities and differences in localization, maturation, stability and degradation of C-terminally GFP-tagged V2R mutants, we stably expressed nine mutants in polarized MDCK cells. The mutants V2R-L44P, -Δ62-64, -I130F, -S167T, -S167L and -V206D were mainly expressed in the endoplasmic reticulum (ER) as immature proteins. These mutants had relatively short half-lives due to proteasomal degradation, except for V2R-Δ62-64. In contrast, V2R-R113W, -G201D and -T204N were expressed in the ER and in the basolateral membrane as immature, high-mannose glycosylated, and mature complex glycosylated proteins. The immature forms of V2R-R113W and T204N, but not V2R-G201D, were rapidly degraded. The mature forms varied extensively in their stability and were degraded by only lysosomes (V2R-T204N and wt-V2R) or lysosomes and proteasomes (V2R-G201D, -R113W). These data reveal that most missense V2R mutations lead to retention in the ER and suggest that mutations that likely distort a transmembrane domain or introduce a charged amino acid close to it make a V2R mutant more prone to ER retention. Since six of the mutants tested showed significant increases of intracellular cAMP levels upon transient expression in COS cells, activation of these six receptors following rescue of cell surface expression might provide a cure for NDI patients.

Keywords: GPCR, endoplasmic reticulum, MDCK, protein degradation

Introduction

To increase renal water reabsorption in states of hypernatremia or hypovolemia, pituitary-released arginine-vasopressin (AVP) binds to its vasopressin V2 receptor (V2R) in the basolateral membrane of polarized principal cells. This inter-action induces the binding, activation and cleavage of a trimeric Gs protein, of which the stimulatory αs subunit activates adenylate cyclase to generate cAMP. Through several steps, this eventually redistributes Aquaporin-2 (AQP2) water channels from intracellular vesicles into the apical membrane, rendering this membrane permeable to water. Following an osmotic gradient over the epithelium, water can then be reabsorbed from the pro-urine to the blood.

In the rare inheritable disorder Nephrogenic Diabetes Insipidus (NDI), patients are unable to concentrate their urine in response to AVP, resulting in the excretion of large volumes of dilute urine. Mutations in the AVPR2 gene have shown to be causative for approx. 90% of all NDI cases, and are inherited in an X-linked fashion. Autosomal NDI, either recessive or dominant, accounts for the remainder 10% of cases and is caused by mutations in the AQP2 gene (10). To date, over 180 mutations have been described in the AVPR2 gene, most of them being missense mutations.

Since an effective treatment of patients depends on the cellular defect introduced by the mutation, Zeitlin classified mutations of the cystic fibrosis transmembrane

conductance regulator (CFTR) gene according to their cellular fate into five different classes (27). Recently, we adapted this classification to G-protein coupled receptors in general and the V2R in particular (5).In this classification, class I mutations lead to defects in the synthesis of stable mRNA, resulting in absence of protein. Promoter alterations, aberrant splicing, exon skipping, and most frame-shift and nonsense mutations fall into this category. Class II mutations lead to fully translated proteins, but are trapped in the ER, as they are misfolded. Class III mutants are normally transported to their site of action, but at that location are disturbed in their activation or regulation. For the V2R, mutations on the cytosolic side of the protein that interfere with G-protein binding and thus prevent intracellular signaling, like the R137H mutation (22) fall into this category. Class IV mutations also do not affect protein trafficking, but impair or decrease the receptor's ability to bind ligand, as has been reported for V2R-ΔR202 (1) and V2R-R181C (19). Finally, class V mutations lead to proteins that are not disturbed in any of the above, but are missorted to another organelle in the cell.

Knowledge of the cellular and molecular cause of mutant proteins in diseases is essential for the development of rational-based treatment. To prevent laborious cell biological analyses of all mutants, a prediction on the cellular fate of the mutant protein made on basis of the identified mutation would be useful. However, for mutant proteins in general and the V2R in particular, it is at present unclear whether particular amino acid substitutions or locations of the mutations within the protein make the mutant likely to be of a particular class. In addition, most data on the cellular cause of V2R mutants in NDI have been obtained from transiently transfected cells. Although possibly useful, these cells are not polarized, as renal principal cells, and proteins may rout differently from polarized cells (13). Moreover, due to the high expression levels in transient assays, the mutants are often expressed at several sites, which make interpretation of the proper cellular location difficult. Recently, we generated an MDCK cell line in which exogenous V2R is expressed and regulated by dDAVP as can be anticipated for the V2R in vivo (21). To address the issues above, we selected nine V2R mutations that cause NDI and which are distributed along the V2R protein. Subsequently, we stably expressed these V2R mutants in MDCK cells, and thoroughly analyzed their functionality, localization, degradation pathway and stability.

Materials and Methods

Materials

MG-132 was from Calbiochem (La Jolla, CA); chloroquine diphosphate, cycloheximide, IBMX and dDAVP, were from Sigma Aldrich (St. Louis, MO). The pEGFP-N1-V2R plasmid encoding wt-V2R, C-terminally-tagged with the Green Fluorescent Protein (GFP) (25) was kindly provided by Dr. Alexander Oksche (FMP, Berlin).

Expression constructs

With a three-steps PCR reaction or quick-change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), each mutation was introduced into the human V2R cDNA sequence, using pEGFP-N1-V2R as a template. Primers used were CGCTGCCATCCATAGTCTTTGTGG for L44P, CTGGTGCTGGCGGC CCGGGGCCGGCGGGG for Δ62-64, CCTGTGTTGGGCCGTGAAGTATC

for R113W, GCCTCCTCCTACATGTTCCTGGC for I130F, CCTTCACACTCCTTCTCAGCCTGC for S167T, CCTTCTTGCTCCTTCTCAGCCTGC for S167L, CTGGGATCGTCGCACCTATGTCAC for G201D, GTCGCAACTATGTCA CCTGGATTGC for T204N, and GTCGCACCTATGATACCTGGATTGC for V206D, and their complementary antisense primers. After digesting correct clones with BglII/PstI (L44P, Δ62-64, R113W) or PstI/HindIII (I130F, R137H, S167T, S167L, G201D, T204N and V206D), the mutation-containing fragments were isolated and cloned into the corresponding sites of wtV2R-GFP. Sequence analysis of selected clones confirmed that only the desired mutations were introduced.

MDCK cells

MDCK cells were maintained and stably transfected, and immunocytochemistry and confocal laser scanning microscopy (CLSM) were performed as described (7). As primary antibodies, 1:100-diluted rabbit anti protein disulfide isomerase (PDI; a kind gift of Dr. I. Braakman, Utrecht, The Netherlands), 1:200-diluted mouse anti lysosome associated membrane protein 2 (LAMP-2) antibodies (15); a kind gift of Dr. Le Bivic, Marseille, France) or 1:100-diluted rat anti E-cadherin (Sigma, St. Louis, MO) were used. As secondary antibodies, 1:100-diluted goat anti-rabbit, goat anti-rat or goat anti-mouse IgGs, all three coupled to Alexa-594, were used (Molecular Probes, Leiden, The Netherlands). Semi-quantification of co-localization of V2R with cellular markers was done as described (21).

Stability and degradation experiments

To determine receptor stability, cell lines were incubated for either 0, 2, 4, or 8 hours in culture medium containing 50 μM cycloheximide to block protein synthesis, after which the cells were lysed in 1X Laemmli sample buffer and subsequently analyzed by immunoblotting. To determine the degradation pathway of the receptors, cells were incubated in culture medium containing 50 μM cycloheximide, supplemented with 20 μM MG-132, 100 μM chloroquine, or both. Cells incubated in normal culture medium were used as control. After eight hours, cells were lysed in 1X Laemmli sample buffer and subsequently analyzed by immunoblotting.

Immunoblotting

Protein samples were prepared and analyzed on a 10% PAAG and subsequently blotted as described (7). For detection of V2R-GFP, 1: 5,000 diluted rabbit anti-GFP antiserum (3) (kindly provided by Dr. B. Wieringa, UMCN, Nijmegen) or 1: 20,000 mouse anti-β-actin (Sigma Aldrich, St. Louis, MO) were used. As secondary antibodies, goat anti-rabbit or sheep anti-mouse IgGs coupled to horseradish peroxidase (Sigma, St. Louis, MO) were used at a 1:5000 dilution. Immunoblot signals were semi-quantified using Image-Pro software (Media Cybernetics, Silver Spring, MD) as described(21). Data of at least three experiments were used for quantification.

cAMP measurement, [^{3}H]AVP binding and cell surface biotinylation

COS-M6 cells were seeded in six-wells plates and grown to 80% confluence. Subsequently, 5 mg plasmid DNA encoding either wild type or mutant V2R-GFP were transfected into the cells using lipofectamine. The next day, cells were seeded at 80% confluence and grown overnight in DMEM with 10% fetal calf serum in a humidified 37°C incubator under 5% CO_2. The cells were subsequently treated with prewarmed

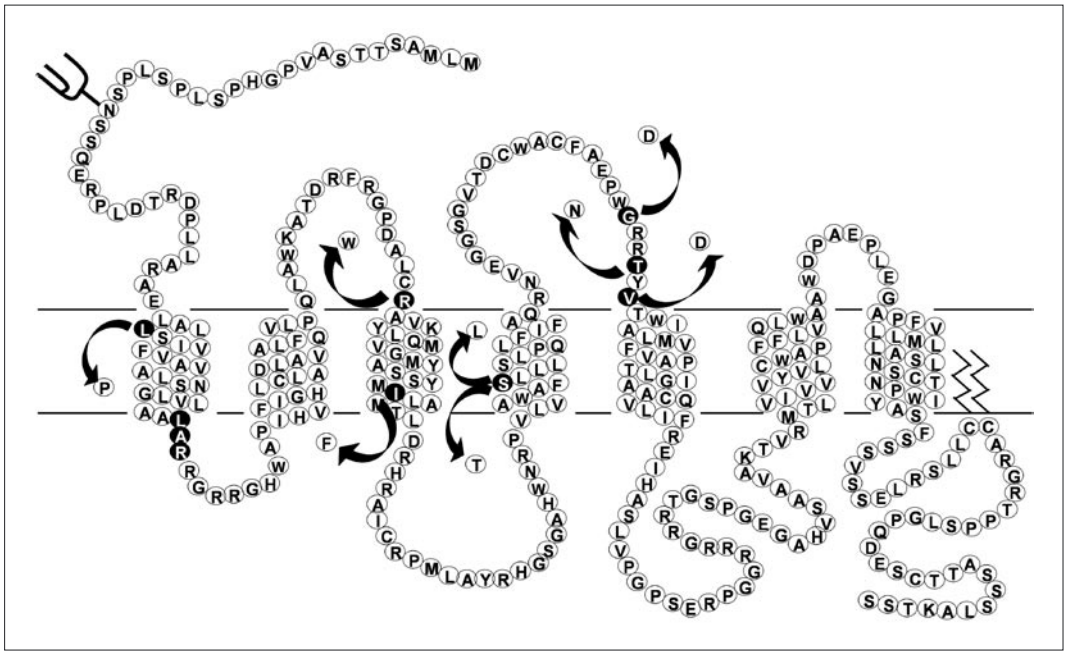

Figure 4-1 **Location of mutations in the V2R protein**
Topology map of the human V2R. Mutated residues are indicated in black; introduced amino acids are indicated by arrows.

medium containing 100 nM dDAVP for 5 minutes in the absence of IBMX. After washing the cells briefly in prewarmed PBS-CM, the cells were lysed in 100 ml of 0.1 M HCl, and cAMP was measured using a fluorescent cAMP assay kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Assay plates were read in the Model 3550-UV Microplate Reader (Bio-Rad, Hercules, CA) using an excitation wavelength of 405 nm. Measurements were performed at least in triplo.

For radioligand binding assays, transfected COS-M6 cells were grown overnight, seeded at a density of 5.0 x 10^5 cells/cm^2 in 12-wells plates and again grown overnight. Radioligand labeling was performed as described(21). Averaged data of at least three independent experiments were used.

For cell surface biotinylation, transfected COS-M6 cells were grown for 2 days, and subsequently biotinylated as described (7). Instead of filters, COS-M6 cells were grown on plastic support.

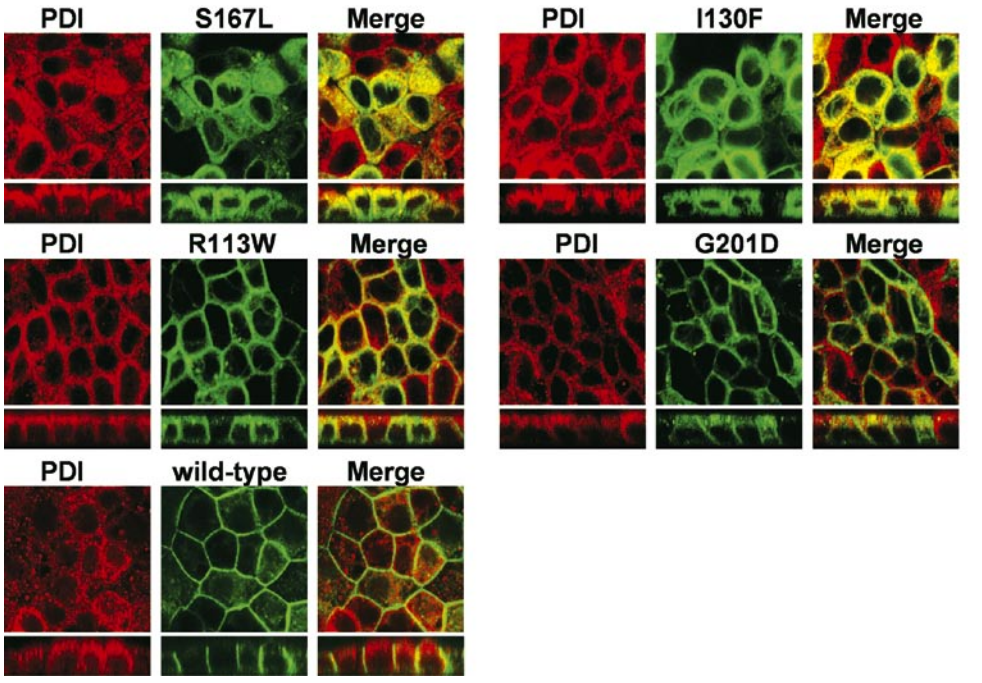

Figure 4-2 **Localization of NDI causing V2R mutants.**
MDCK cell lines stably expressing V2R-S167L, -I130F, -R113W, -G201D or wild-type V2R, were grown to confluence and fixed. Immunocytochemistry was done for protein disulphide isomerase (PDI) to stain the ER. The signals for PDI and the V2R are shown in red and green, respectively.

Results

Localization of the V2R mutants.

The nine V2R mutations L44P, R113W, S167L, T204N, V206D (11), Δ62-64 (2), I130F (20), S167T (17) and G201D (23), which are dispersed over the V2R protein (Fig.1), have been shown to cause NDI. In MDCK type I cells, the localization, maturation and dDAVP-induced internalization of V2R-GFP mimics that anticipated for V2R in principal cells to a great extent (21). Therefore, we stably-transfected MDCK cells with expression constructs encoding the V2R-GFP mutants mentioned. Multiple positive clones were isolated for each mutant. Immunocytochemistry and subsequent CLSM analysis of V2R-S167L and -I130F showed a nearly complete co-localization with the ER marker PDI (Fig. 2, upper panel). Semi-quantification revealed a co-localization of 94.1 +/- 4.5% and 94.3 +/- 4.2%, respectively. The mutants V2R-L44P (92.4 +/- 5.1%), -Δ62-64 (94.4 +/- 3.4%), -S167T (92.3 +/- 3.9% and -V206D (95.7 +/- 3.6%) showed similar results (not shown).

Confocal analysis of V2R-R113W and -G201D (Fig. 2, middle panel), however, showed only a partial co-localization with the ER marker PDI (53.3 +/- 5.5% and 50.3 +/- 8.5 %, respectively). The remainder co-localized with the basolateral marker E-cadherin in the lateral membrane (44.8 +/- 6.9% and 46.1 +/- 8.1%, respectively) or with the late endosomal/lysosomal marker LAMP-2 (4.2 +/- 2.4% and 5.6 +/- 3.1%; not shown). Similar results were obtained for V2R-T204N (51.3 +/- 7.9% with PDI; 43.2 +/- 5.1% with E-cadherin; 5.1 +/- 2.3% with LAMP-2; not shown). As reported (21), wt-V2R was expressed predominantly in the lateral plasma membrane of MDCK cells (75%), and to a lesser extent in late endsomes/lysosomes (25%), and did not show any co-localization with the ER marker protein (Fig. 2, lower panel).

Maturation state of the V2R mutants.

To test the maturation states of the different V2R mutants, total cell lysates were undigested, digested with endo H, which removes N-linked high-mannose sugar groups, or with PNGase F, which removes all N-linked sugar moieties, and immunoblotted. Recently, we have reported that V2R-GFP is mainly expressed as complex-glycosylated proteins of 75 kD (Fig. 3, lower panel) (21). With endo-H, the majority remained unaffected, while some 60 kD core-glycosylated V2R appeared. With PNGase F, the 75 kD band was reduced to 67 kD, which might represent O-glycosylated V2R.

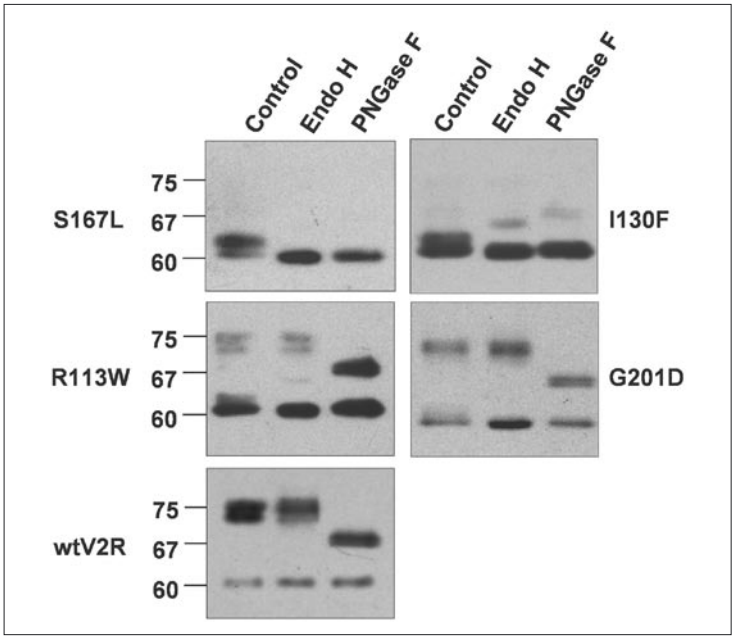

Figure 4-3 **Maturation of NDI causing V2R mutants.**
To determine the glycosylation state of V2R-S167L, -I130F, -R113W, -G201D or wt-V2R (indicated), confluent MDCK-V2R-GFP cells were lysed in Laemmli buffer, treated without any enzyme (control), or with endo H or PNGase F, and analyzed by immunoblotting using anti GFP antibodies. Estimated masses (in kD) are given on the left.

Western blot analysis of undigested V2R-S167L (Fig. 3, upper left panel) showed a strong 60 kD core protein and a second band of approximately 63 kD. This latter band disappeared with Endo H (and PNGase F) digestion, which indicated that this band represents high-mannose glycosylated V2R-GFP. These data suggested that most, if not all V2R-S167L is retained in the ER. Similar results were obtained for V2R-L44P, -Δ62-64, -S167T and -V206D (not shown).

V2R-I130F was also mainly expressed as 60 and 63 kD bands, although weak bands of 67 and 75 kD were observed (Fig. 3, upper right panel). Upon digestion with Endo H, the weak 75 kD and strong 60 kD bands remained, while a faint band of approx. 65 kD appeared. The 63 kD band disappeared with endo H. With PNGase F, a weak 67 kD band and strong 60 kD were observed. The presence of the weak 75, 65 and 67 bands indicated that a small fraction of V2R-I130F has left the ER and was partially (65 kD with endo H) or fully (75; 67 kD with PNGase F) matured.

Analysis of the maturation of V2R-R113W revealed similar bands as those observed for V2R-I130F, except here the bands of 75 (control and endo H) and 67 (PNGase F) kD, were stronger for V2R-R113W (Fig. 3, middle left panel). V2R-G201D was even more matured, because the 75 kD and 67 (PNGase F) bands were of similar intensity as the core form (Fig. 3, middle right panel). Similar data as of V2R-G201D were obtained for V2R-T204N (not shown). Semi-quantification of the signals confirmed the differences in maturation, because V2R-I130F, -R113W, -G201D and -T204N are for 7.3 +/- 4.1%, 59.2 +/- 5.9%, 64.3 +/- 7.1%, and 66.6 +/- 4.5% expressed as 75 kD proteins, respectively (n=3), while the remainder comprises core and high-mannose glycosylated proteins. These data demonstrate that the mutants V2R-R113W, -G201D and -T204N are only partially retained in the ER and that a considerable proportion undergoes maturation. Similar patterns were observed for transfected clones with different expression levels of these mutants (not shown), indicating that the observed differences in maturation were related to the mutation and not due to clonal differences in expression levels.

Stability of V2R mutants

As introduced mutations may affect the stability of proteins, we determined the half-lives (T½) of the V2R mutants. Following a protein synthesis block with cycloheximide, the total V2R content of the cells was monitored in time. V2R-S167L (Fig. 4, top panel) showed a rapid decrease of its 60/63 kD bands (here they run as one band). Semi-quantification of the signals (n=3) revealed a T½ of 1.5 +/- 0.4 hours for V2R-S167L. The T½ of V2R-L44P (1.5 +/- 0.3 hours), -S167T (2.4 +/- 0.4 hours), and -V206D (3.2 +/- 0.7 hours) were in the same range (not shown). The 60/63 kD bands of V2R-Δ62-64, however, were much more stable, as it has a calculated T½ of 12.5 +/- 3.4 hours (Fig. 4, 2nd panel). The mutant V2R-I130F (Fig. 4, 3rd panel), also showed a rapid decrease of its 60/63 kD bands, from which we calculated a T½ of 1.9 +/- 0.5 hours. We were not able to quantify the stability of V2R-I130F's complex glycosylated form, as this signal was too weak for this analysis.

As V2R-R113W, -G201D and -T204N are expressed as core-glycosylated (60/63 kD) and complex-glycosylated (75 kD) proteins, we analyzed the stability of both forms. Semi-quantification revealed that complex-glycosylated V2R-R113W had a half-life of 14.4 +/- 3.2 hours. In contrast, the 60/63 kD form had a T½ of only 2.1

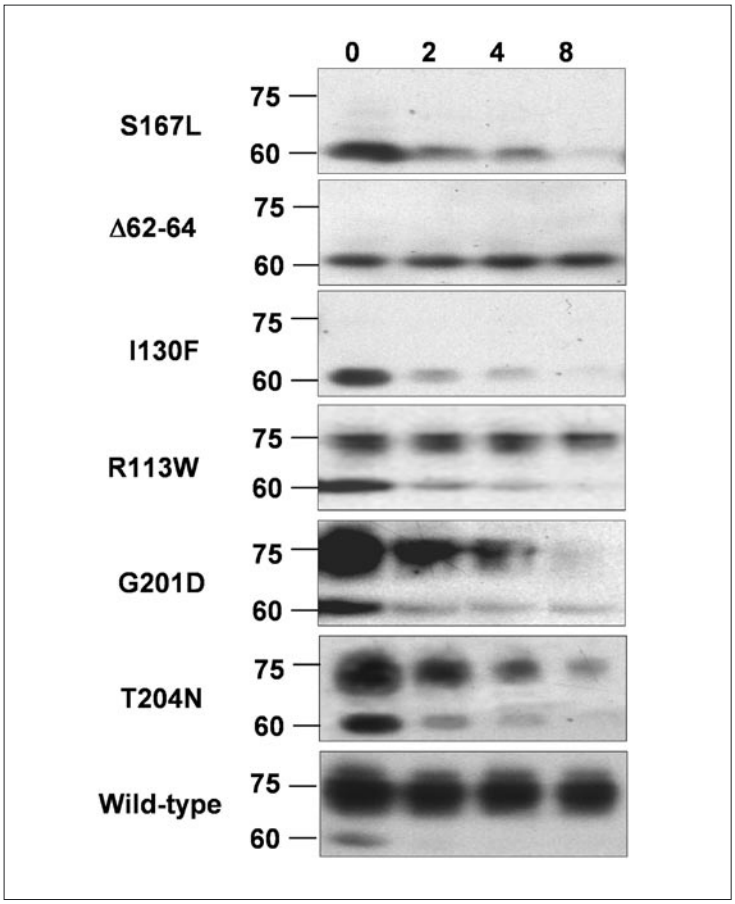

Figure 4-4 **Stability of NDI causing V2R mutants**

MDCK cells expressing V2R-S167L, -Δ62-64, -I130F, -R113W, -G201D -T204N or the wild-type V2R were grown to confluence and subsequently treated with 50 µM cycloheximide to block protein synthesis. At the indicated time points (in hours), cells were lysed in Laemmli sample buffer and analyzed by immunoblotting as indicated in the legend of figure 3. Estimated masses (in kD) are given on the left.

+/- 0.3 hours. For V2R-G201D, the T½ of the complex glycosylated form was only 4.1 +/- 1.5 hours. Although a rapid decrease is observed during the first 2 hours for the high-mannose glycosylated V2R-G201D, hardly any degradation is observed beyond this period, leading to a calculated T½ of 10.2 +/- 3.0 hours. For V2R-T204N, the half-lives of the complex-glycosylated form and the high-mannose glycoyslated form were 2.2 +/- 0.6 and 2.1 +/- 0.2 hours, respectively (Fig. 4, 2nd bottom panel). Again, similar half-lives were obtained from two independent clones per construct, indicating that the differences in half-lives are not due to clonal differences. As reported, wt-V2R has a half-life of 11.5 +/- 2.8 hours (21).

Degradation pathway of the V2R mutants

Proteins can be degraded by the proteasomal pathway, which can be inhibited by MG132, or the lysosomal pathway, which is inhibited by chloroquine and sometimes MG132. Considering the differences in stability of the mutants, we next studied their degradation pathways by incubating the cells for eight hours with cycloheximide alone or combined with MG132, chloroquine, or both. As shown in figure 5 (top and second panel), the degradation of V2R-S167L and - I130F was nearly completely prevented by MG132, while chloroquine had no effect on its own, nor in combination with MG132. Similar results were obtained for the mutants V2R-L44P, -Δ62-64, -S167T, and -V206D (not shown).

The degradation of the complex-glycosylated bands of V2R-R113W was partially prevented by co-incubation with either chloroquine or MG-132 (Fig. 5, middle panel). Incubation with both inhibitors revealed no degradation at all. Strikingly, of the two most prominent bands of complex-glycosylated V2R-R113W, chloroquine stabilized the larger form, whereas MG-132 treatment mainly prevented degradation of the lower band. The 60/63 kD band was only partially prevented from degradation by the combination of MG132 and chloroquine.

For V2R-G201D (Fig. 5, fourth panel), chloroquine treatment did not prevent degradation of the high-mannose or complex glycosylated form. MG-132, however, prevented degradation of both, resulting in similar protein levels as found in the untreated sample. Combined treatment of chloroquine and MG-132 did not show an additional effect compared to MG-132 alone. For V2R-T204N (Fig. 5, 2nd bottom panel), cycloheximide treatment resulted in decreased V2R protein levels of both the complex- and the 60/63 kD forms. Chloroquine treatment completely prevented degradation of the complex-glycosylated form of V2R-T204N, but did not prevent degradation of the 60/63 kD forms. MG-132 partially prevented the degradation of the complex-glycosylated form (about 14%), and completely prevented the degradation of the 60/63 kD forms of the V2R-T204N. Combined MG132/chloroquine treatment did not result in more 60/63 kD or complex-glycosylated V2R-T204N when compared to MG132 or chloroquine alone. Degradation of the complex-glycosylated form of the wtV2R (fig. 5, bottom panel) could completely be prevented by co-treatment with chloroquine, whereas MG132 was able to prevent degradation of the high-mannose glycosylated form.

Receptor functionality

To assess which V2R mutants are functional, the mutant's ability to generate cAMP in response to a dDAVP treatment was determined. As MDCK type I cells express low levels of endogenous V2R (6; 21), receptor functionality assays were performed in COS cells, as these cells do not endogenously express V2Rs. Furthermore, To obtain some mutant receptor expressed at the cell surface, these cells were transiently-transfected as this mostly gives high expression levels. As shown in figure 6A, dDAVP binding of the mutants V2R-L44P, -R113W, -I130F, -S167T, -G201D, and -T204N resulted in significantly increased cAMP levels compared to mock-transfected cells ($p<0.001$; $n\geq3$), indicating that these mutants can translate AVP-binding into a cAMP response. The mutants V2R-_62-64, -S167L and -V206D showed no significant cAMP increase compared to mock-transfected cells. Since the expression of these three mutants was similar or higher than that of functional V2R-S167T (Fig. 6B) and have similar

localization and maturation characteristics (Figs 2 and 3), these three mutants were likely to be interfered in their binding to dDAVP or to activate a Gs protein.

To investigate this further, COS cells expressing V2R-GFP, V2R-Δ62-64, -S167L -V206D, and the functional mutants V2R-L44P or -I130F, were subjected to radioligand binding assays. As shown in figure 6C, V2R-L44P, -I130F and -Δ62-64 bind significantly more [^{3}H]AVP than mock-transfected cells ($p<0.001$; $n\geq3$). No significant binding was observed for cells expressing V2R-S167L and –V206D ($p>0.05$; $n\geq3$). V2R-GFP expressing cells bound radioligand to 19619+/-2453 counts (not shown).

Since the lack of AVP binding and cAMP generation could be due to the lack of expression in the plasma membrane, transfected COS cells were subjected to cell surface biotinylation assays. Immunoblotting of the obtained samples, however, revealed that the plasma membrane expression levels of the non-AVP binding receptors, V2R-S167L and V2R-V206D, were clearly higher than that of the functional V2R-L44P (Fig. 6D). In these biotinylation samples no signals for β-actin were observed (Fig. 6D, lower panel), while strong actin signals were obtained in the corresponding lysate sample of COS cells expressing wtV2R (Fig. 6D) or the mutants (not shown). This indicated that the biotinylation samples did not contain intracellular proteins. Therefore, these data reveal that V2R-S167L and V2R-V206D are unable to generate an intracellular cAMP response due to their inability to bind AVP.

Discussion

All V2R mutants are retained in the ER, but to different levels

To study the cellular fate (summary in table 1) of nine V2R mutants involved in NDI, MDCK cells were stably transfected with expression constructs for these proteins. CLSM analysis revealed that all mutants co-localized with the ER marker protein PDI, indicating their retention in the ER. The level of ER retention, however, differed between mutants. Whereas the mutants V2R-L44P, -Δ62-64, -S167T, -S167L and -V206D were restricted to the ER (group 1), a considerable fraction of V2R-R113W, -G201D and -T204N was expressed in the basolateral membrane (group 2; Fig. 2). Their level of maturation was consistent with the observed localizations, as the members of group 1 were only expressed as non-glycosylated 60 kD or core-glycosylated 63 kD proteins, while the group 2 receptors were expressed as immature 60/63 kD proteins and mature complex glycosylated proteins (Fig. 3). The pronounced ER retention has also been reported for V2R-Δ62-64 and –S167L in HEK293 cells (9) and for the V2R mutants L292P, DV278 and R337X in polarized MDCK cells (26).

An exception is formed by V2R-I130F, which seemed restricted to the ER with immunocytochemistry, but of which immunoblot data of the Endo H or PNGase F digestions revealed a small fraction of intermediate or complex glycosylated receptor. Of V2R-I130F, only 7.3% is expressed in a mature form, whereas 60-70% of V2R-R113W, -G201D and -T204N is expressed in the mature form. Therefore, mature V2R-I130F may possibly be present in the basolateral membrane at levels that could not be detected by immunocytochemistry. Taken together, these data indicate that the level of ER retention and maturation of V2R-I130F is in between that of the two groups.

Our finding that all mutants are –at least partially- retained in the ER illustrates the high sensitivity of the ER quality control mechanism to identify mutant proteins. Strikingly, based on the proposed topology of the V2R (24), all V2R mutations leading to a strong ER retention are located in either one of the transmembrane domains (TMD; L44P, I130F, S167T, S167L) or change a hydrophobic into a charged amino acid at the edge of a TMD (L into R in V2R-Δ62-64, V206D). These mutations might interfere with the proper folding or positioning of the hydrophobic TMDs of which luminal or cytosolic exposure is thought to render proteins prone to recognition by the ER quality control mechanism, ER retention and subsequent degradation (8; 12). The slightly better maturation of V2R-I130F compared to the others might be due to exchange of a hydrophobic amino acid for another in the TMD of V2R-I130F. In contrast, with V2R-L44P, the introduced proline is likely to introduce a knick and therefore distort the a-helical structure of TMDI.

Our finding that V2R mutants of which the mutations are located in the third extracellular loop (G201D and T204N) or introduce a hydrophobic amino acid at the edge of the first extracellular loop and transmembrane (TM) domain III (R113W), suggests that mutations located on the ER luminal side of V2R are

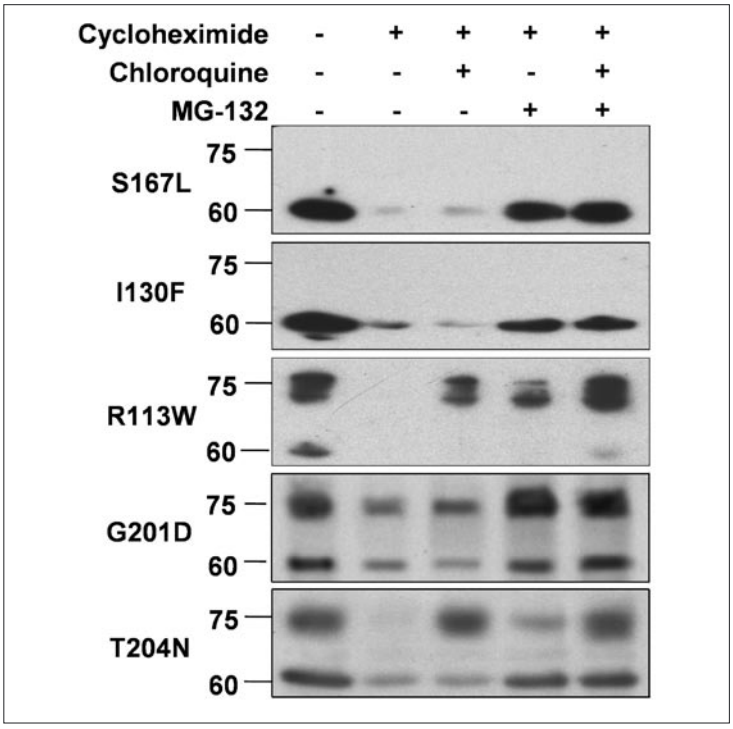

Figure 4-5 **Degradation pathways of NDI causing V2R mutants**
Cell lines stably expressing V2R-S167L, -I130F, -R113W, -G201D –T204N or wtV2R were treated for 8 hours as indicated in the figure. Western blot analysis was performed as described in the legend of figure 3. Estimated masses (in kD) are given on the left.

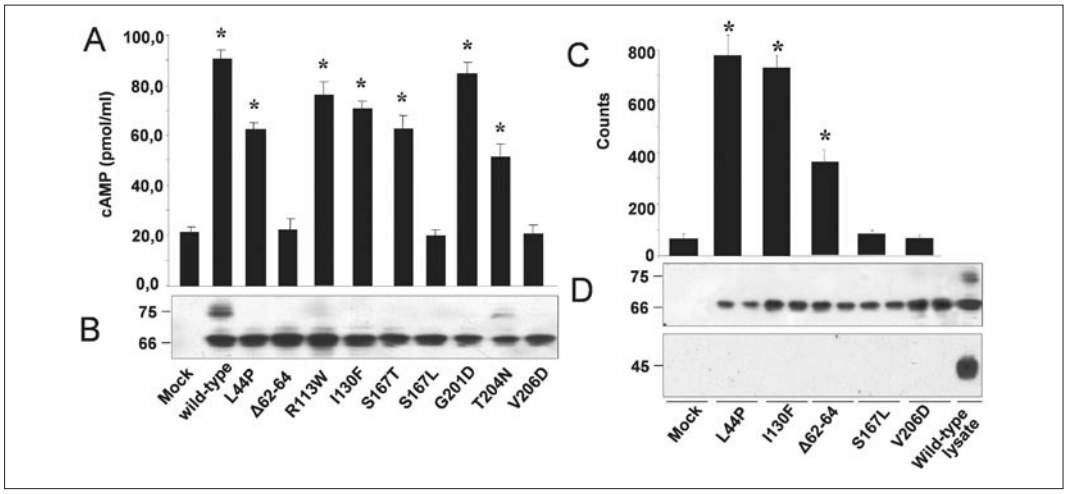

Figure 4-6 **Functionality of NDI causing V2R mutants**

A) cAMP generation. COS-M6 cells transiently expressing either wild-type or mutant V2R proteins were treated for 5 minutes with 100 nM dDAVP, washed in PBS-CM and lysed in 0.1 M HCl, followed by cAMP measurement in a fluorescence-based assay. Samples significantly differing from mock-transfected cells are indicated by an asterisk. ($p<0.001$; $n\geq3$)

B) V2R protein expression levels. Cell equivalents from the experiment under (A) were lysed in Laemmli buffer and subjected to immunoblotting as described under figure 3.

C) AVP binding. Transiently transfected COS cells were incubated for 1 hour at 4°C with 100 nM [^{3}H]AVP in PBS-CM followed by washing, scraping, and counting in a scintillation counter. Significant differences in radioligand binding compared to mock-transfected cells are indicated by an asterisk ($n=3$, $p<0.01$)

D) Cell surface expression of V2R proteins. Transiently transfected COS cells from experiment C were subjected to cell surface biotinylation. Cell surface proteins were subjected to immunoblotting for V2R (top) or β-actin (bottom) as described under figure 3. As a control for the antibodies, the utmost right lane contains a total lysate sample of COS cells expressing wtV2R.

causing less severe misfolding of the receptor protein, compared to mutations in the TM domains. Alternatively, they might be less well recognized by ER quality control proteins than transmembrane or cytosolic segments. Definite answers to these issues can only be given when the atomic structure of the V2R has been elucidated.

Stability and degradation of V2R mutants.

To further study the fate of the V2R mutant proteins, their stability and degradation pathways were investigated (Figs. 4,5; table 1). Compared to the half-life of complex-glycosylated wt-V2R, the stability of ER-retained mutants was in general drastically reduced (up to 8.5-fold). Also, degradation of the ER-retained V2R forms (60/63 kD) occurred for most mutants through the proteasomal pathway, as MG132, which interferes proteolysis by the proteasome, greatly stabilized most 60/63 kD V2R mutants. These data are consistent with the current view that wild-type or mutant proteins targeted for degradation from the ER are processed via the so-called ER as-

sociated degradation (ERAD) pathway, which involves the recognition of misfolded proteins by molecular chaperones, their transport from the ER to the cytoplasm through an ER translocon and degradation in the cytosol by proteasomes. V2R-G201D and V2R-Δ62-64 seem to form exceptions to this rule. Although a considerable portion of the 60/63 kD V2R-G201D proteins was degraded within 2 hours, a small fraction appeared very stable (8 hours; Fig. 5). This might indicate that, whereas most V2R-G201D is recognized as misfolded and degraded, a small portion might be resistant to recognition by molecular chaperones, transport from the ER and/or degradation by proteasomes. More interesting, however, the half-life of V2R-Δ62-64 was similar to that of wt-V2R. Since this is the only mutant in which amino acids are deleted instead of exchanged, it might suggest that the ERAD pathway is more sensitive to missense mutations than to a lack of amino acids. Alternatively, since we only studied one deletion mutant, the difference might be just coincidental. Therefore, analysis of more V2R deletion mutants is required to address this issue.

For the complex glycosylated forms of the mutants (i.e. V2R-R113W, -G201D, T204N), the stability and degradation pathways vary considerably and appear unrelated to the stability and degradation pathways acting on the immature receptors. Mature V2R-GFP was degraded only by the proteasomal pathway, as only chloroquine inhibited its degradation. V2R tagged at its N-terminus with an HA-tag showed a similar stability (T½ = 12.2 +/- 3.8 h) and degradation pathway as V2R-GFP (not shown), which indicated that neither the tag (GFP or HA) nor its site of coupling (N- or C-terminal) affected V2R degradation. This was different for the V2R mutants. While V2R-T204N is also only degraded by the lysosomal pathway, V2R-R113W degradation was inhibited by MG132 and chloroquine, while V2R-G201D degraded was only inhibited by MG132. These results might indicate that mutations that are located at the luminal side of the membrane can induce the degradation of mature V2R by proteasomes, which are thought to degrade proteins from the cytosolic side. Alternatively, these V2R mutants are also degraded in lysosomes, as MG132 is also a highly potent inhibitor of various cysteine proteases and cathepsins [29,30]. The degradation pathway used for the mature mutants, however, does not appear to be indicative for their stability, because V2R-G201D and V2R-T204N are 3-6 times less stable than wt-V2R, whereas mature AQP2-R113W is as stable as wt-V2R. Complex-glycosylated proteins are formed from core-glycosylated proteins in the Golgi complex and, therefore, the observed stabilities of the V2R proteins might be overestimated. In the same line, the observed high stability of V2R-R113W compared to V2R-G201D and –T204N might be due to a higher level of immature V2R-R113W processed through the Golgi complex. Although this can not be excluded, the similar or low stability of immature V2R-R113W compared to those of V2R-G210D and –T204N render this possibility rather unlikely. More likely, the observed differences are due to different impacts of the mutations on the structure of the V2R. How mature V2R mutants are targeted for proteasomal and/or lysosomal degradation, however, remains unclear.

Functionality of V2R mutants

All mutants tested, with the exception of V2R-Δ62-64, -S167L and -V206D, showed significantly increased cAMP levels compared to non-transfected COS cells in a transient over-expression system, and were thus designated as being functional. Of the "non-functional" mutants, only V2R-Δ62-64 was able to bind AVP. V2R-S167L and –V206D did not bind detectable levels of AVP, although their membrane expression lev-

els were higher than that of the AVP-binding mutant V2R-L44P, which indicated that the lack of V2R-S167L and –V206D to mediate a cAMP response is due to a reduced ability to bind AVP. In contrast to us, Morello et al. found a cAMP response with rescued V2R-Δ62-64 (14). This discrepancy might be caused by their use of the PDE inhibitor IBMX, their 100-fold higher dDAVP concentration, the longer cAMP accumulation time (20 min versus 5 min here) and/or increased expression of this mutant receptor due to its rescue to the cell surface by the V2R antagonist SR121463A. Both data, however, are in line with the hypothesis of Oksche et al., that the 1st intracellular loop of the V2R is involved in Gs protein binding and that this is greatly reduced by the Δ62-64 mutation, thereby reducing the activation of adenylate cyclase (16). With the V2R-V206D mutant, we have a discrepancy with the data from Postina et al., who observed a residual cAMP formation upon stimulation, but no AVP binding. Here, it might be due to the higher amount of DNA in their transient COS transfection experiments (nearly 1 ug DNA/cm2 compared to 0.5 ug/cm2), the presence of IBMX, the long incubation time with dDAVP (40 min compared to 5 min) and/or the higher dDAVP concentration used (1 uM). However, our data are consistent with the computer model of Czaplewski et al., which hypothesizes that V206 is important for AVP binding (4).

Classification of mutations in the V2R

In line with our cell biological data, patients encoding V2R mutants that are severely ER retarded in our cell model (V2R with L44P, Δ62-64, I130F, S167T, S167L, V206D) do not increase their urine concentration in response to administration of dDAVP (2; 11; 18; 20). In contrast but consistent with the observed maturation and partial basolateral membrane expression in MDCK cells, patients encoding V2R-G201D (23) or -T204N (NVAM Knoers, unpublished results) are able to increase their urine concentrating ability upon administration of a high dose of dDAVP. For patients carrying the R113W mutation no increased urine concentrating ability after dDAVP administration has been reported in patients. Overall, these parallels with in vivo findings provide further support that stably-transfected polarized MDCK cells are a good model to study the cell biological features of wt and mutant V2R proteins.

In conclusion, our data reveal that all nine V2R mutations studied are of class II, because all V2R mutants studied are retained in the ER. The V2R-R113W, -G201D and -T204N mutants, however, are also partially expressed in the basolateral membrane of MDCK cells and initiate a cAMP response following AVP binding, which reveals that these mutations are also of class IV. It has to be noted, however, that we can not exclude the possibility that the mutations studied result in unstable V2R (pre-)mRNAs in vivo (i.e. class I mutations).

Recently, Morello et al. showed that the cell surface expression of several V2R mutants of class II could be rescued by cell permeable V2R antagonists (14). Here, we found that all nine studied V2R mutations were of this class of which six (L44P, R113W, I130F, S167T, G201D, T204N) turned out to be able to translate AVP binding into a cAMP response. Therefore, identifying cell permeable pharmacological chaperones that are able to rescue these six mutants, may eventually lead to a specific treatment to relieve NDI patients harboring functional class II V2R mutations from their disease.

	L44P	Δ62-64	S167T	S167L	V206D	I130F	R113W	G201D	T204N	wtV2R
Location of the mutation	TM I	ICL 1	TM IV	TM IV	ECL 2	TM III	ECL 1	ECL 2	ECL 2	N.A.
Cellular Localization	ER	ER	ER	ER	ER	ER	ER, PM, LE	ER, PM, LE	ER, PM, LE	PM, LE
Glycosylation	HM	HM	HM	HM	HM	HM /(C)	HM /C	HM/C	HM/C	C
Half-life (hours)	1.5	12.5	2.4	1.5	3.2	1.9	2.1/14.4	10.2/4.1	2.1/2.2	11.5
Degradation	MS	MS	MS	MS	MS	MS	MS/CS	MS/CS	MS/CS	CS
AVP binding	+	+	N.A	-	-	+	N.A.	N.A.	N.A.	+++
cAMP response	+	-	+	-	-	+	++	+++	++	+++
Mutant Class	II	II	II	II	II	II	II and IV	II and IV	II and IV	

Table 4-1 **Localization, maturation, functionality and classification of wild type and mutant V2R.**

Summary of the data from figs. 1-6. Abbreviations used: TM, transmembrane domain; ICL, intracellular loop; ECL, extracellular loop; PM, plasma membrane; LE, late endosomes and lysosomes; ER, endoplasmic reticulum; C, complex-glycosylated; HM, high-mannose glycosylated; MS, MG132-sensitive degradation; CS, chloroquine-sensitive degradation; N.A., non-applicable. Mutants were classified according to Deen et al. (5)

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Chapter 5

Rescue of vasopressin V2 receptor mutants by chemical chaperones: specificity and mechanism

Abstract

As missense mutations in genetic diseases of membrane proteins often result in endoplasmic reticulum (ER) retention of functional proteins, drug-induced rescue of their cell surface expression and understanding the underlying mechanism is of clinical value. To study this, we tested chemical chaperones and SERCA pump inhibitors on Madin-Darby canine kidney cells expressing nine ER-retained vasopressin type-2 receptor (V2R) mutants involved in Nephrogenic Diabetes Insipidus. Of these nine, only V2R-V206D showed improved maturation and plasma membrane rescue with glycerol, DMSO, thapsigargin/curcumin, and ionomycin, but not with other osmolytes or growth at 27°C. This revealed that rescue is mutant-specific and that this mutant is prone to rescue by multiple compounds. Rescue did not involve changed expression of molecular chaperones calnexin, HSP70 or HSP90. V2R antagonist SR121463B treatment revealed that V2R-V206D and V2R-S167T were rescued and matured to a greater extent, suggesting that the rescuing activity of a pharmacological versus chemical chaperone is broader and stronger. Calcium measurements showed that rescue of V2R-V206D by thapsigargin, curcumin and ionomycin was due to increased $[Ca^{2+}]_{cyt}$, rather than decreased $[Ca^{2+}]_{ER}$. The molecular mechanism underlying rescue by DMSO, glycerol and SR121463B is different, as with these compounds intracellular calcium levels were unaffected.

Introduction

In 50 percent of congenital diseases, such as cystic fibrosis (20), long QT syndrome (11) or Nephrogenic Diabetes Insipidus (NDI)(4), the underlying cause is the presence of missense mutations in the gene involved. Cell culture expression of the corresponding mutants revealed that the majority of these proteins are misfolded and retained by molecular chaperones of the quality control machinery in the endoplasmic reticulum (ER), after which they are usually targeted for proteasomal degradation. Despite the mutation, several studies have reported that these so-called class II mutants may be functional on a molecular level, which implicates that compounds that restore the routing of such mutants to the cell surface may be useful as a therapeutic.

A group of compounds known as 'chemical chaperones' is able to facilitate the escape of mutant proteins from the ER quality control mechanism, leading to their translocation to the proper subcellular location (24). Among these compounds are osmolytes, such as glycerol, di-methyl sulphoxide (DMSO) and tri-methyl-amine-N-oxide (TMAO) (17; 21; 22; 25) that may facilitate rescue by stabilizing a mutant protein's conformation or by inducing a stress response, leading to upregulation of molecular chaperones (8; 24). Also, inhibitors of sarco- and endoplasmic reticulum calcium ATPases (SERCA), such as thapsigargin and curcumin, have been reported to induce plasma membrane rescue (6; 9; 10). As these SERCA pumps help maintaining high $[Ca^{2+}]_{ER}$ levels, the inhibitors cause decreased ER calcium levels, which are thought to affect the action of ER molecular chaperones, thereby allowing mutant proteins to leave the ER (2). Finally, factors that increase expression of heat-shock proteins (HSPs), such as 4-phenyl butyric acid (4-PBA) or growth at reduced temperature, are sometimes able to rescue the cell surface expression of ER retained proteins (3). Although rescued cell surface expression has been reported for several chemical chaperones it is still unclear whether rescue is mutation-specific, and whether certain mutants are more prone to

be rescued. In addition, the mechanisms by which chemical chaperones rescue the cell surface expression of ER-retained mutants are still largely unknown.

Nephrogenic Diabetes Insipidus (NDI) is a disease in which the kidney is unable to concentrate urine in response to vasopressin, resulting in polyuria and polydipsia. The autosomal dominant and recessive inheritable forms are caused by mutations in the gene encoding the water channel aquaporin-2, whereas X-linked recessive NDI involves mutations in the vasopressin V2 receptor (V2R) gene (4). Recently, we showed that in polarized Madin-Darby Canine Kidney (MDCK) cells, stably-expressed human V2R coupled to green fluorescent protein (wtV2R-GFP) is mainly localized in the basolateral membrane and is regulated as can be anticipated to occur in vivo (15). Also, we found that nine V2R missense mutants in NDI were predominantly retained in the ER when stably expressed in polarized MDCK cells (16). To address the issues above, we tested seven chemical chaperones and incubation at decreased temperature for their ability to rescue the plasma membrane expression of these nine V2R mutants. In addition, we set out to determine the molecular mechanism underlying the role of cytosolic and ER calcium levels in the rescue of V2R mutants.

Materials and Methods

Materials

Glycerol was from Invitrogen (Carlsbad, CA), DMSO, TMAO, curcumin, thapsigargin and probenecid were from Sigma (St. Louis, MO), 4-PBA was from Aldrich (Gillingham, UK) FURA-2-AM, BAPTA-AM and Pluronic were from Molecular Probes (Leiden, The Netherlands). SR121463B was kindly provided by Dr. C. Serradeil-Le Gal (Sanofi Synthélabo, Toulouse, France).

Expression constructs

Using the quick-change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), mutations were introduced into the human V2R cDNA sequence, using pEGFP-N1-V2R (18) as a template, the primers GTCGCACCTATC TCACGTGGATTGCCCTGATG (V206L), CCGTCGCACATATGAAACCTGG ATTGCCCTG (V206E), and CGTCGCACCTACAGAACCTGGATTGCCCTG (V206R), and their complementary antisense primers. After digestion of mutagenized clones with PstI/HindIII, the mutation-containing fragments were isolated and cloned into the corresponding sites of pEGFP-N1-V2R. Sequence analysis of selected clones confirmed that only the desired mutations were introduced.

Cell culture and chemical chaperone treatments

MDCK type I cells stably expressing the GFP tagged wt-V2R or its mutants V2R-L44P, -Δ62-64, -R113W, -I130F, -S167T, -S167L, -G201D, -T204N or –V206D were generated and maintained as described. Stable transfection of MDCK I cells, isolation, analysis and selection of clones was performed as described (15; 16). For translocation studies, cells were seeded on Costar filters at a density of 300.000 cells/cm^2 and grown for three days. Next, cells were treated for 16 hours with 4% glycerol, 1% DMSO, TMAO, 5 mM 4-PBA, or 1 μM SR121463B at 37°C or grown for 16 hours at 27°C. Alternatively, cells were treated for 2 hours at 37°C with 1 μM thapsigargin, curcumin, or ionomycin in culture medium unless indicated otherwise.

Immunoblotting and immunocytochemistry

Poly-acrylamide gel electrophoresis, western blotting and immunodetection were performed as described. (5; 15). Immunocytochemistry (ICC), confocal laser scanning microscopy (CLSM) and data quantification was performed as described (16). Mouse anti-HSP70 and -HSP90 antibodies were kindly supplied by Dr. David Toft (Mayo Clinic, Rochester, MN). Rabbit anti-calnexin antibodies were kindly supplied by Dr. I. Braakman (UMC Utrecht, Utrecht, The Netherlands)

Calcium measurements

To measure cytosolic calcium levels, cells were seeded in 35/22 mm glass bottom dishes (Wilco Wells, Amsterdam, The Netherlands) at a density of 100.000 cells/cm^2 and grown O/N. Subsequently, the cells were loaded with 3 μM FURA-2 in the presence of 100 nM pluronic and 500 μM probenecid in culture medium and incubated for 30 minutes at 37°C. As the phenol red in Dulbecco's Modified Eagle Medium (DMEM) interferes with the FURA-2 measurement, measurements were performed in Hepes/Tris buffer. The cells were then washed in Hepes/Tris buffer (132.6 mM NaCl, 5.8 mM glucose, 10 mM Hepes, 4.2 mM KCl, 1 mM $MgCl_2$), followed by addition of 500 μM Hepes/Tris buffer with 500 μM probenecid, supplemented with either 1 mM EGTA or different concentrations of $CaCl_2$ and one of the chemical chaperones. The FURA-2 fluorescence emission ratio at 492 nm was monitored as a measure of $[Ca^{2+}]_{cyt}$ after alternating excitation at 340 and 380 nm using MetaFluor software (Universal Imaging Corp.). Averaged data of 25 individual cells was used per experiment. Measurements were performed on an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) equipped with a Zeiss 40x/1.3 NA F Fluar objective coupled to a CoolSNAP HQ monochrome CCDcamera (Roper Scientific, Vianen, The Netherlands). Experiments were performed at least in threefold.

Results

Chemical chaperones and their specificity

Recently, we reported that the V2R mutants -L44P, -del62-64, -I130F, -S167T, -S167L, and -V206D were fully ER retained when stably expressed in polarized MDCK cells, whereas-R113W, -G201D, and -T204N were partially ER retained (16). Consistently, the ER-retained proteins are expressed as 60-63 kD immature proteins, whereas the mature forms were expressed as 75 kD proteins. As the maturation state of the V2R mutants is an indication for their level of plasma membrane localization, these mutant-expressing cell lines were incubated with different chemical chaperones and subjected to V2R immunoblotting to test for increased receptor maturation. MDCK-V2R-V206D cells showed an increased amount of mature V2R when treated with 4% glycerol or 1% DMSO (vol/vol) for 16 hours, or with 1 μM thapsigargin for 2 hours (Fig. 1, top panel). Incubation with TMAO, 4-PBA, or growth at 27°C for 16 hours increased the amount of immature V2R proteins, but did not induce receptor maturation. For the mutants V2R-S167T (Fig.1, middle panel), -L44P, -del62-64, -R113W, -I130F, -S167L, -G201D, and -T204N (not shown), however, no significant ($p<0.05$) increases in maturation were observed. Immunodetection of beta-actin revealed equal levels for all lanes (Fig. 1, bottom panel), which indicated that the cells were not affected by the given treatments. These effects were observed for 2 independent clones per mutant, indicating that the effects were V2R mutant dependent and not due to clonal differences. Altogether,

our data show that chemical chaperones induce maturation of only a limited number of V2R mutants (i.e. only V2R-V206D) and that the maturation of this particular mutant can be induced by different chemical chaperones.

Chemical chaperones induce translocation of V2R-V206D to the plasma membrane

To determine whether chemical chaperones and thapsigargin rescue the cell surface expression of V2R-V206D, cells grown and treated as above were subjected to CLSM, following immunocytochemistry. Co-localizations with the organelle marker proteins PDI (ER) and E-Cadherin (basolateral membrane) were semi-quantified using densitometry. As reported (16), V2R-V206D in untreated cells is almost completely localized in the ER (94.5 +/- 5.3%; Fig. 2A top panel), whereas no V2R-V206D is found in the basolateral membrane. When treated with DMSO (Fig. 2A, middle panel) or glycerol (not shown), the localization of V2R-V206D indeed shifted from the ER to the basolateral membrane, as PDI co-localization decreased to 44.2 +/- 6.4% or 54.5 +/- 7.4%, respectively, whereas E-Cadherin co-localization (not shown) increased to 52.5 +/- 7.1% or 49.6 +/- 8.3%, respectively.

Upon treatment with 1 μM thapsigargin for 2 hours (Fig. 2A, bottom panel), localization of V2R-V206D to the ER was reduced to 46.2 +/- 6.5%, while the remainder co-localized with E-Cadherin in the basolateral membrane (53.8 +/- 7.0%; not shown). This effect was slightly weaker with 1 μM curcumin,

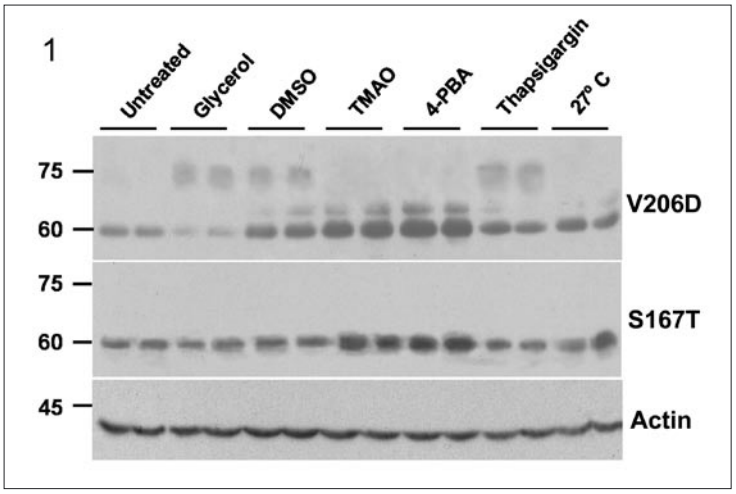

Figure 5-1 **Maturation of V2R-V206D upon treatment with chemical chaperones**
Confluent V2R-V206D or V2R-S167T expressing MDCK cells wereuntreated, incubated for 16 hours with 4% glycerol, 1% DMSO, 1% TMAO, 5 mM 4-PBA or at 27°C, or treated for 2 hours with 1 μM thapsigargin, lysed in Laemmli sample buffer, loaded on a 10% PAAG and subjected to immunoblotting. V2R-V206D or –S167T were detected using anti-GFP antibodies (top and middle panel, respectively). To ensure equal loading of the samples, blots were incubated with β-actin antibodies (bottom panel). Duplicate samples are shown. Mass indications in kD are given on the left.

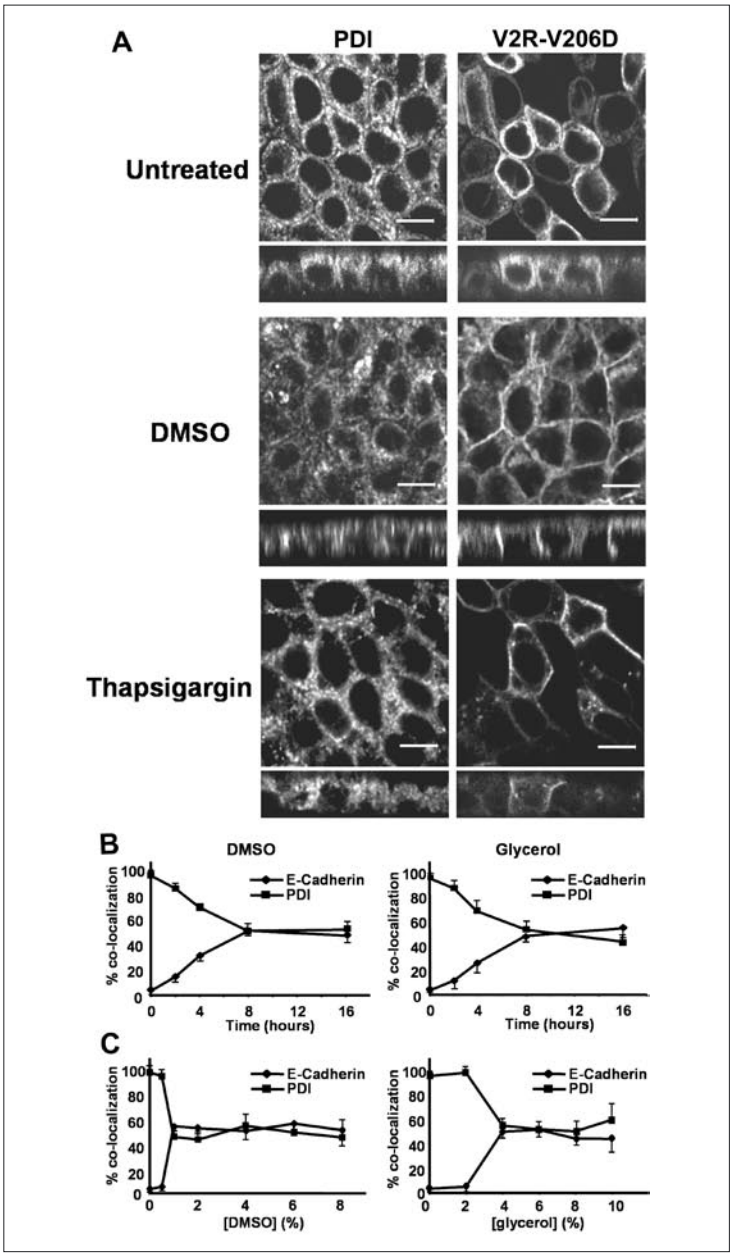

Figure 5-2 **Rescued cell-surface expression of V2R-V206D upon treatment with chemical chaperones**

A. MDCK-V2R-V206D cells were seeded on filters, grown to confluence and left untreated, or incubated with 1% DMSO or 1 μM thapsigargin (indicated). Cells were subsequently fixed and subjected to immunocytochemistry using anti PDI antibodies to stain the ER, followed by

confocal analysis. Signals for PDI are shown in the left panel, while signals for the GFP tagged V2R-V206D are shown in the right panel. Bar, 10 µm

B. Confluent MDCK-V2R-V206D cells were treated for increasing time periods with 1% DMSO or glycerol (indicated), followed by fixation, immunocytochemical staining, and confocal analysis as described under A. The percentage co-localization of V2R-V206D with E-Cadherin (basolateral membrane marker) or Protein Disulfide Isomerase (PDI; endoplasmic reticulum marker) were quantified for each time point (n>3) and plotted as a function of time.

C. Confluent MDCK-V2R-V206D cells were treated for 16 hours with the indicated concentrations of DMSO (left panel) or glycerol (right panel), followed by fixation, staining, and analysis as described under B. The percentage co-localization of V2R-V206D with E-Cadherin or PDI were quantified for each concentration (n>3) and plotted.

as 2 hours treatment with this drug decreased the ER localization of V2R-V206D to 64.6 +/- 7.3%, while its basolateral membrane localization increased to 32.8 +/- 6.7% (not shown). Within this time frame, the rescuing effect with DMSO and glycerol was much lower. To determine the speed of V2R-V206D rescue by DMSO and glycerol, we performed a time-response curve, and semi-quantified the corresponding CLSM data (Fig. 2B). In time, co-localization with PDI gradually decreases, which is accompanied with increased co-localization with E-Cadherin. For DMSO, the values obtained for t=8 hours were not significantly (p>0.05) different from the values for t=16 hour described above. For glycerol, however, a significantly increased rescue effect was observed in this time period (p=0.03).

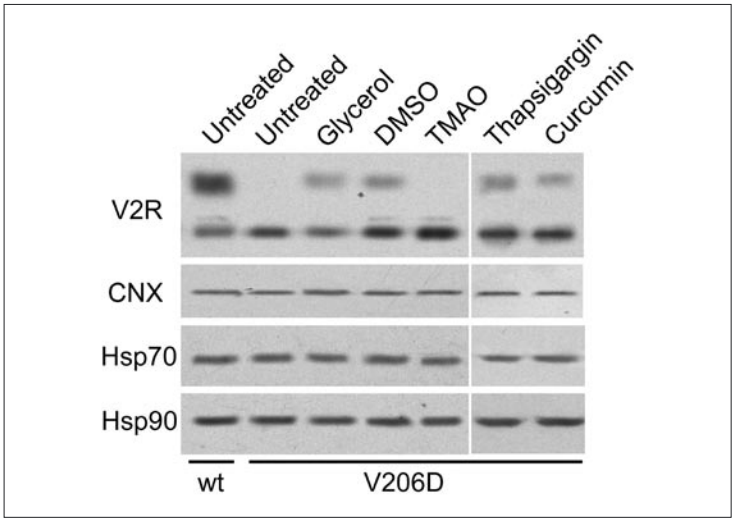

Figure 5-3 **The expression of molecular chaperones is not affected by osmolyte treatment**

MDCK-wtV2R or -V2R-V206D cells were left untreated or incubated for 16 hours with 4% glycerol, 1% DMSO or 1% TMAO, or 2 hours with 1 µM thapsigargin or 1 µM curcumin (indicated), lysed and subjected to immunoblotting as described in the legend of figure 1. As primary antibodies, rabbit-anti-GFP (top panel), rabbit-anti-calnexin (CNX), mouse-anti-Hsp70 (HSP70) or mouse-anti-Hsp90 (Hsp90) were used. Per lane, 10 µg total cell lysate was loaded.

In all cases, the translocation effect was observed for approximately 70% of the cells expressing V2R-V206D. The remaining cells did not show any translocation effect. There was no correlation between the expression levels of individual cells and the occurrence of rescue. Also, consistent with the absence of maturation, none of the other V2R mutants showed any rescued cell surface expression with any of the chemical

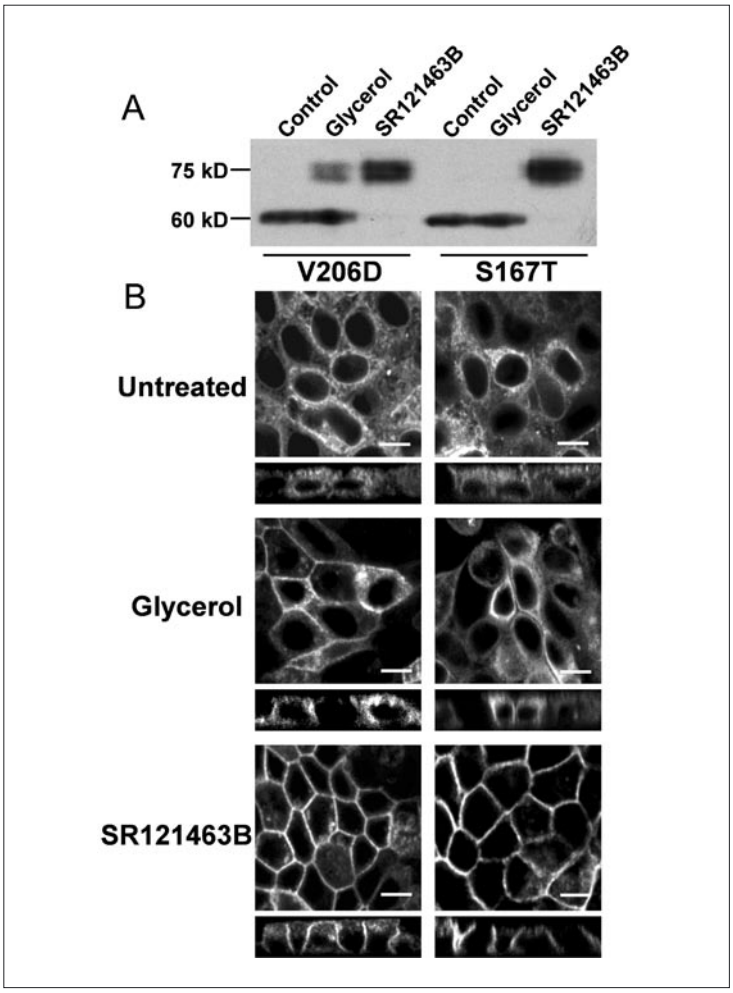

Figure 5-4

Rescue of V2R mutants by pharmacological and chemical chaperones

A. Confluent V2R-V206D or –S167T expressing MDCK cells (indicated) were left untreated, or incubated for 16 hours with 4% glycerol, or 1 μM SR121463B. Subsequently, cells were lysed and analyzed by immunoblotting as described in the legends of figure 1.

B. V2R-V206D or –S167T expressing MDCK cells (indicated) were seeded on filters, grown to confluence and were subsequently left untreated, or incubated for 16 hours with 4% glycerol, or 1 μM SR121463B. Cells were subsequently fixed and analyzed by confocal microscopy. Bar, 10 μm.

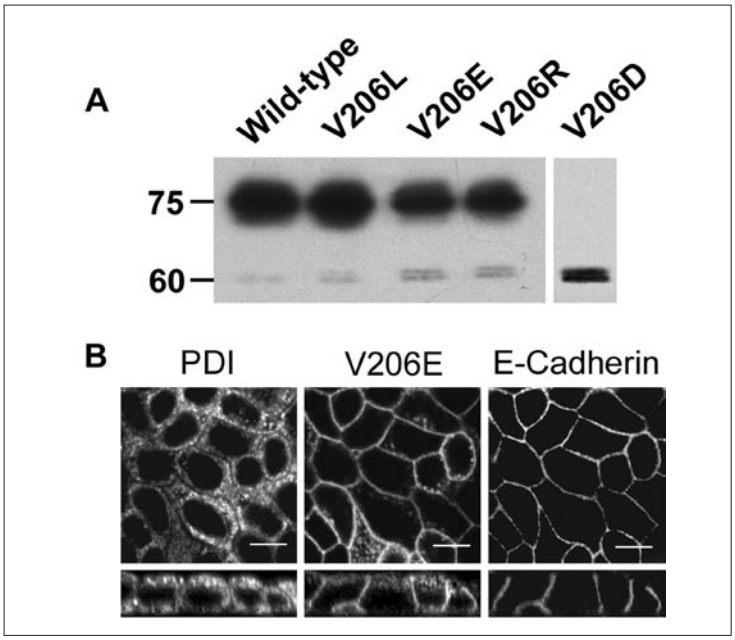

Figure 5-5 **V2R-V206L, -V206E, and V206R are expressed as mature proteins**

A. MDCK cell lines stably expressing either wild-type V2R, V2R-V206L, -V206E, -V206R or -V206D (indicated) were grown to confluence, lysed and analyzed by immunoblotting for GFP. The masses of the immature (60 kD) and mature (75 kD) V2R proteins are indicated on the left. B. MDCK cells stably expressing the GFP tagged V2R-V206E were grown to confluence, fixed, immunocytochemically stained using anti-PDI and anti-E-Cadherin antibodies, and analyzed by CLSM as described in the legend of figure 2. Bar, 10 µm.

chaperones or thapsigargin (not shown). Incubation with 2, 4, 6, or 8% DMSO, or 6, 8, or 10% glycerol, also induced translocation of V2R-V206D, but semi-quantification of CLSM data (Figure 2C) revealed that the observed effect was not significantly increased compared to treatment with 1% DMSO, or 4% glycerol. At concentrations above 10% DMSO or 12% glycerol, the compounds induced severe morphological changes, or showed cytotoxic effects (as revealed by the reduced number of remaining cells; not shown).

As shown in figure 3, no differences in expression were observed for calnexin, HSP70 or HSP90 between MDCK cells expressing wtV2R, or V2R-V206D. In addition, their expression was not altered in response to treatment with the rescue-inducing osmolytes glycerol and DMSO, or with TMAO.

Chemical chaperones vs. cell permeable ligands

Recently, Morello et al. showed that the plasma membrane expression of several, but not all, V2R mutants in HEK293 cells could be rescued by the cell permeable V2R antagonist SR121463B(13). To determine whether V2R mutants shows a different

sensitivity in rescue towards this pharmacological chaperone as compared to a chemical chaperone, we treated MDCK cells expressing V2R-V206D or V2R-S167T for 16 hours with 1 μM SR121463B or 4% glycerol. As shown in figure 4A, treatment with SR121463B drastically induced the maturation of both V2R mutants, which co-incided with the disappearance of the 60 kD band. In contrast, glycerol treatment increases receptor maturation of V2R-V206D only. In line with these data, immunocytochemistry revealed that, while both mutants localized to the ER when untreated (Fig. 4B, upper panel) and glycerol rescued the plasma membrane expression of V2R-V206 only (Fig. 4B, middle panel), SR121463B rescued the basolateral plasma membrane localization of both V2R-V206D and –S167T (Fig. 4B, lower panel). These data indicate that SR121463B has a more pronounced rescuing effect on more V2R mutants than glycerol.

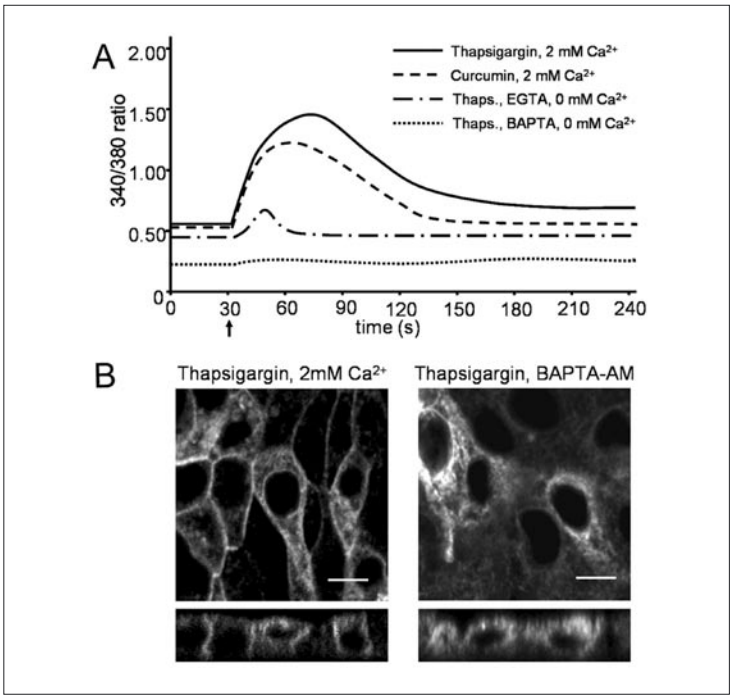

Figure 5-6 **Cytosolic calcium responses to treatment with SERCA inhibitors**

A. MDCK type 1 cells were seeded at 100.000 cells/cm^2 in glass-bottom culture dishes and grown for 24 hours. Cytosolic calcium levels were measured using FURA-2. Cells were incubated in Hepes/Tris buffer with the indicated levels of Ca^{2+}, or EGTA with or without BAPTA (indicated), to allow the baseline to settle. 30 seconds after the start of the measurement (indicated with an arrow), 1 μM thapsigargin or curcumin (indicated) was added to the cells followed by measurement of the 340/380 nm ratio. Each data series comprises the averaged data of 25 cells that were measured simultaneously. The figures show data of a representative experiment (n=3).

B. Confluent MDCK-V2R-V206D cells were incubated for 2 hours in Hepes/Tris buffer with 1 μM thapsigargin, supplemented with 2 mM Ca^{2+} (left panel), or 5 mM BAPTA-AM and without Ca^{2+} (right panel). Subsequently, cells were fixed and analyzed by confocal microscopy. Bar, 10 μM.

The V206D mutation specifically induces ER retention

To elucidate whether the ability to restore the plasma membrane localization of V2R-V206D depends on the charge of the introduced mutation, V206 was changed into a negatively-charged glutamic acid (V206E), a hydrophobic leucine (V206L), or a positively-charged arginine (V206R), and stably-expressed in MDCK type I cells. Immunoblot analysis of expressing clones, however, showed that all these mutants were mainly expressed as mature 75 kD proteins (Fig. 5A), suggesting that these mutants were hardly ER-retained. Indeed, immunocytochemistry on two clones of each cell line revealed that V2R-V206E (Fig. 5B), V2R-V206R and V2R-V206L (not shown) mainly localize in the basolateral membrane and co-localized only to a minor extent with PDI. These data indicated that the ER retention of V2R-V206D is specific for aspartic acid and that no information could be obtained on the amino acid specificity of rescue by chemical chaperones.

Cytosolic calcium is involved in the rescue of V2R-V206D

Thapsigargin-induced emptying of ER Ca^{2+} stores, resulting in decreased free $[Ca^{2+}]_{ER}$, has been postulated to be the determining factor causing rescue of the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator (CFTR) (9). To determine how alterations in intracellular Ca^{2+} levels affect translocation of V2R-V206D to the plasma membrane, we determined the changes in $[Ca^{2+}]_{cyt}$ upon drug treatment using the Ca^{2+} sensitive fluorophore FURA-2-AM. Addition of thapsigargin to MDCK-V2R-V206D cells in Hepes/Tris buffer with 2 mM $CaCl_2$ showed a rapid increase of $[Ca^{2+}]_{cyt}$, which peaked after approx. 30 seconds, followed by a gradual decrease to a steady state level that was slightly increased compared to the starting situation (Fig. 6A). Addition of curcumin showed similar results, although the peak value and the final plateau phase were decreased compared to thapsigargin. In Ca^{2+} free buffer with 1 mM EGTA, base levels of $[Ca^{2+}]_{cyt}$ were lower than in Ca^{2+}-containing the buffer, which did not change with the addition of thapsigargin, except for a small peak right after addition, which is likely caused by Ca^{2+} diffusing from the ER lumen. Supplementary incubation with the cytosolic Ca^{2+} chelator BAPTA-AM resulted in a low $[Ca^{2+}]_{cyt}$ throughout the experiment. Immunocytochemistry revealed a similar rescue effect of V2R-V206D with thapsigargin (Fig. 6B, left panel) and curcumin in Hepes/Tris buffer with 2 mM Ca^{2+} as in DMEM. Without Ca^{2+} and independent of the presence of BAPTA, however, no V2R-V206D rescue was observed (Fig. 6B, right panel).

These experiments could indicate that increased $[Ca^{2+}]_{cyt}$ instead of reduced $[Ca^{2+}]_{ER}$ mediated rescue of V2R-V206D to the plasma membrane. To further investigate the roles of $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_{cyt}$ in rescue of V2R-V206D, we used the ionophore ionomycin. This drug renders cell membranes permeable to Ca^{2+}, which allowed us to clamp $[Ca^{2+}]_{ER}$. In eukaryotic cells, the resting $[Ca^{2+}]_{ER}$ is about 300 μM Ca^{2+} (1; 7). Incubation of MDCK-V2R-V206D cells in buffer with 2 mM Ca^{2+} did not affect its ER localization (Fig. 7, top panel), but addition of 1 μM ionomycin for 2 hours induced a clear translocation of V2R-V206D to the basolateral membrane (Fig 7, 2nd top panel. As shown in Fig. 7, 3rd top panel, incubation of the cells for 2 hours with 1 μM ionomycin in buffer with 300 μM Ca^{2+} also induced a translocation of V2R-V206D to the basolateral membrane. In Ca^{2+}-free buffer with EGTA and/or BAPTA, however, ionomycin was not able to rescue the cell surface expression of V2R-V206D (Fig. 7, bottom panel).

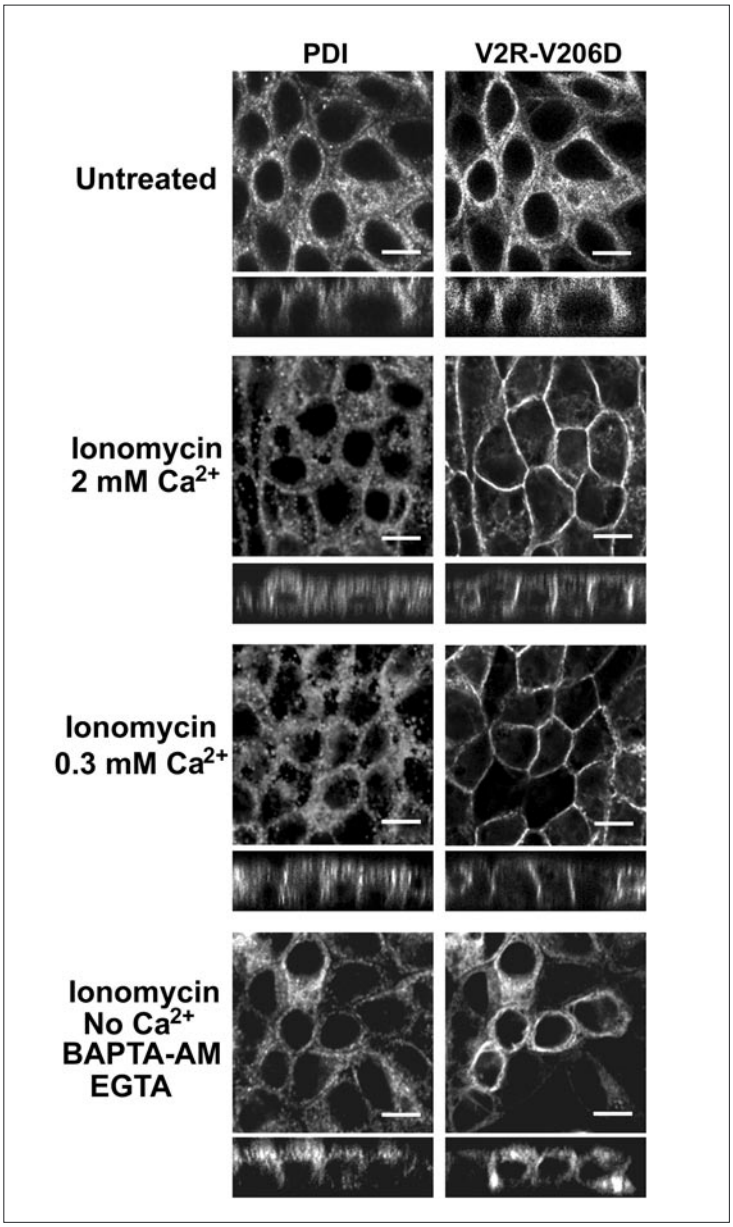

Figure 5-7 **Effect of ionomycin and calcium on the localization of V2R-V206D**

Confluent cell layers of MDCK cells stably expressing GFP-tagged V2R-V206D were incubated for 2 hours in Hepes/Tris buffer with 2 mM Ca^{2+}, 2 mM Ca^{2+} and 1 µM ionomycin, or 300 µM Ca^{2+} and 1 µM ionomycin, or in buffer without Ca^{2+} in the presence of 1 µM ionomycin, 5 mM BAPTA-AM and 1mM EGTA (indicated). Subsequently, the cells were fixed, immunocytochemically stained and analyzed by CLSM as described in the legend of figure 2. Bar, 10 µm.

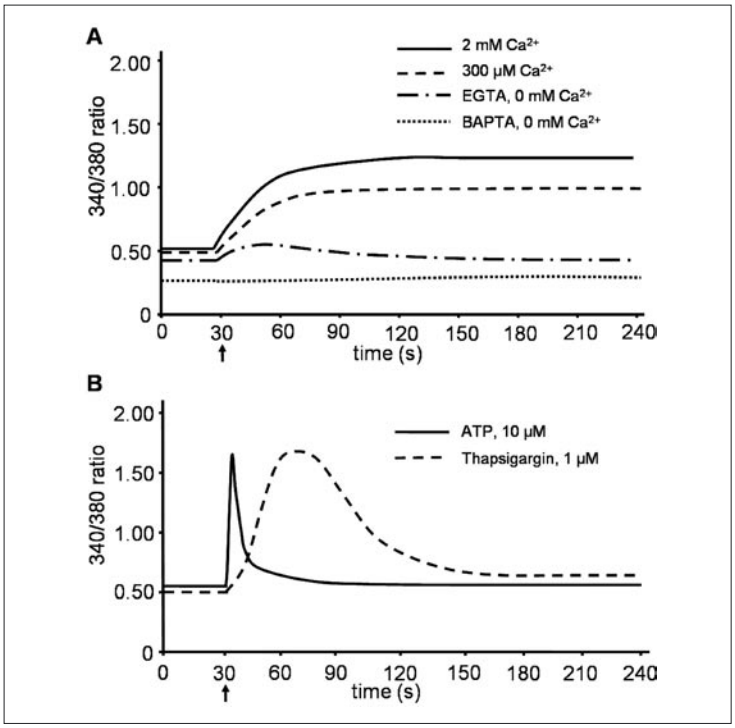

Figure 5-8 **Cytosolic calcium levels in MDCK cells upon treatment with ionomycin.**
MDCK cells were grown as described in the legend of figure 4.A. Cells were incubated in Hepes/Tris buffer containing the indicated levels of Ca^{2+}, or EGTA with or without BAPTA (indicated), to allow the baseline to settle. 30 seconds after the start of the measurement (indicated with an arrow), ionomycin was added to the cells, followed by measurement of the 340/380 nm ratio.
B. Cells were incubated in Hepes/Tris buffer containing 2 mM Ca^{2+} to allow the baseline to settle. 30 seconds after the start of the measurement (indicated with an arrow), 1 µM thapsigargin or 10 µM ATP (indicated) was added. Each data series comprises the averaged data of 25 cells that were measured simultaneously. The figures show data of a representative experiment (n=3).

Corresponding FURA-2 measurements showed a steady state increase in $[Ca^{2+}]_{cyt}$ with ionomycin when either 2 mM or 300 µM Ca^{2+}, which reached a plateau phase after approx. 30 seconds (Fig. 8A). In Ca^{2+}-free buffer with 1 mM EGTA, a small increase in the $[Ca^{2+}]_{cyt}$ was observed, which decreased to below base level after approx. 2 minutes. In Ca^{2+}-free buffer with BAPTA and EGTA, $[Ca^{2+}]_{cyt}$ again remained low throughout the experiment.

Several hormones, such as ATP, also induce an increase in cytosolic calcium via the phospholipase C pathway. To compare the intracellular calcium flux patterns of such a hormone with those induced by thapsigargin, curcumin and ionomycin, we added

ATP to MDCK cells and measured intracellular calcium. As shown in Fig. 8B, ATP appeared to induce an increase in intracellular calcium, but with less total Ca^{2+} mobilization, and different kinetics compared to the drugs mentioned above. As anticipated, ATP did not induce a translocation of V2R-V206D to the plasma membrane (not shown).

Discussion

Rescue of V2R mutants is mutation-specific and induced by several chemical chaperones

We have previously shown that in polarized cells, many V2R mutants involved in the X-linked NDI are retained in the ER. Out of nine of these mutants, only V2R-V206D showed a rescued plasma membrane expression and increased receptor maturation upon treatment with chemical chaperones or calcium-affecting drugs, whereas all other mutants tested did not (Figs. 1 and 2). This illustrates that plasma membrane rescue is not a general phenomenon for a particular protein, but is specific for a limited set of mutations in a particular protein. This is in line with the results of Delisle et al., who reported that the potassium channel mutants HERG-G601S and -F805C, found in long QT syndrome, were rescued in their cell surface expression by thapsigargin, whereas the localization of HERG-N470D was not changed (6). Although we tested only one pharmacological chaperone, this class of compounds seems to induce a stronger level of rescue of V2R mutants, because the level of maturation of V2R-V206D and V2R-S167T with SR121463B was better than obtained with the most optimal concentrations of the chemical chaperones glycerol or DMSO (Fig. 4). Moreover, the pharmacological chaperone seems to act on more V2R mutants than chemical chaperones or SERCA inhibitors, because it also rescued the basolateral expression of V2R-S167T. This is in line with the finding of Morello et al.(13) who found that 8 out of 15 V2R mutants were rescued by SR121463B, although they used different V2R mutants and did not test chemical chaperones. In addition to their possible clinical applicability, this makes cell permeable antagonists, and the mechanism by which they facilitate rescue, promising subjects for more detailed investigation, which will be the subject of further studies.

The V2R-V206E/R/L mutants were only ER-retained to a minor extent (Fig. 4), which may indicate that V206D is a subtle mutation, which allows proper folding upon slight structural changes induced by chemical chaperones. However, as V2R-R113W, –G201D, and –T204N are only partially ER retained (16), it is likely that these mutants are also not severely misfolded. It is, therefore, striking that the latter three mutants did not respond to any of the chemical chaperones. Likely, the relationship between the location and type of a mutation in the V2R, and the mutant's ability to be rescued by chemical chaperones has to await the atomic structure of the V2R.

Our study furthermore revealed that V2R-V206D cell surface expression is rescued by the chemical chaperones DMSO and glycerol, the SERCA-inibitors thapsigargin and curcumin, and the Ca^{2+} ionophore ionomycin, while none of these compounds changed the localization of any of the other V2R mutants. These data indicate that if a particular mutant is rescued by one chemical chaperone, it seems to be more prone to be rescued by others.

Mechanism of rescue by chemical chaperones

Rescue of ER-retention of mutant membrane proteins in eukaryotic cells by osmolytes, such as DMSO and glycerol, has been postulated to be due to increased expression or changed functionality of stress-sensitive molecular chaperones(3; 12). Despite rescue of V2R-V206D, however, glycerol and DMSO treatment did not affect the expression level of the ER lectin calnexin (Fig.3), which has been suggested to be involved in ER retention of V2R mutants (14). Also, while increased HSP70 expression levels has been shown to promote rescue of CFTR-ΔF508 cells (3), its level remained unaltered with V2R-V206D. In addition, HSP90 expression was unchanged. Although our data reveal that a changed expression of these molecular chaperones does not contribute to V2R-V206D rescue, it does not exclude effects of a changed activity of these proteins or the involvement of other folding proteins in this process.

Besides these explanations, DMSO and glycerol have been suggested to increase the relative hydration around a polypeptide, thereby inducing a tighter packing of the protein and a stabilization of the protein's conformation (19; 24). However, whereas the effects on 3-hydroxy-3-mehtylglutaryl-CoA reductase in yeast is accomplished in minutes, rescue of V2R-V206D by DMSO and glycerol is only fully effective after 8-16 hours, while there is hardly any rescue noticeable after 2 hours (Fig. 2B). Although we can not exclude it, a direct effect of the osmolytes on V2R-V206D to explain its rescue is therefore rather unlikely. It remains to be established which mechanism underlies rescue of V2R-V206D plasma membrane expression by chemical chaperones.

Mechanisms of rescue by ER calcium modifying drugs

The mechanism underlying rescue by thapsigargin, curcumin or ionomycin seems to be different in that these compounds efficiently rescued the cell surface expression of V2R-V206D within 2 hours. Also, these drugs induced a raise in intracellular calcium levels (Figs. 6 and 8), which was not observed with DMSO, glycerol or SR121463B (not shown).

Low cytosolic calcium levels are mainly maintained by the SERCA ATPases, which pump leaked calcium back into the ER, and by plasma membrane calcium ATPases (PMCAs), which pump calcium out of the cells. From the combined FURA-2 measurements and V2R-V206D translocation studies, the following information can be deduced: First, cells incubated with thapsigargin, ionomycin or curcumin in the absence of extracellular calcium show a small and transient increase in intracellular calcium in contrast to cells with extracellular calcium. Cell surface expression of V2R-V206D is only obtained under the latter condition, which indicates that an influx of extracellular calcium is needed for the rescue of V2R-V206D.

Second, if the mechanism by which V2R-V206D is rescued is identical for thapsigargin, ionomycin and curcumin, our data indicate that this is rather due to increased cytosolic, instead of decreased ER calcium levels. Inhibition of SERCA pumps by thapsigargin, and to a lower extent curcumin, result in a cytosolic calcium spike of about 90 seconds, which likely results from an initial extracellular calcium entry and a reduced ER calcium entry. The following decrease of cytosolic calcium to the observed slightly-increased basal levels is likely caused by activated PMCAs. Under this condition, however, $[Ca^{2+}]_{ER}$ will be decreased. The calcium ionophore ionomycin renders the ER and plasma membranes permeable for calcium (23). Under this condition, PMCAs are

not able to compensate the extracellular calcium influx, which is shown by the sustained high cytosolic calcium levels (Fig.8A). Therefore, $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{ER}$ will be similar to the extracellular $[Ca^{2+}]$. Extracellular calcium levels similar (300 μM) or well above (2 mM) ER resting levels co-incided with a rescued cell surface expression of V2R-V206D, which indicated that this rescue was due to increased $[Ca^{2+}]_{cyt}$ levels. The absence of V2R-V206D rescue with ATP indicates that a short transient increase in cytosolic calcium is not sufficient to mediate rescue. It remains unclear, however, whether the larger calcium peak, the increased basal calcium level or both are involved in this rescue.

As the V206D mutation is exposed to the ER luminal side, it is unlikely that altered $[Ca^{2+}]_{cyt}$ levels directly affect the mutation. More likely, the observed increased $[Ca^{2+}]_{cyt}$ levels may affect cytosolic folding proteins, such as HSP70 or HSP90, to facilitate V2R-V206D folding or induce its release from the ER. If so, this was not mediated by changed expression levels of these proteins, as these were unchanged (Fig.3). Possibly, the functionality of the transmembrane ER protein calnexin may be directly or indirectly affected by changes in $[Ca^{2+}]_{cyt}$, as this molecular chaperone was shown to have prolonged interaction with an ER retained V2R mutant compared to wtV2R (14).

Interestingly, thapsigargin and curcumin also rescue the cell surface expression of CFTR-ΔF508 (9; 10). In contrast to V2R-V206D, however, CFTR-ΔF508 was rescued in the presence of BAPTA-AM, which indicates that cytosolic calcium has no role in CFTR-ΔF508 rescue and that CFTR-ΔF508 rescue is mediated through another mechanism. However, in the experiments of Egan et al., a 1 hour thapsigargin and BAPTA-AM treatment was followed by a 1.5 hour recovery time. As thapsigargin, but not BAPTA-AM, is difficult to wash out, it can not be excluded that CFTR-ΔF508 rescue occurred in the last 1.5 hour due to thapsigargin alone. Unfortunately, we were not able to obtain CFTR-ΔF508 or CFTR expression in MDCK cells to test this further.

In conclusion, we have shown that plasma membrane rescue by chemical chaperones and altered Ca^{2+} levels only occurs for V2R-V206D, but that its cell surface expression is rescued by the chemical chaperones glycerol and DMSO. In addition, the SERCA-inhibitors thapsigargin and curcumin as well as the Ca^{2+}-ionophore ionomycin are able to induce translocation of V2R-V206D to the plasma membrane by increasing $[Ca^{2+}]_{cyt}$, rather than by decreasing $[Ca^{2+}]_{ER}$. Increased insight in the molecular mechanisms that facilitate restoration of the plasma membrane localization of class II mutants may aid in developing therapies for diseases caused by such mutant proteins.

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Chapter 6

Functional rescue of vasopressin V2 receptor mutants in MDCK cells by pharmacological chaperones: Relevance to therapy of Nephrogenic Diabetes Insipidus.

Abstract

Intracellular retention of functional vasopressin V2 receptors (V2R) is a major cause of congenital nephrogenic diabetes insipidus (NDI) and rescue of V2R mutants by non-peptide antagonists may restore their basolateral membrane (BM) localization and function. However, the criteria for efficient functional rescue of G-protein coupled receptor (GPCR) mutants at clinically-feasible antagonist concentrations are unknown.

We found that the four non-peptide antagonists SR49059, OPC31260, OPC41061 and SR121463B induced maturation and rescued the BM expression of eight out of nine different V2R mutants, stably-expressed in physiologically-relevant polarized cells. The extent of maturation and rescued BM expression correlated with the antagonists' concentration and affinity for the V2R. Displacement of the antagonists by AVP and subsequent cAMP generation inversely correlated with the antagonists' affinities for the V2R, but is partially influenced by antagonist-specific aspects. Despite limited increases of maturation and cell-surface expression of V2R mutants, the low-affinity SR49059 optimally induced functional rescue at high concentrations, due to its easy displacement by vasopressin. At clinically-feasible antagonist concentrations, however, only the high-affinity antagonists OPC31260 and OPC41061 induced functional rescue, as at these concentrations the extent of BM expression became limited.

In conclusion, functional rescue of mutant V2Rs at clinically-feasible concentrations is most effective with high-affinity antagonists. As OPC31260 and OPC41061 are clinically safe, they are promising candidates to relieve NDI. Moreover, as numerous other diseases are caused by ER-retained GPCRs for which cell-permeable antagonists become available, our finding that high-affinity antagonists are superior is anticipated to be important for pharmacotherapy development of these diseases.

Introduction

The synthesis, maturation and routing of plasma membrane proteins are extremely complex processes that require specific interactions between many different intracellular components. It is not surprising, therefore, that flaws in these processes are responsible for many diseases, which are often caused by mutations in genes encoding membrane proteins. In the last two decades, numerous mutations have been identified in the coding sequences of such genes, of which about 50% are missense mutations involving only one or a few nucleotides. For example, in cystic fibrosis (CF), a severe disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, more than 300 unique missense mutations have been described (http://www.genet.sickkids.on.ca/cftr/). Cell expression studies revealed that most of these mutations lead to fully synthesized proteins that fail to pass the quality control mechanism of the endoplasmic reticulum (ER) as the protein is misfolded(10). Based on this cellular fate, these gene defects are so-called class II mutations, giving rise to 'conformational diseases'(8). Usually, ER-retention of such proteins is followed by their degradation by proteasomes(22).

As numerous studies revealed that ER retained mutant proteins are often functional, research of the last decade has focused on the identification of compounds that can rescue the cell surface expression of such proteins. In this respect, the vasopressin type 2 receptor (V2R) is the prototype protein, as it was the first receptor for which the exciting discovery was made that cell-permeable antagonists (CPAn, known as 'pharmacological chaperones' or 'pharmacoperones') can promote cell surface trafficking of its ER-retained mutants(18).

V2R mutations cause the X-linked form of nephrogenic diabetes insipidus (NDI), a disorder in which patients are unable to concentrate their urine in response to the antidiuretic hormone arginine-vasopressin (AVP)(13). Morello and co-workers showed that pretreatment with the high-affinity cell-permeable V2R antagonist SR121463A rescued the cell surface expression of 8 out of 15 ER-retained V2R mutants (rescued cell surface expression), which could subsequently be activated by AVP(18) (i.e. functional rescue). Since then, the concept by which CPAns rescue V2R mutants has been the subject of several studies(4; 31; 34). As indicated above, a crucial aspect necessary for functional rescue is, besides rescued cell surface expression of the mutant, displacement of the V2R-bound antagonist by AVP to generate a cAMP response. Likely based on this requirement, a V1 receptor CPAn SR49059 was recently tested for its ability to increase the urine concentrating abilities in NDI patients(5). For three patients encoding the partially ER-retained V2R-R137H mutant, a significant urine volume reduction was obtained, thereby providing the proof of principle of the disease-curing effect of pharmacological chaperones in vivo. In patients encoding the fully ER-retained mutants V2R-W164S and V2R–del62-64, however, SR49059 was less effective.

To be of clinical value, functional rescue of V2R mutants should occur at low concentrations of antagonists and AVP and should last as long as possible. At present, however, it is unclear which features of CPAn are important to give the best functional rescue of V2R mutants under such conditions. Moreover, as the V2R is expressed in the basolateral membrane of renal principal cells, and proteins can traffic or function differently in non-polarized versus polarized cells(17), such studies are best performed in polarized renal epithelial cells.

Recently, we generated Madin-Darby canine kidney (MDCK) cells stably expressing V2R tagged with a green fluorescent protein (GFP)(23). In these cells, V2R-GFP was localized and regulated as can be anticipated to occur for V2R in vivo. Moreover, we found that several V2R mutants in NDI stably expressed in MDCK cells are ER-retained(24). To determine which CPAn is likely the optimal pharmacological chaperone to relieve NDI in patients, we thoroughly tested a V1 receptor antagonist, a medium-affinity V2R antagonist, and two high-affinity V2R antagonists for their ability to rescue the cell surface expression and activity of several V2R mutants.

Materials and Methods

Pharmacological chaperones

The V2R antagonist SR121463B(28) and the V1R antagonist SR49059(29) were kindly supplied by C. Serradeil-Le Gal (Sanofi Synthélabo, Toulouse, France). The V2R antagonists OPC31260 and OPC41061(14; 35) were kindly provided by

Koji Komuro (Otsuka Pharmaceutical Co., Tokushima, Japan). All compounds were dissolved in dimethylsulfoxide as 0.01 M stock solutions, and diluted in culture medium as indicated.

Expression constructs, cell culture and transfection

Expression constructs encoding the wild-type V2R or the NDI causing mutants –L44P, -I130F, -S167T or -S167L fused at their C-terminus to enhanced GFP were as described(24). MDCK type II cells, which lack endogenous V2R expression, were kindly provided by Dr. Alexander Oksche (FMP, Berlin, Germany). MDCK type II cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowittaker, Verviers, Belgium) supplemented with 5% fetal bovine serum (PAA Laboratories, Karlsruhe, Germany), gentamicin, l-glutamine, sodium carbonate, and 1% nonessential amino acids. Calcium phosphate transfection and isolation of clones were done as described for MDCK type I cells(9).

Immunoblotting and immunocytochemistry

Poly-acrylamide gel electrophoresis, western blotting and immunodetection were performed as described(9; 23). For detection of V2R-GFP, 1:5000 diluted rabbit anti-GFP serum was used (kindly provided by prof. B. Wieringa, RUN-MC, Nijmegen, The Netherlands). As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit IgGs (Sigma) were used. Immunocytochemistry (ICC), confocal laser scanning microscopy (CLSM) and data quantification were performed as described(24). As primary antibodies, 1:100-diluted rat anti-E-cadherin (Sigma, St. Louis, MO), or rabbit anti-PDI antibodies (kindly provided by Dr. I. Braakman, Utrecht University, Utrecht, The Netherlands) were used. As secondary antibodies, 1:100-diluted goat anti-rat IgG or goat anti-rabbit IgG, both coupled to Alexa-594, were used (Molecular Probes, Leiden, The Netherlands).

[^{3}H]AVP competition assay

Cells were seeded on 12 multiwell filters (Costar) at a density of 150.000 cells/cm^2 and grown for 3 days. Cells were subsequently treated with the antagonists for 16 hours, followed by three 3 washes with ice-cold phosphate buffered saline containing 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-CM). The cells were then incubated for 1 hour on ice with [^{3}H]AVP (Perkin-Elmer Life Sciences, Boston, MA) and the antagonist diluted in PBS-CM. Cells were washed three times with ice-cold PBS-CM, followed

Compound	Abbreviation	Ki (nM)	
SR49059	SR4	275±50	V1R antagonist
OPC31260	OPC3	9.42±0.90	V2R antagonist
OPC41061	OPC4	0.43±0.06	V2R antagonist
SR121463	SR1	0.54± 0.08	V2R antagonist

Table 6-1 **Characteristics of pharmacological chaperones**
The values of the inhibitory constant (Ki) for OPC3(1260), OPC4(1061), SR4(9059) and SR1(21463) on the human V2R as described(28-30; 36).

by excision of the filters and counting of the radioactivity as described(23). Triplicate samples were measured, and every experiment was performed at least in threefold.

cAMP measurements

MDCK II cells were seeded on 24 multiwell filters at a density of 150.000 cells/cm^2, grown to confluence, and treated with the antagonists as indicated. Subsequently, cells were briefly washed in PBS-CM, followed by incubation for 10 minutes in culture medium supplemented with 250 µM 3-Isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO) to prevent cAMP degradation by phosphodiesterases. Cells were then challenged for 10 minutes with dDAVP on the basolateral side in the presence of IBMX. After three washing steps with PBS-CM, cells were lysed in 100 µM 0.1 M HCl and cAMP was measured using a fluorescent cAMP assay kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Triplicate samples were measured, and experiments were performed at least in threefold.

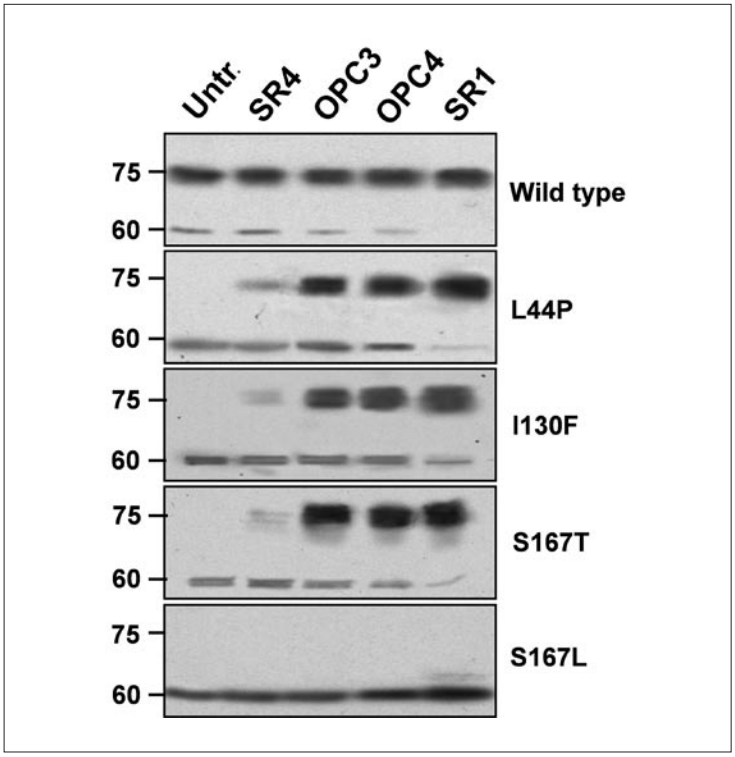

Figure 6-1 **Maturation of mutant V2R upon antagonist treatment is compound- and mutation specific**

MDCK II cells expressing either wt-V2R, V2R-L44P, -I130F, -S167T or –S167L fused to GFP were seeded on filters and grown to confluence, after which they were treated for 16 hours with 1 µM of the indicated cell permeable V2R antagonists. Untreated cells were used as a control (indicated). Cells were lysed and subsequently analyzed by SDS-PAGE followed by immunoblotting using anti-GFP antibodies. Molecular masses (in kDa) are indicated on the left.

Results

Maturation of V2R mutants upon antagonist treatment

During folding in the ER, V2R is expressed in its high-mannose glycosylated form. As it traverses the Golgi compartment on its way to the basolateral membrane (BM), it matures to complex- and O-glycosylated proteins. We have previously shown that missense V2R-GFP proteins in NDI that are trapped in the ER (class II) do not undergo maturation and are therefore visible as immature proteins of 60-63 kDa when expressed in MDCK I cells (24). Type I cells, however, endogenously express low levels of V2R and are thus not suitable for functional testing of mutant receptors. As MDCK II cells lack V2Rs(20), we stably expressed wild-type (wt) V2R, the functional mutants V2R-L44P, -I130F, -S167T, and the non-functional V2R–S167L in these cells. As found for MDCK I cells, wt-V2R was mainly expressed in the mature 75 kDa form, while the missense mutants were present as immature proteins of 60-63 kDa (Fig. 1, untreated samples). In addition, wt-V2R was mainly expressed in the BM, whereas the mutants were trapped in the ER (Fig. 2, untreated cells).

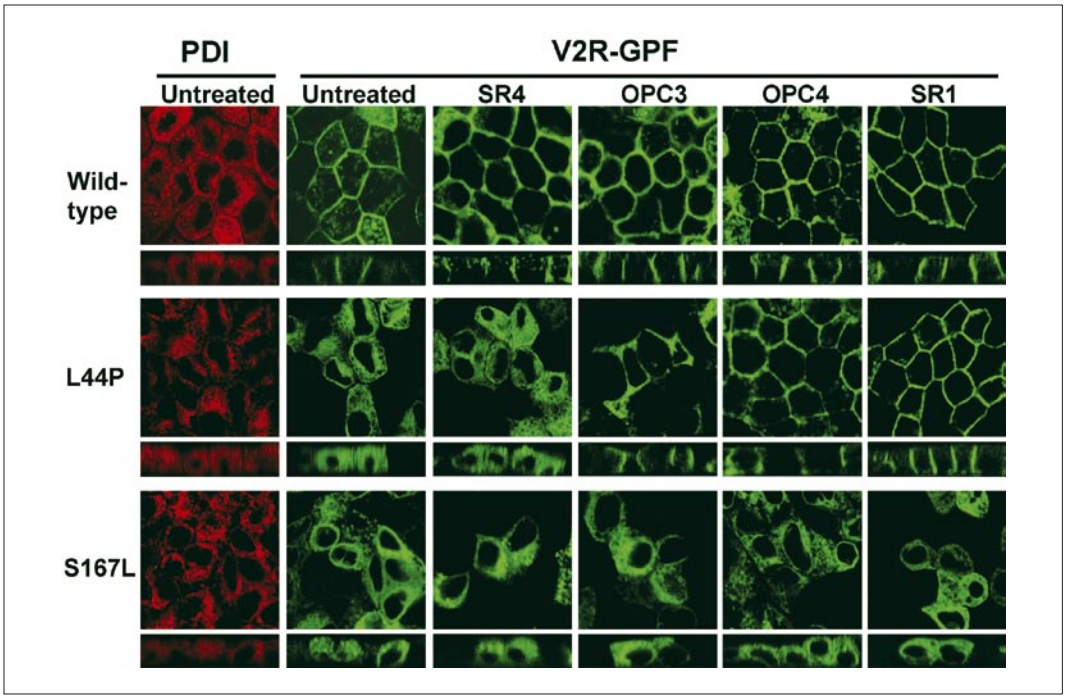

Figure 6-2 **Selective restoration of the plasma membrane expression of V2R-L44P by pharmacological chaperones**

MDCK II cells expressing wt-V2R, V2R-L44P or V2R-S167L (indicated) were seeded, grown and treated for 16 hours with 1 µM of the indicated cell permeable V2R antagonists. Untreated cells were used as control (indicated). Cells were fixed, subjected to immunocytochemistry to stain for the ER marker protein disulfide isomerase (PDI, indicated in red) and analyzed by confocal laser scanning microscopy. V2R-GFP is indicated in green.

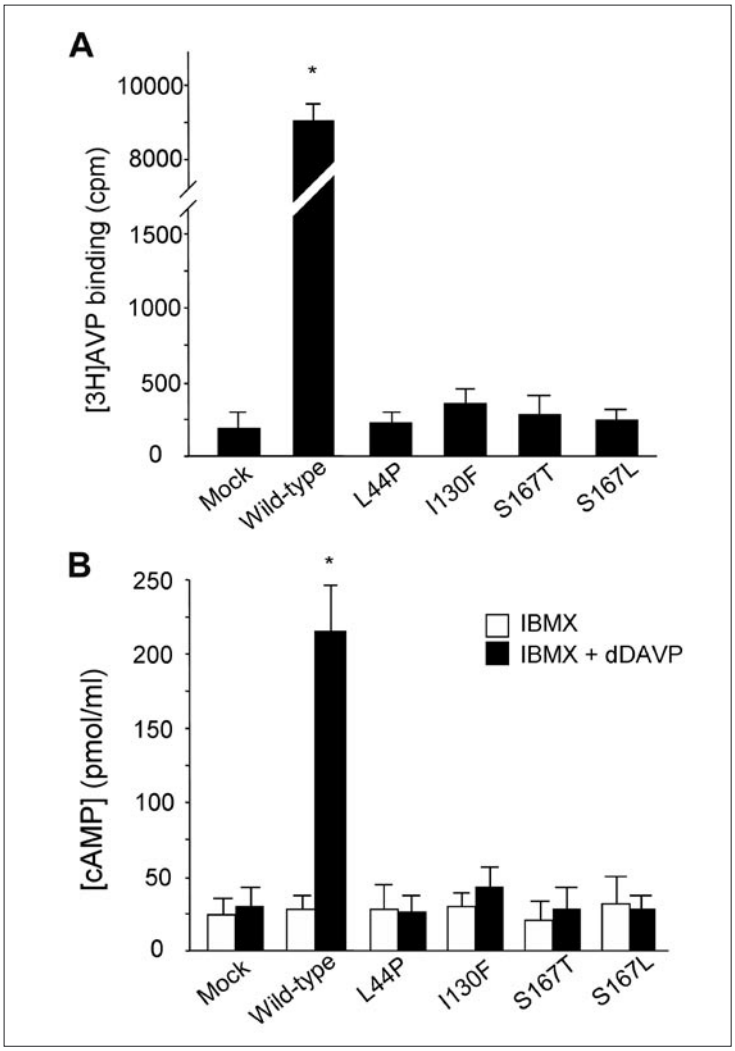

Figure 6-3 **Effect of dDAVP on MDCK cells expressing wtV2R or mutants in NDI**

(A) MDCK II-wtV2R, V2R-L44P, -I130F, -S167T or –S167L (indicated) were seeded on filters, grown to confluence, washed with ice-cold PBS-CM and subsequently incubated for 1 hour with 100 nM [^{3}H]AVP for 1 hour at 4°C to allow radioligand binding to cell-surface receptors. Subsequently the filters with the cells were washed three times with ice-cold PBS-CM, excised and counted in a scintillation counter.

(B) MDCK II-wtV2R, V2R-L44P, -I130F, -S167T or –S167L (indicated) were seeded on filters, grown to confluence and subsequently treated with IBMX alone, or in combination with 100 nM dDAVP on the basolateral side. Subsequently, cells were lysed and cAMP accumulation measured using a fluorescent cAMP assay kit.

Triplicate samples were measured, and experiments were performed at least in threefold. Samples indicated with an asterisk were significantly ($p<0.01$) different from untreated samples.

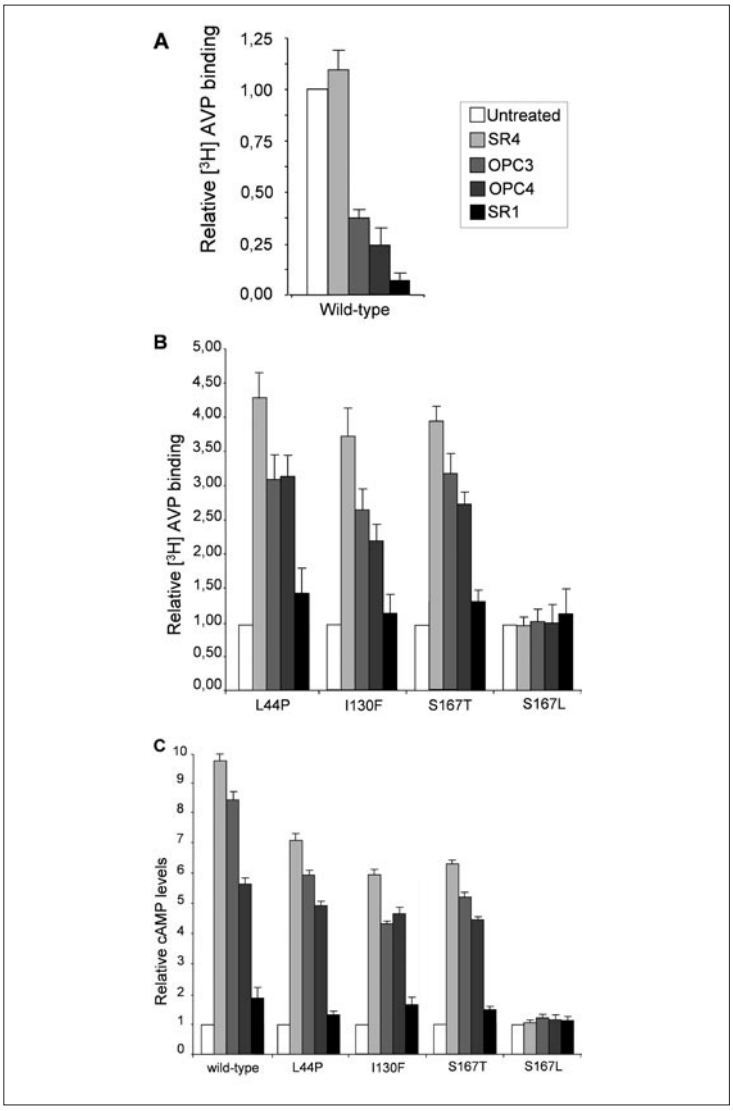

Figure 6-4 **Functional rescue of V2R mutants**

MDCK II-wt-V2R (A) or V2R-L44P, -I130F, -S167T or –S167L (indicated; B) cells were seeded on filters, grown to confluence followed by 16 hour treatment with 1 μM of the indicated pharmacological chaperones or left untreated. Next, cells were washed with PBS-CM followed by labeling with 100 nM [^{3}H]AVP for 1 hour at 4˚C. Filters were washed three times with PBS-CM, excised and counted in a scintillation counter. Values for untreated samples were set to 1. **(C)** MDCK II-wt-V2R, MDCK-V2R-L44P, -I130F, -S167T or –S167L cells (indicated) were seeded, grown, and treated as described above. Cells were subsequently washed in PBS-CM, and challenged for 10 minutes with 100 nM dDAVP in the presence of IBMX. cAMP accumulation was measured using a fluorescent cAMP assay kit. All experiments were performed at least in threefold.

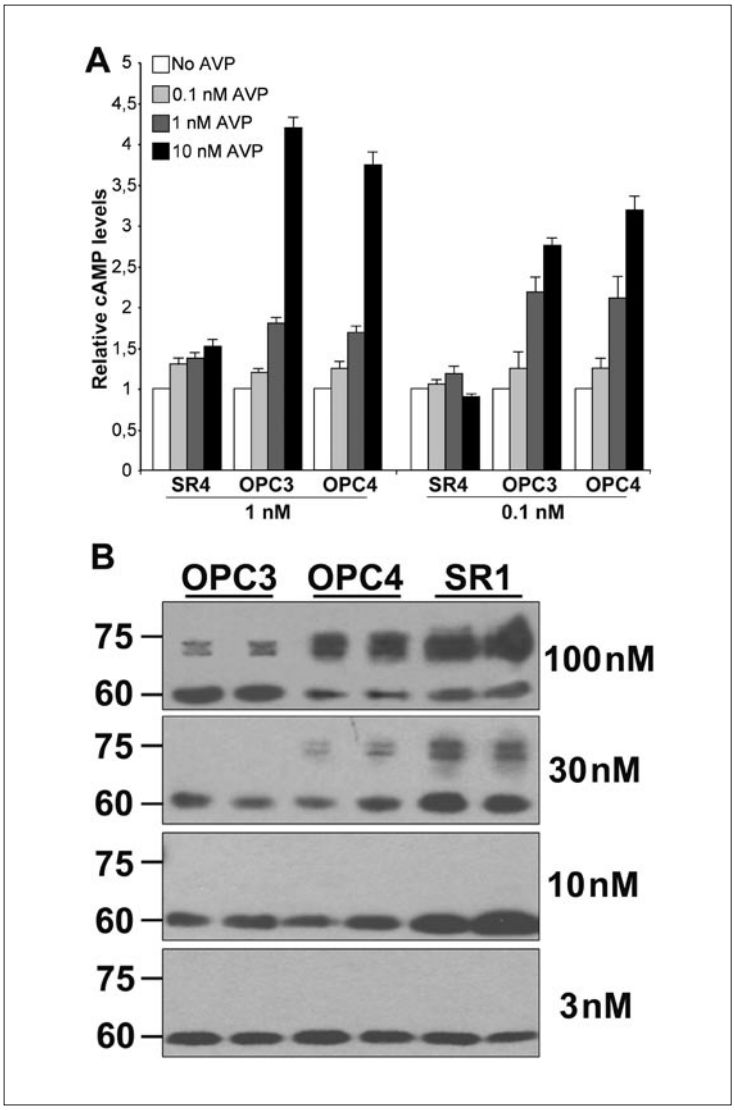

Figure 6-5 **Concentration- wdependent functional rescue of V2R-L44P**

MDCK II-V2R-L44P cells were seeded and grown to confluence, followed by a 16 hours treatment with the indicated concentrations of SR4, OPC3 or OPC4.

(A) Cells were washed with PBS-CM followed by a 10 minute challenge with different concentrations of AVP (indicated) in the presence of IBMX. Subsequently, cells were lysed and cAMP accumulation measured using a fluorescent cAMP assay kit. All experiments were performed at least in threefold.

(B) Cells were lysed and subsequently analyzed by SDS-PAGE followed by immunoblotting using anti-GFP antibodies. Molecular masses (in kDa) are indicated on the left. All experiments were performed at least in threefold.

As maturation can serve as a read-out for translocation of mutant receptors to the BM, the cell-permeable antagonists were tested for their ability to induce receptor maturation. As shown in figure 1, O/N treatment with 1 μM of the V1R antagonist SR4(9059), or the V2R antagonists OPC3(1260), OPC4(1061) and SR1(21463) did not significantly ($p>0.05$) increase the 75 kDa signal of wt-V2R. Treatment with OPC4 and SR1, however, caused a decrease or complete disappearance of the 60 kDa signals, respectively, suggesting that these compounds somewhat stabilize and increase receptor maturation of wt-V2R. Treatment of MDCK cells expressing V2R-L44P, -I130F or -S167T (Fig.1, middle three panels) with the four compounds resulted in increased maturation of all receptor mutants. However, the extent of maturation differed, as less matured V2R proteins were observed with SR4 as compared to the other compounds. Moreover, especially for SR1, increased receptor maturation was accompanied by a decrease of the 60 kDa signal. Maturation of the non-functional mutant V2R-S167L was not increased by treatment with any of the compounds tested, although its expression was somewhat increased with SR1 (fig.1, bottom panel). In MDCK type I cells, similar effects on maturation for the four compounds were observed for V2R-L44P, -del62-64, -R113W, -I130F, -G201D, -T204N (not shown), and V2R-S167T and -V206D(26).

Rescue of V2R mutant plasma membrane expression upon antagonist treatment

To determine whether increased receptor maturation coincided with increased basolateral membrane localization, the cells were also subjected to confocal laser scanning microscopic (CLSM) analysis. As reported for MDCK type I cells(23), wt-V2R was predominantly present in the basolateral membrane of untreated MDCK II cells. Its localization was not affected by treatment with any of the compounds (Fig.2, top row). Without treatment, V2R-L44P, -I130F, -S167T and –S167L were retained in the ER (Fig.2 for V2R-L44P, -S167L), where they co-localized with the ER marker protein disulfide isomerase (PDI).

Treatment for 16 hours with 1 μM SR4 did not visibly change the localization of V2R-L44P (Fig.2, middle row). Treatment with OPC3, OPC4 or SR1, however, resulted in a clear translocation of V2R-L44P to the BM (Fig.2, middle row), after which the localization was similar to that of wt-V2R. V2R-I130F and -S167T proteins responded similarly to the antagonist treatments as –L44P (not shown). V2R-S167L, however, did not translocate to the BM upon antagonist treatment, but remained trapped in the ER (Fig.2, bottom row). The lack of a visible translocation of the V2R mutants by SR4, whereas maturation was clearly observed (Fig.1), indicates that CLSM is less sensitive than immunoblotting.

Functional rescue of V2R mutants upon antagonist treatment

Following rescue to the plasma membrane, the antagonists need to be displaced by an agonist in order to have functional rescue. To study the rate of displacement, we used radioactively labeled AVP, as this most closely resembles the natural ligand of the V2R. When untreated, the amount of [^{3}H]AVP bound by mock-transfected cells was low compared to wtV2R-expressing cells (Fig. 3A). Also, [^{3}H]AVP binding to untreated MDCK II cells expressing V2R-L44P, -I130F, -S167T or -S167L was not significantly ($p>0.05$) different from binding to mock-transfected cells. To further exclude the presence of endogenous V2R in these cells, or the presence of low levels of V2R mutants in the plasma membrane, we determined whether dDAVP induces cAMP generation

in these cell lines. However, treatment of mock-transfected MDCKII cells with 100 nM dDAVP did not result in a cAMP response, whereas cells stably-expressing wtV2R showed an approximately 10-fold increase in intracellular cAMP levels compared to untreated cells. Also, the cell lines expressing the mutants V2R-L44P, -I130F, -S167T and -S167L did not respond to dDAVP treatment. In addition, basal cAMP levels were not significantly different between all cell lines and clones tested. Together, these data reveal that without a rescued cell surface expression of V2R mutants, these cells lack the ability to bind AVP or generate cAMP in response to dDAVP.

This was different upon a rescued cell surface expression. Pre-treatment of MDCK-V2R cells with 1 μM SR4 did not interfere with binding of AVP at all, as a similar amount of AVP was bound as found for non-pretreated control MDCK-V2R cells (Fig.4A). In contrast, both OPC3 and OPC4 treatment reduced the amount of available binding sites for the wild-type receptor to approximately 30% of the non-pretreated control MDCK-V2R cells, indicating that both compounds are displaced by AVP to some extent. Finally, pre-treatment with SR1 decreased the amount of available wt-V2R binding sites by 95% compared to control cells, indicating that this compound is hardly displaced with 100 nM [^{3}H]AVP.

Subsequently, AVP binding was tested on the V2R mutants treated with the antagonists. Although we observed no BM localization, but some maturation, for V2R-L44P, -I130F or –S167T upon treatment with SR4, this compound increased the number of AVP binding sites 4.2 fold (Fig.4B). OPC3 and OPC4 treatment, which clearly increased V2R mutant BM localization and maturation, increased the amount of binding sites for these three mutants 2-3 fold. In contrast, despite the clear BM localization and maturation of V2R-L44P, -I130F or -S167T upon treatment with SR1, incubation with this drug did not lead to a significant increase of binding sites for these mutants ($p>0.05$, $n=3$; Fig.4B). No significantly increased amount of AVP binding sites was measured for the non-functional mutant V2R-S167L with any of the treatments (Fig.4B). To test whether AVP binding also leads to intracellular signaling, cAMP measurements were performed following the same treatments as for the binding experiments. The relative cAMP levels generated (Fig.4C) were in line with the obtained levels of AVP binding (Fig.4B).

Functional rescue at reduced antagonists and AVP concentrations.

In line with the choice for the use of a V1R antagonist in patients(5), our data above suggest that SR4 is most effective to functionally rescue mutant V2R in patients. However, the used concentrations of the antagonists (1 μM) and AVP (100 nM) will be difficult to obtain in patients. Therefore, we tested functional rescue of the V2R mutants at decreased antagonist concentrations, and measured cAMP levels after stimulation with 0.1-10 nM concentrations of AVP. As shown in Fig.5A, pre-treatment of V2R-L44P expressing cells with 1 or 0.1 nM SR4 did not yield a cAMP response anymore upon stimulation with any of the used AVP concentrations. Pre-treatment with 1 or 0.1 nM OPC3 or OPC4, however, led to a 2-4 fold increase in cAMP levels when stimulated with 1 or 10 nM AVP, respectively. Similar results were obtained for V2R-I130F and -S167T (not shown). Pretreatment with SR1 did not result in significantly increased cAMP levels ($p>0.05$) when tested in the conditions above (not shown).

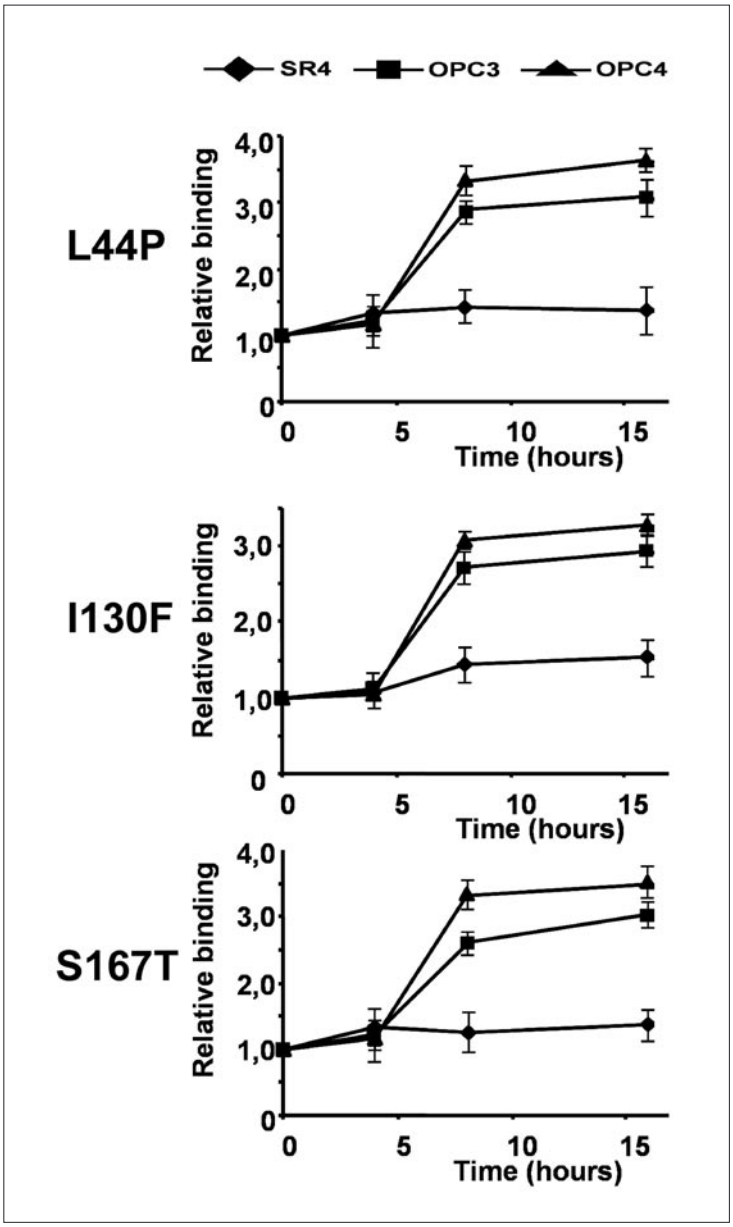

Figure 6-6 **Time-resolved rescue of V2R-L44P, -I130F and –S167T**

MDCK II-wtV2R (A), V2R-L44P (B), –I130F (C), or –S167T cells were seeded and grown to confluence, and treated for 0, 4, 8 or 16 hours with 0.1 nM SR4 (diamonds), OPC3 (squares), or OPC4 (triangles). Subsequently, cells were incubated for 1 hour with 1nM [^{3}H]AVP at 4°C followed by three washes with ice-cold PBS-CM, excision of the filters, and counting in a scintillation counter.

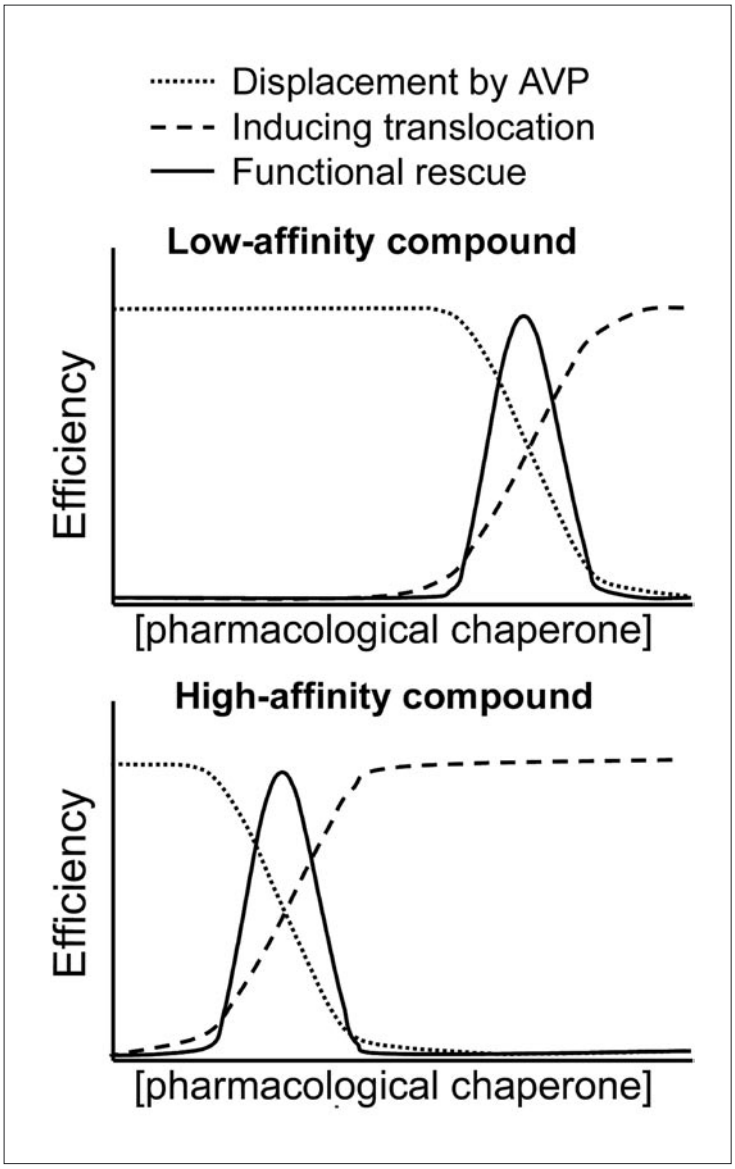

Figure 6-7 **Functional rescue is a balance between protein translocation and pharmacological chaperone displacement by AVP**
Schematic model for the efficiency of functional rescue for low- or high-affinity pharmacological chaperones. Increasing the pharmacological chaperone concentration enhances translocation of mutant receptors to the basolateral membrane, whereas displacement of the pharmacological chaperone by AVP will decrease. Functional rescue is optimal when both translocation and displacement takes place, and occurs at a high concentration for low-affinity pharmacological chaperones, and at a low concentration for higher-affinity pharmacological chaperones.

To determine the level of rescued cell surface expression at lower CPA concentrations, cells expressing V2R-L44P, -I130F, -S167T were treated for 16 hours with 100-to-3 nM concentrations of OPC3, OPC4 or SR1 and immunoblotted (Fig.5B). At 100 and 30 nM concentrations, the extent of maturation (75 kDa versus 60 kDa signals) of V2R-L44P was highest for SR1 and OPC4, whereas OPC3 showed only a limited amount (100 nM) or no (30 nM) mature V2R. A further decrease of the concentration of the CPAns to 10 nM did not reveal any maturation anymore, but showed an increased V2R-L44P expression for SR1 treated cells only. This SR1-specific effect on V2R-L44P expression was also found with 30 nM and 100 nM concentrations (Fig.5B, upper two panels). Treatment with 3 nM (Fig.5B, bottom panel) of any of the CPAns showed no effect anymore on V2R-L44P maturation or expression level. Similar data were found for the mutants V2R-I130F and -S167T (not shown).

Time-resolved functional rescue of V2R mutants.

Upon administration to patients, the blood concentrations of the antagonists will not be stable in time. Therefore, it is important to know how long it takes for the antagonists to confer a functionally-rescued V2R phenotype, and whether this is different between antagonists. To study this, cells expressing V2R-L44P, –I130F and –S167T were treated for different time points with 0.1 nM of the pharmacological chaperones, followed by [^{3}H]AVP labeling to semi-quantify the available AVP binding sites at the cell surface. Treatment of V2R-L44P, –I130F, or -S167T cells with OPC3 and OPC4 increased the available binding sites up to 3 fold, which became apparent after 8 hours of treatment and did not further increase when between 8 and 16 hours of treatment (Fig. 6). Consistent with the absence of any rescue at the used low concentrations, SR4 treatment did not significantly ($p>0.05$) affect [^{3}H]AVP binding at any of the time points. These data indicated that in cell culture, between 4-8 hours treatment with 0.1 nM antagonist is needed for a maximal functional rescue of the V2R mutants.

Discussion

Pharmacological chaperones rescue a broad spectrum of V2R mutants

By definition, class II mutant proteins are ER retained due to misfolding. Binding of an antagonist to a mutant receptor can reverse the distorting effect of the mutation, and thus aid in protein folding(3; 32). Indeed, our study reveals that all nine V2R mutants in NDI tested, except for V2R-S167L, are stabilized by the used antagonists, resulting in different levels of receptor maturation. In line with the finding of Tan et al. that achieving the proper complex glycosylation state is necessary for V2R to reach the BM(31), maturation of the V2R mutants on immunoblot coincides with BM expression as detected by CLSM. Consistently, V2R-S167L did not mature with any of the ligands and failed to leave the ER, which has been suggested to be due to severe distortion of the structure(34). Our data indicate, however, that V2R-S167L can be bound by the V2R antagonist SR1, as administration of this drug increased the amount of immature proteins (Fig.1). A similar ER-stabilizing effect has been observed for the non-peptide antagonist naltrexone on immature forms of the δ-opioid receptor(21). In line with the data from Morello et al.(18), the rescue of multiple V2R mutants reveals the high efficacy by which non-peptide antagonists stabilize ER-retained mutant proteins. These effects were observed for different clones obtained in MDCK I (not shown) and MDCK II cell lines, indicating that the effects were inherent to the V2R mutant and not due to clonal differences. In contrast, using the same V2R mutants, a subset of chemical chaperones

restored the BM localization of only one mutant, V2R-V206D(26). In addition, the level of maturation and cell surface rescue of V2R-V206D upon treatment with chemical chaperones was also less than observed for the antagonists OPC3, OPC4 and SR1 (not shown). Likely, this is due to their different mode of action, as cell-permeable antagonists stabilize the receptor's conformation through direct interaction, whereas chemical chaperones may evoke a stress response, modify the activity of folding proteins, or dehydrate the mutant's environment(33).

In general, the extent of cell surface rescue of V2R mutants is determined by the affinity of the antagonists

Brothers et al. suggested that C-terminal fusion of GFP to a receptor might induce plasma membrane expression (6). However, our data reveal that this is not the case here, as the MDCK cells expressing the GFP-tagged V2R mutants have no significant radioligand binding or cAMP signaling after agonist stimulation (Fig. 3). Following rescue to the cell surface, however, most of the V2R mutants are able to bind AVP and consequently elicit a cAMP response. Our data show that SR1 and OPC4 induce maturation of V2R-L44P, -I130F and –S167T the best, followed by OPC3 and, much less, SR4 (Figs.1, 5B). Similarly, at 1 μM concentrations, SR1, OPC4 and OPC3 induce a robust cell surface rescue, whereas SR4 induced no detectable translocation (Fig. 2). A similarly-reduced rescue for V2R-S167T and V2R-del62-64 by SR4 compared to SR1 was found by others(34). With the exclusion of severely distorted receptors (V2R-S167L), our data indicate that the level of maturation and translocation of V2R mutants in general is directly related to the antagonists' affinities for V2R (table 1), as similar relative effects were observed for eight out of nine V2R mutants tested.

Functional rescue is a balance between membrane expression and displacement by (dD)AVP

Once at the plasma membrane, functional rescue can only occur if the pharmacological chaperone is displaced by an agonist, thereby allowing receptor activation and induction of the signaling cascade(12). Our data reveal that for functional rescue of V2R mutants, the pharmacological chaperone should fit two contradictory criteria: it should have a sufficient high affinity to facilitate the mutant receptor's stabilization and translocation to the plasma membrane, but its binding should not be so strong as to interfere with its displacement by AVP. Moreover, our data confirm that the extent of functional rescue critically depends on the used concentration(12; 18).

At high concentrations (1 μM), SR4 showed a weak cell surface rescuing effect for the V2R mutants, whereas OPC3, OPC4 and SR1 rescued large amounts of receptor to the BM (Figs. 1, 2). Nevertheless, subsequent AVP binding and cAMP generation is considerably lower for OPC3, OPC4 and SR1 compared to SR4 treated cells and correlates largely with their affinities for the V2R (Fig.4B,C; table 1). These data indicated that the extent of displacement by (dD)AVP is of major importance at these concentrations. A better functional rescue is obtained with a few rescued receptors which are fully available for AVP binding (SR4) than when many V2R mutants are rescued, which are limitedly available for AVP binding (OPC3, OPC4, SR1). At low concentrations, however, pre-treatment with (sub)nanomolar concentrations of OPC3, OPC4 or SR4, followed by stimulation with 1-10 nM AVP, only resulted in increased cAMP levels for OPC3 and OPC4 (Fig.5). As shown in Fig. 5B, low concentrations of OPC3 and OPC4 are still able to induce cell surface trafficking and maturation, whereas

SR4 is not. Therefore, at low antagonist concentrations, the extent of rescued cell surface expression becomes critical.

The absence of functional rescue upon SR1 treatment seems to contradict data published by Morello et al., who found up to 15 fold increased cAMP levels with V2R mutants pretreated with SR1. This difference is most likely due to the higher agonist/antagonist ratio and concentrations use by Morello et al, which were 10-fold and 100-fold higher as compared to our 'high concentration amounts', respectively. This, however, provides additional support that the observed effect on cAMP generation depends on the used concentrations and ratios of antagonist versus agonist.

Surprisingly, OPC4 and SR1 have similar affinities for the V2R (Table 1), but OPC4 was easier displaced by AVP than SR1 (Fig.4A) and consequently yielded better functional rescue at any concentration used (Figs.4B,C). This difference was not caused by a reduced V2R mutant cell surface expression with SR1, as at low concentrations this was similar to, or better than, that of OPC4 or OPC3. Possibly, the different effects observed for SR1 and OPC4 might be due to differences in their V2R binding sites, as recently established(15). This is underscored by our finding that SR1, but not OPC4, stabilizes the ER retained form of V2R-S167L (Fig.1). These data reveal that compound-intrinsic factors other than their affinities influence their extent of displacement by AVP and ability to confer functional rescue.

Optimal pharmacological chaperone to treat congenital NDI

Treatment with SR4 showed a significant increase in urine concentration in three NDI patients encoding V2R-R137H, thereby providing the proof of principle that pharmacological chaperones can relieve NDI(5). In two other patients encoding V2R-W164S and del62-64 (185-193del), however, the response to SR4 treatment was weaker. Interestingly, V2R-R137H is only a partial class II mutant, as a considerable portion of this mutant is fully matured, but is constitutively internalized from the plasma membrane (class V)(1), whereas V2R-W164S and del62-64 are fully retained in the ER(5). The reduced ER-retention suggests a low level of misfolding of V2R-R137H, and the difference in the extent of ER-retention between V2R-R137H and other V2R mutants may underlie the observed effects of SR4 in the NDI patients(4). Likely, due to its low maximal blood plasma concentration of 30 nM (D. Bichet, personal communication; erratum in press), SR4 does not effectively rescue full class II mutants at low concentrations (Fig.5A). As OPC3 and OPC4 allow functional rescue of fully ER-retained V2R mutants at nanomolar concentrations and NDI patients harbouring full class II mutations are much more common (25), these compounds are anticipated to relieve NDI better than SR4 and in more NDI patients. Moreover, and in line with the adopted strategy by Bernier et al. (5), continuously elevated levels of the antagonists are needed, as it takes >4 hours before a functional rescue is obtained (Fig. 6). Since non-peptide antagonists remain active in vivo up to 8 hours (11; 27), this would require the administration of at least 3 doses per day. The analyses in patients will be the subject of future studies.

In conclusion, we have demonstrated that cell-permeable V2R antagonists can rescue the cell surface expression of a broad spectrum of ER-retained V2R mutants and that functional rescue is a balance between a cell-permeable antagonist's ability to rescue the cell surface expression of the V2R mutant and its ability to be displaced by

AVP. Moreover, we show that at low concentrations the functional rescue occurs most efficiently by antagonists with a relatively high affinity for the receptor (Fig.7). Our findings that a large number of V2R mutants are rescued by pharmacological chaperones, and that functional rescue of mutant V2Rs at low antagonist concentrations is most effective with relatively high-affinity antagonists are anticipated to become of importance for other diseases, such as hypogonadotrophic hypogonadism(12), early-onset obesity(16), or hypothyroidism(7), in which mutations in GPCRs are causal and for which cell-permeable antagonists are, or may become, available.

Concerning NDI patients with V2R mutations, of the four compounds tested, OPC3(1260) and OPC4(1061) combine cell surface rescue and displacement by AVP best when tested with low antagonist and near-physiological AVP concentrations. While other high-affinity V2R antagonists might be as suitable, OPC31260 is currently being tested as a treatment for polycystic kidney disease(2), while OPC41601 is under trial to treat hyponatremia and congestive heart failure in man(19). Since negative side- or toxicity-effects have not been reported in these studies, OPC31260 and OPC41061 represent safe and promising candidates to treat NDI in patients with type II mutations in the V2R.

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Chapter 7

Intracellular activation by non-peptide agonists rescues the function of mutant vasopressin V2 receptors in Nephrogenic Diabetes Insipidus

Abstract

Mutations in the vasopressin V2 receptor (V2R) cause the urine concentration defect Nephrogenic Diabetes Insipidus. Often, V2R missense mutants are functional on the molecular level, but ER-retention prevents them to bind vasopressin (AVP). We tested the ability of three non-peptide agonists (OCP51803, FE999088 and FE999089) to functionally rescue six ER-retained V2R mutants, stably expressed in polarized kidney cells. Unlike cell-permeable antagonists, that increase the basolateral membrane localization of mutant V2R, non-peptide agonists did not induce receptor maturation or plasma membrane trafficking of the mutants. However, brief incubation with the non-peptide agonists, but not with a peptide agonist, increased intracellular cAMP levels, indicating that the mutant receptors initiate the cAMP-cascade from the ER. In addition, the induced cAMP response stimulated the insertion of aquaporin-2 water channels in the apical plasma membrane. Thus, non-peptide agonists represent a novel class of therapeutics to functionally rescue intracellularly retained GPCRs via signaling from the ER.

Introduction

Misfolding of membrane proteins due to the presence of a mutation often leads to the retention of such mutant proteins in the endoplasmic reticulum (ER). Mutations in genes encoding G protein-coupled receptors (GPCRs), are the molecular cause of many genetic diseases, including a genetic form of mental retardation (32), retinitis pigmentosa (15), hypogonadotropic hypogonadism (10), ovarian dysplasia (1), obesity (30), hypothyroidism (7), and X-linked nephrogenic diabetes insipidus (NDI) (27; 31).

In the kidney, the vasopressin V2 receptor (V2R) is predominantly expressed in the basolateral membrane (BM) of the principal cells of the collecting duct, where it regulates water reabsorption. Binding of the antidiuretic hormone arginine-vasopressin (AVP) to the V2R induces a cAMP-cascade via the stimulatory G protein ($G\alpha s$) and adenylate cyclase. Subsequent activation of protein kinase A induces phosphorylation of aquaporin-2 (AQP2) water channels, leading to their translocation to the apical membrane, rendering this plasma membrane water-permeable. Following an osmotic gradient across the principal cells, water reabsorption and urine concentration is achieved. Patients suffering from congenital NDI are unable to concentrate their urine in response to AVP, resulting in polyuria and, consequently, polydipsia. In approximately 10% of these patients, NDI is caused by mutations in the AQP2 gene. In the majority of patients, however, V2R mutations lead to the absence of functional V2R in the basolateral membrane of the principal collecting duct cells.

Recently, we generated and characterized polarized cell lines stably expressing V2R missense mutants, and classified them according to their cellular fate. Class II mutations, which form the most prominent class of V2R mutations in NDI, induce ER retention of the mutant V2R, which precludes their binding to AVP at the BM (11; 25). However, several V2R mutants may be functional on a molecular level, i.e. they are able to bind ligand and activate G proteins (6; 18; 25; 33). In the search to cure NDI, research has focused on identifying compounds that allow the escape of V2R mutants from the ER quality control and restore their plasma membrane localization. Most promising

have been the so-called pharmacological chaperones (5; 18; 26; 29; 33) which are cell-permeable antagonists that are able to bind and stabilize the conformation of mutant receptors in the ER. As a result, they escape the quality control mechanism of the ER, mature in the Golgi compartment, and traffic to the BM (4). In order for the receptor to be able to signal, the antagonist then has to be displaced by AVP, which, depending on the characteristics of the antagonist, may require supra-physiological concentrations of AVP (Chapter 6).

Application of cell-permeable agonists to functionally rescue mutant GPCRs seems a logic solution to overcome displacement, as the bound agonist will lead to receptor activation as soon as the receptor reaches the plasma membrane. In a recent study, Petäjä-Repo et al. reported that non-peptide agonists of the δ-opioid receptor (DOR) are able to induce stabilization and maturation of the wild-type DOR (21), which is poorly processed in HEK293 cells (22). To asses whether non-peptide V2R agonists could function in a similar way on immature ER-retained V2R mutants in NDI, we tested the ability of the non-peptide V2R agonists FE999088 (FE88), FE999089 (FE89) and OPC51803 (OPC51) to induce receptor maturation, its translocation to the BM and rescue the function of class II V2R mutants in NDI. In theory, the use of cell-permeable agonists would possibly constitute a better strategy to relieve NDI than currently adopted methods.

Materials and Methods

Agonists

The V2R agonist OPC51 (19) was kindly provided by Dr. Komuro (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan). The V2R agonists FE88 and FE89 (patent WO01/29005) were kindly provided by Dr. Haigh and Wittendorf (Ferring Research Ltd., Southampton, UK). SR121463B (SR1) (28) was kindly provided by Dr. Serradeil-Le Gal (Sanofi Synthélabo Recherce, Toulouse, France) All compounds were dissolved in dimethylsulfoxide as 0.01 M stock solutions, and diluted in culture medium as indicated. dDAVP was from Sigma (St. Louis, MO).

Expression constructs

Expression constructs encoding the wild-type (wt) V2R or the NDI causing mutants –L44P, -I130F, -S167T, or S167L fused at their C-termini to enhanced GFP were as described (25). Using the quick-change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), mutations were introduced into the human V2R cDNA sequence using pEGFP-N1-V2R as a template. Primers used were GGTGGGCATGTACGCG TCCTCCTCCATGATCCTGG for Y128S, GTGGTCGTCTGTGTGCTGTGCT GGGCCCCCTTCTTCC for Y280C, and CCTCAACAGCTGCACGAATTCCT GGATCTATGC for P322S, and their complementary antisense primers. 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.

Generation of recombinant lentivirus

To construct a recombinant lentivirus for transient transduction of MDCK II cells with AQP2, the AQP2 cDNA was cut from pBSII-KS(+)wtAQP2 using SpeI

and XhoI, and cloned into the corresponding sites of pRRL-CMV (Tronolab, Geneva, Switzerland) to yield pRRL-CMV-wtAQP2. Subsequently, pRRL-CM-wtAQP2 was digested with MluI, blunt-ended using the Klenow fragment, and cut with NheI. The 994 bp insert containing the wtAQP2 coding sequence was cloned into the NheI and the blunt-ended AgeI site of pLV-PGK (Tronolab, Geneva, Switzerland) to yield pLV-PGK-wtAQP2. Recombinant lentiviruses were generated by cotransfecting pLV-PGK-wtAQP2, with the pMD2G, pMDLg and pRSV-REV (Tronolab, Geneva, Switzerland) plasmids into HEK293T cells using the calcium phosphate method. Two and three days post-transfection, the supernatant containing recombinant viruses was collected followed by centrifugal concentration of the viruses and subsequent titration using an ELISA assay based on measurement of viral DNA. 1 ng viral DNA on 2,500 cells corresponds to a multiplicity of infection (MOI) of 1 (http://tronolab.epfl.ch/page58122.html).

Cell culture, transfection and infection

MDCK type II cells lacking endogenous V2R expression were kindly provided by Dr. Oksche (FMP, Berlin, Germany). MDCKII cell maintenance, transfection and isolation of clones were done as described for MDCK type I cells (12). Experiments were performed at least on two clones per transfectant. Maintenance, transient transfection of COS-M6 cells were done as described (25). For transient expression of AQP2 in MDCK cells, 70-80% confluent cells were trypsinized, and cells were mixed with the PGK-AQP2 lentivirus at a MOI of 2. Cells were then seeded at a density of 300.000 cells/cm^2 on Costar filters in presence of the lentivirus and incubated for 16 hours at 37^{o}C. Subsequently, the medium was replaced with normal culture medium and the cells were grown for an additional two days, treated as indicated, and subjected to apical cell surface biotinylation (2) or immunocytochemistry (12) as described.

Immunoblotting and immunocytochemistry

Poly-acrylamide gel electrophoresis, western blotting and immunodetection were performed as described (12; 24). Immunocytochemistry (ICC), confocal laser scanning microscopy (CLSM) and data quantification were performed as described (Robben et al., 2005) .

cAMP measurements

MDCKII cells were seeded on 24 multiwell Costar filters at a density of 150.000 cells/cm^2, and grown to confluence. Subsequently, cells were briefly washed in PBS-CM, followed by incubation for 10 minutes in culture medium supplemented with 250 μM 3-Isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO) to prevent

Compound	Abbreviation	(Ant)agonist	(Non-)peptide
1-desamino-8-D-AVP	dDAVP	agonist	peptide
FE999088	FE88	agonist	non-peptide
FE999089	FE89	agonist	non-peptide
OPC51803	OPC51	agonist	non-peptide
SR121463B	SR1	antagonist	non-peptide

Table 7-1 | Overview of the used compounds

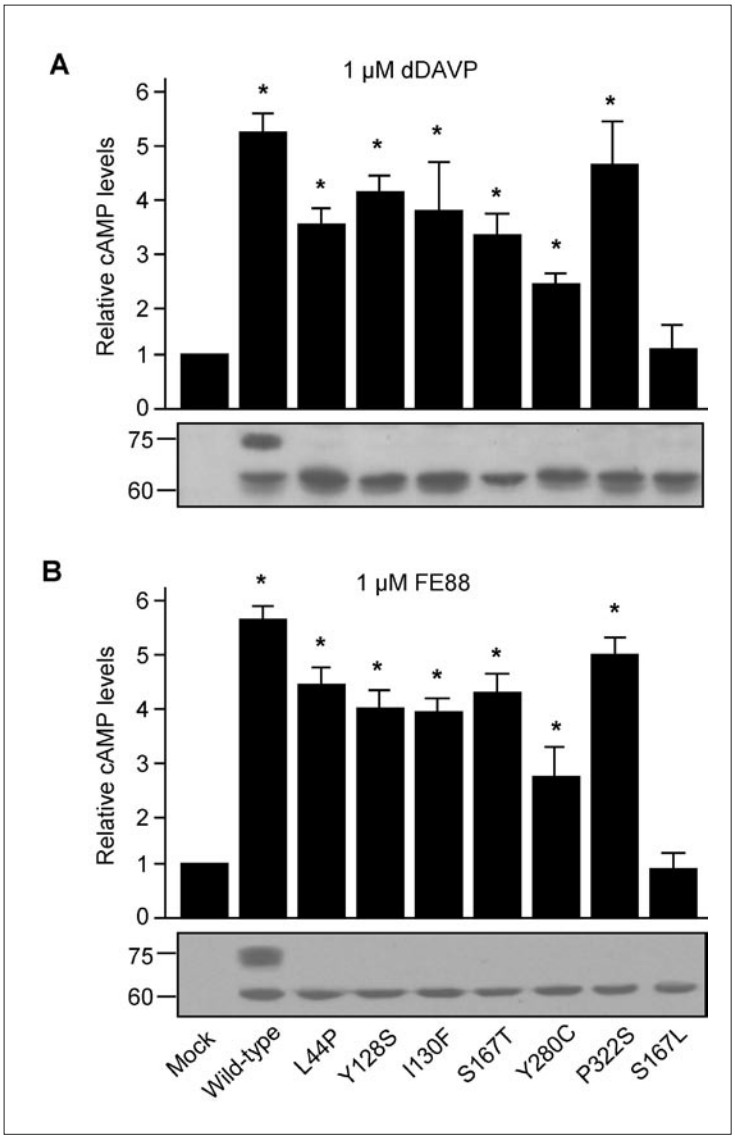

Figure 7-1 **Functionality of NDI causing V2R mutants**

COS-M6 cells transiently expressing wtV2R or the indicated mutants were treated for 10 min. with 1 µM dDAVP (A) or 1 µM FE88 (B), washed, lysed in 0.1 M HCl and cAMP levels were measured using a fluorescent assay kit. The untreated samples were set to 1, and relative changes are shown on the vertical axis. Triplicate samples were measured and experiments were performed at least in threefold. Significantly-increased cAMP increased ($p<0.01$) compared to mock-transfected cells are indicated by an asterisk. In parallel, cell equivalents from these experiments were lysed in Laemmli buffer, run on a 10% PAAG and analyzed by immunoblotting using anti-GFP antibodies. Protein masses are indicated in kDa.

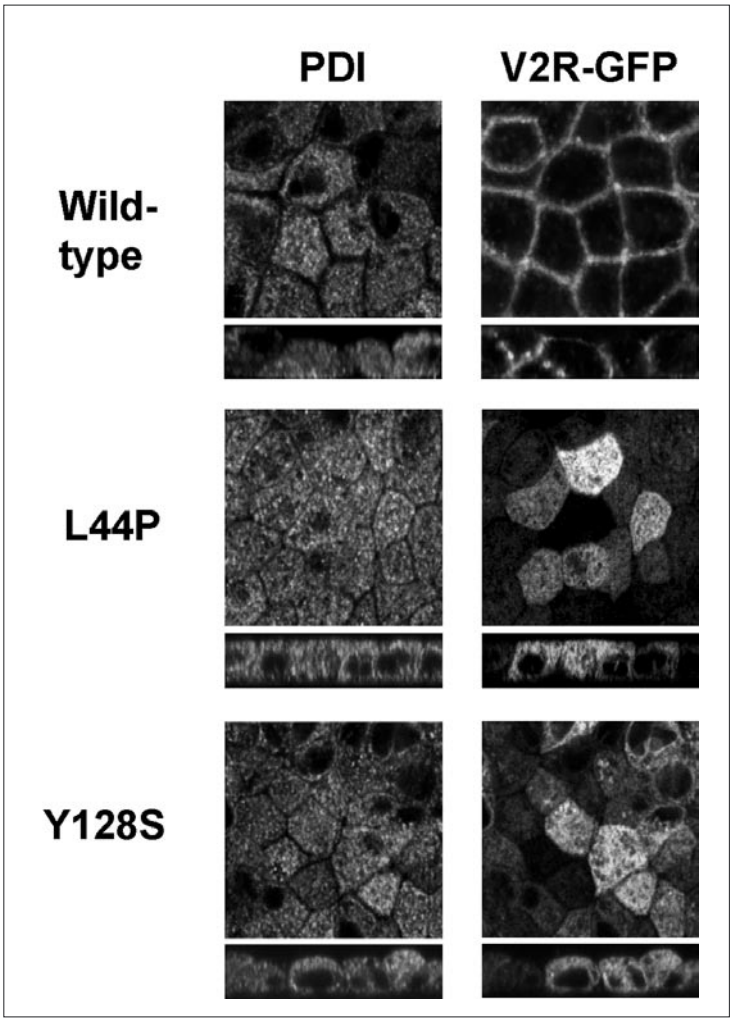

Figure 7-2 **Subcellular localization of wtV2R and V2R mutants in NDI**
MDCK cells expressing wtV2R, or the mutants V2R-L44P, or –Y128S were grown to confluence on filters. Subsequently, the cells were fixed, subjected to immunocytochemistry using anti-PDI antibodies to stain the endoplasmic reticulum (left panel), and analyzed by CLSM.

cAMP degradation by phosphodiesterases. Cells were then challenged for 10 minutes with agonists on the apical and basolateral side in the presence of IBMX. After three washing steps with PBS-CM, cells were lysed in 100 μM 0.1 M HCl and cAMP was measured using a fluorescent cAMP assay kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Measurement of intracellular cAMP in transiently transfected COS-M6 cells was performed as described (25). Triplicate samples were measured, and experiments were performed at least in threefold.

Results

Functionality of V2R mutants in NDI

The first prerequisite for functional rescue is a receptor that is functional on the molecular level, i.e. it should be able to bind agonist, and subsequently activate G proteins. To assess whether mutants in NDI are functional, we used a previously successful strategy (25), by which putative ER-retained V2R mutants are transiently overexpressed in COS cells, thereby forcing a subset of otherwise ER-retained receptors to the plasma membrane. Besides the wild-type (wt) V2R and the non-functional mutant V2R-S167L (25), the V2R mutants -L44P, -Y128S, -I130F, –S167T, -Y280C

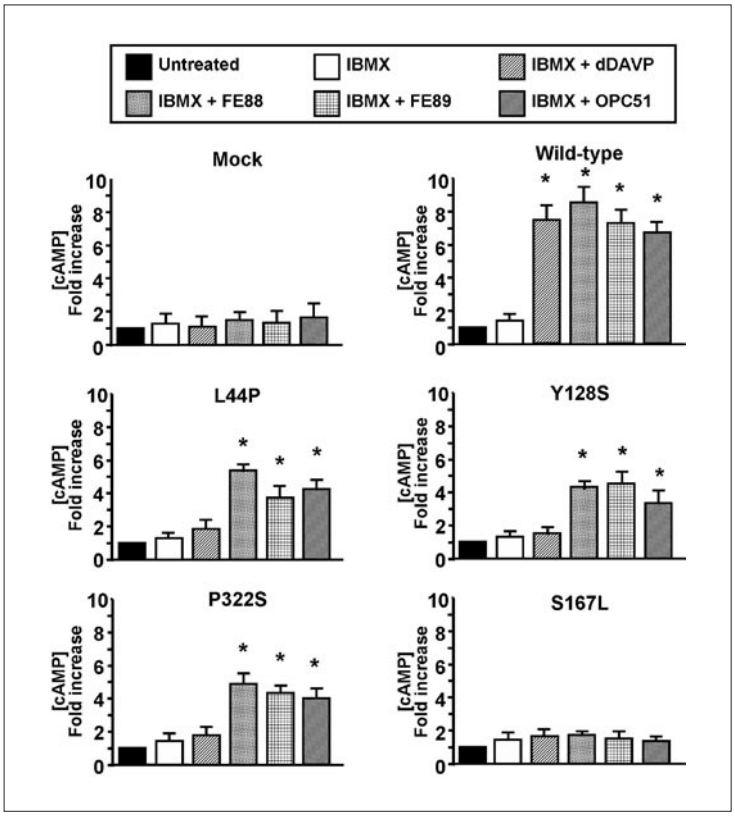

Figure 7-3

Functionality of ER retained V2R mutants upon treatment with non-peptide agonists

Filter-grown confluent MDCK cells grown expressing wtV2R or the mutants V2R-L44P, -Y128S, -P322S, or –S167L were left untreated, treated for 10 minutes with IBMX alone, or in combination with 1 μM dDAVP, FE88, FE89, or OPC51 (indicated). Subsequently, cells were washed, lysed in 0.1 M HCl, and cAMP levels were measured. The untreated samples were set to 1, and relative changes are shown on the vertical axis. Triplicate samples were measured, and experiments were performed at least in threefold. Bars indicated with an asterisk were significantly increased ($P<0.05$) compared to IBMX alone.

and -P322S were overexpressed in COS cells. Subsequent stimulation with 1 μM of the synthetic non cell- permeable AVP analogue dDAVP for 10 minutes significantly ($p < 0.05$) increased cAMP levels for wtV2R (5-6 fold), whereas stimulation of all the mutants, except for V2R-S167L increased cAMP levels approximately 3-4 fold. Expression of wtV2R and the V2R mutants was confirmed by immunoblotting (Fig. 1A). In a parallel experiment, stimulation with 1 μM FE88 for 10 minutes resulted in similar cAMP increases as observed for dDAVP (Fig. 1B). V2R-S167L did not respond to FE88 treatment. Expression of wtV2R and the mutants was confirmed by immunoblotting. Similar results were observed for treatment with FE89 and OPC51 (data not shown).

Subcellular localization of wtV2R and mutants

MDCK type II cells are a polarized cell model for the collecting duct that lack endogenous expression of V2R (20), and are suitable to study protein localization as well as for functional experiments. In stably-transfected MDCK cells, the C-terminal GFP tag fused to V2R does not interfere with the function and localization of the wtV2R,

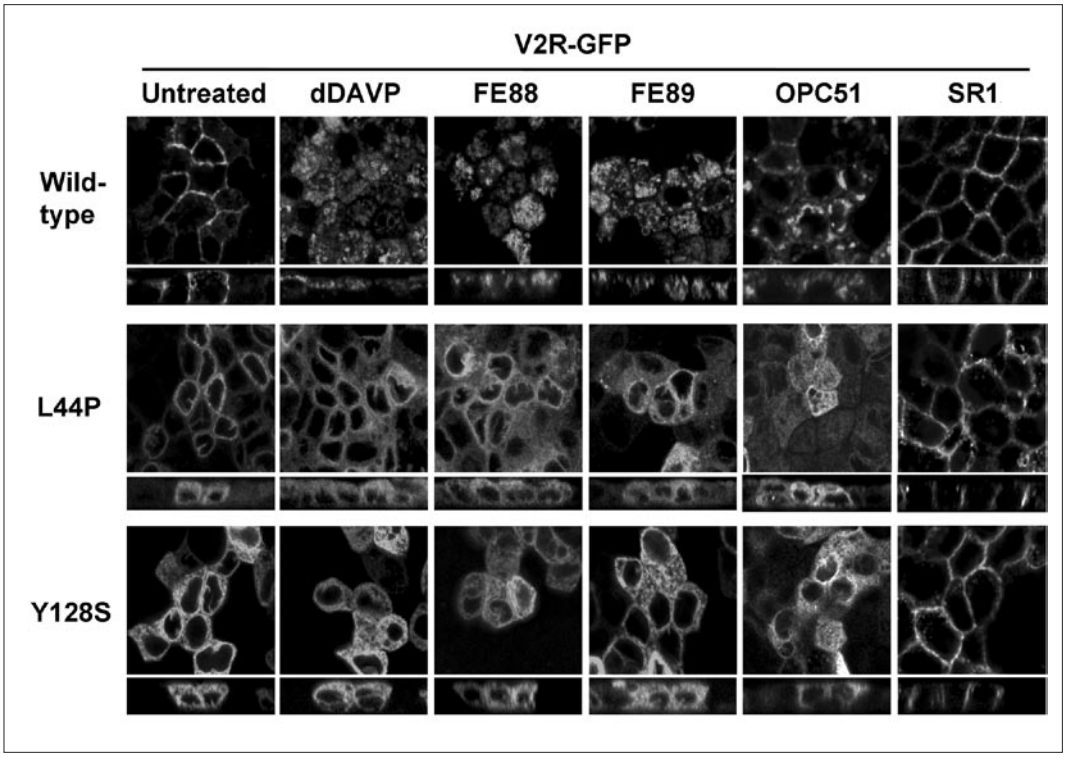

Figure 7-4 **Peptide and non-peptide agonist action on the localization of wtV2R-GFP and V2R mutants in NDI**

MDCK cells expressing wtV2R, or the mutants V2R-L44P or -Y128S, were grown to confluence on filters, and subsequently left untreated, or treated with 1 μM of the peptide agonist dDAVP, the non-peptide agonists FE88, FE89 or OPC51, or the non-peptide antagonist SR1 for 16 hours. Subsequently, cells were fixed and analyzed by CLSM.

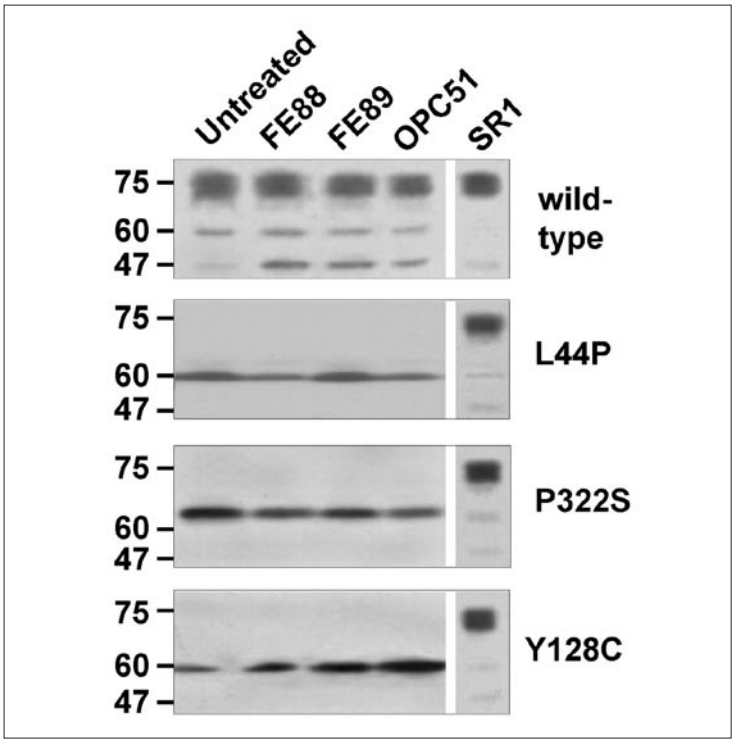

Figure 7-5 **Effect of non-peptide V2R agonists on expression level and maturation of V2R mutants**

MDCK cells expressing wtV2R-GFP, or the mutants V2R-L44P, -P322S, or -Y128S were left untreated, treated with the non-peptide agonists FE88, FE89 or OPC51, or with the non-peptide antagonist SR1 for 16 hours. Subsequently, cells were lysed in Laemmli sample buffer, run on a 10% PAAG and analyzed by immunoblotting using anti-GFP antibodies.

which was expressed predominantly in the basolateral membrane, and did not co-localize with the ER marker protein disulfide isomerase (PDI; Fig. 2). However, the mutants V2R-L44P, Y128S, -I130F, -S167T, -S167L, -Y280C and P322S were expressed in the ER, where they co-localized with PDI (Shown for V2R-L44P and –Y128S; Fig. 2).

Functional rescue of V2R mutants by non-peptide agonists

Next, we studied whether any of the three non-peptide agonists was able to induce a cAMP response in the cell lines expressing wtV2R or the mutants. To test whether the compounds acted as agonists on MDCK cells expressing exogenous V2R-GFP, mock-transfected and wtV2R expressing cells were stimulated with 1 μM dDAVP or the three non-peptide agonists. As shown in figure 3, the peptide and non-peptide agonists induced a robust cAMP response, which was approx. 8-9 fold over the basal cAMP level. Administration of 1 μM dDAVP did not induce a significant cAMP response in cells expressing V2R-L44P, -Y128S, -P322S, -S167L (Fig. 3), -I130F, -S167T and Y280C (data not shown). Incubation for 10 minutes in the presence of 1

μM FE88, FE89 or OPC51, however, caused a significant increase of cAMP for V2R-L44P, -Y128S, -P322S (fig. 3. P<0.01), -I130F and –S167T (data not shown) of 4-5 fold. For V2R-Y280C, the increase of intracellular cAMP was 3-4 fold (data not shown; P<0.05). cAMP levels of cells expressing the non-functional mutant V2R-S167L did not significantly (P>0.05) change upon treatment with any of the compounds.

Subcellular localization of V2R mutants upon treatment with non-peptide agonists

Next, we tested whether the non-peptide agonists were able to change the subcellular localization of wtV2R or the mutants. In the absence of agonists, wtV2R was predominantly localized in the BM. Incubation for 16 hours with 1 μM of the peptide agonist dDAVP or any of the non-peptide agonists induced internalization of wtV2R into late endosomal and lysosomal vesicles. In contrast, the non-peptide antagonist SR1 did not alter the BM localization of wtV2R compared to untreated cells (Fig. 4, top row). Treatment with the non-cell permeable V2R agonist dDAVP did not affect the ER localization of the V2R mutants V2R-L44P, -Y128S (Fig. 4) or any of the five other mutants (data not shown). Also, treatment with any the non-peptide agonists did not affect protein localization for any of the mutants. In contrast, treatment with the non-peptide antagonist SR1 clearly increased the BM localization of these mutants (Fig. 4, right panel).

Non-peptide agonists affect protein expression levels, but not maturation of V2R mutants

We have previously shown that CLSM analysis is less sensitive compared to immunoblotting to detect receptor maturation upon treatment with non-peptide ligands (Chapter 6). Therefore, we analyzed whether non-peptide agonists induce maturation of the V2R mutants from their immature 60 kDa form to mature 75 kDa proteins. Overnight treatment with the non-peptide agonists FE88, FE89 or OPC51 did not significantly (p>0.05) affect the expression level or maturation of wtV2R (Fig. 5, top panel), but increased the signal of a 47 kDa V2R-GFP degradation product, which is likely due to increased degradation as reported for dDAVP treatment of wtV2R expressing cells (24).

V2R-L44P was expressed as an immature protein of approx. 60 kDa in MDCKII cells (Fig. 5, 2nd panel). Overnight treatment with FE88 and OPC51 resulted in approximately 2-fold decreased expression levels compared to untreated cells (P<0.05), and did not increase receptor maturation. FE89 treatment did not affect the expression level of V2R-L44P compared to untreated cells, but also failed to induce receptor maturation. Similar results were obtained for V2R-Y128S, -I130F and -S167T (data not shown). For unknown reasons, the mass of V2R-P322S (Fig. 5, 3rd panel) was slightly larger compared to the other mutants, but responded similar to treatment with the non-peptide agonists and antagonist as V2R-L44P. V2R-Y128S, however, which was expressed as an immature protein of approx. 60 kDa, was increased in its expression with all three compounds, but did not show maturation (Fig.5, bottom panel). The extent of the increase varied between different V2R-Y128S clones. As published, and in contrast to the above, treatment of V2R-Y128S, –L44P and -P322S cells with SR1 increased the levels of the mature 75 kDa form (Chapter 6).

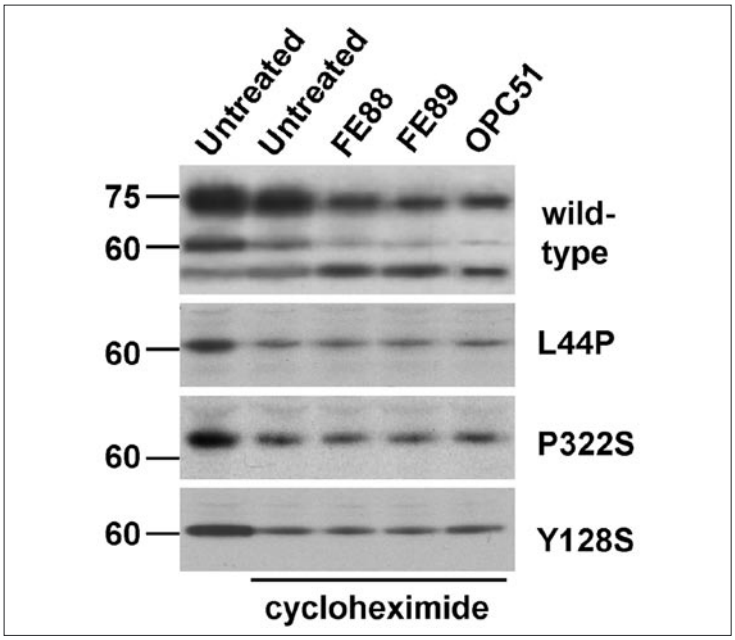

Figure 7-6 **Effect of non-peptide agonists on post-translational stability of wtV2R and V2R mutants**

MDCK cells expressing wtV2R-GFP or the mutants V2R-L44P, -P322S, or -Y128S were seeded on filters, and grown to confluence. Then, cells were left untreated, treated with 50 μM cycloheximide alone, or in combination with 1 μM FE88, FE89 or OCP51 (indicated) for 6 hours. Subsequently, cells were lysed in Laemmli sample buffer, run on a 10% PAAG and analyzed by immunoblotting using anti-GFP antibodies.

Receptor stability

From the data above, it is clear that non-peptide agonists can bind and activate wtV2R at the plasma membrane and V2R mutants in NDI in the ER. As we and others previously reported that activation of the wtV2R by dDAVP leads to its lysosomal degradation (9; 24), we investigated whether also the ER-retained mutants would become increasingly degraded upon agonist stimulation. When wtV2R cells were treated for 6 hours with 50 μM cycloheximide in order to block protein synthesis, the 60 kDa signal markedly decreased, whereas the 75 kDa signal decreased only slightly (Fig. 6, top panel). Co-treatment with the agonists FE88, FE89 or OPC51, however, lead to a further decrease of the 75 kDa signal, which coincided with an increased intensity of the 47 kDa band. Incubation of the mutants V2R-L44P, -P322S, -Y128S (Fig. 6), -I130F, -S167T, -S167L and -Y280C (data not shown) with cycloheximide for 6 hours also decreased their expression level, but co-incubation with any of the agonists did not lead to a further decrease of the signals. With the mutants, a 47 kDa signal as found for wtV2R upon agonist treatment was not observed.

Translocation of AQP2

In order to relieve NDI in patients, the cAMP response that is induced as a result of the administration of non-peptide agonists should be sufficiently strong to activate PKA and allow trafficking of AQP2 to the plasma membrane. To study this, MDCKII-V2R-L44P cells were infected with recombinant lentiviruses that contain a wtAQP2 expression cassette. As shown in figure 7A, AQP2 was expressed intracellularly, consistent with a vesicular localization, when treated with the cyclooxygenase inhibitor

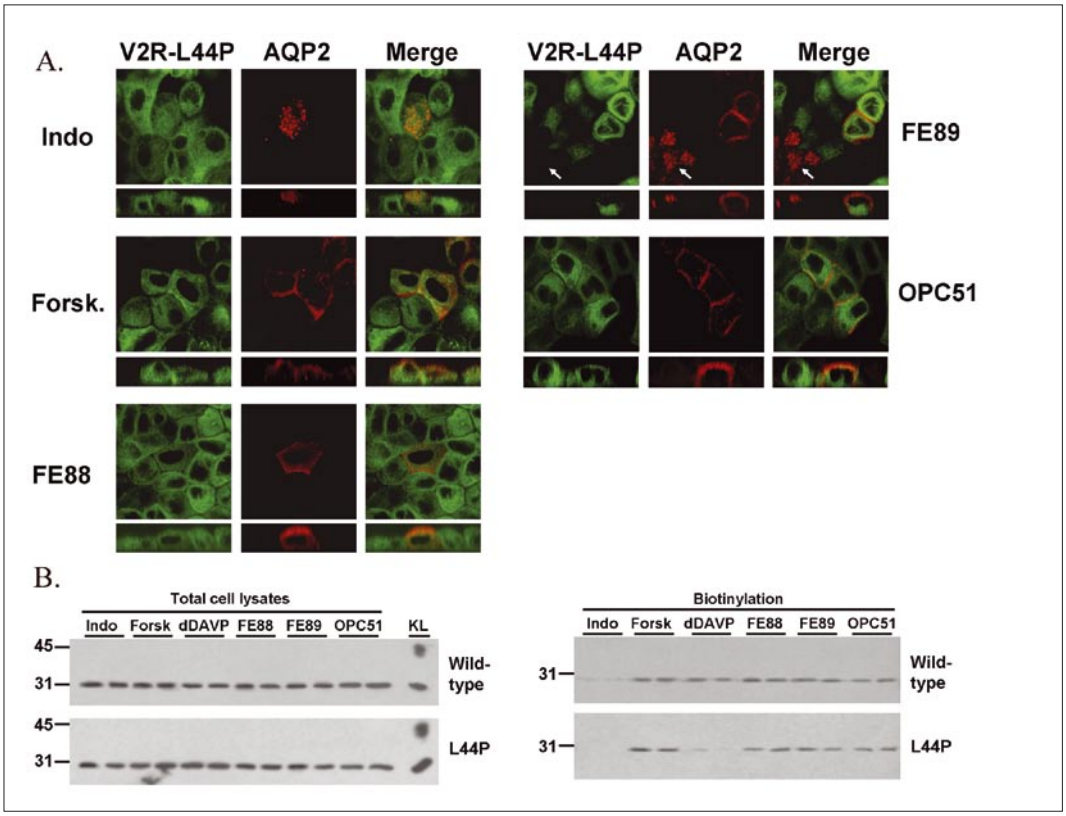

Figure 7-7

Agonist induced translocation of AQP2

MDCKII-V2R-L44P cells were trypsinyzed, infected with a lentivirus containing an AQP2 expression cassette, seeded and grown to confluence in three days.

(A) Subsequently, cells were incubated overnight with 50 µM indomethacin. Then, cells were kept on indomethacin alone, or supplemented with 10 µM forskolin (Forsk.), 1 µM FE88, FE89, or OPC51 for 2 hours. Next, the cells were fixed, permeablilized and subjected to immunocytochemistry using rabbit anti-AQP2 antibodies and subjected to CLSM.

(B) In parallel, MDCKII-wtV2R and V2R-L44P cells were pretreated as under A, and subsequently subjected to cell surface biotinylation followed by cell lysis and streptavidin extraction. Protein samples of total cell lysates and the biotinylation (indicated) were run on a 12% PAAG and analyzed by immunoblotting using anti-AQP2 antibodies. A rat kidney cortex lysate (KL) is shown as a positive control for immunoblotting.

indomethacin. Co-incubation with $5*10^{-5}$ M of the adenylate cyclase activator forskolin, or 1 μM of the non-peptide agonists FE88, FE89 or OPC51 for 3 hours, however, drastically increased translocation of AQP2 to the apical and, to a lesser extent, basolateral membrane. All cells co-expressing the V2R-L44P and AQP2 responded to treatment with the non-peptide agonist, whereas in cells lacking V2R-L44P, AQP2 did not traffic to the plasma membrane, but the vesicular staining pattern remained (e.g. arrow in Fig. 7A, FE89 treatment). As the apical signal for AQP2 was diffuse in these MDCK II cells, apical cell surface biotinylation was performed to investigate whether AQP2 was translocated to the apical membrane, and not just in the apical domain of the cell. In MDCKII-wtV2R and V2R-L44P cells in wt AQP2 was expressed predominantly as a 29 kDa protein (Fig. 7B, total lysates) and the expression levels were similar under all conditions tested. Cell surface biotinylation experiments (Fig. 7B, biotinylation) showed that treatment with indomethacin alone resulted in the detection of only low amounts of AQP2 in the apical membrane for the wtV2R and -L44P cells and co-incubation with forskolin, increased the signals for AQP2 in both cell lines. Co-incubation with the peptide agonist lead to an increased AQP2 for the wtV2R cells, but not for the V2R-L44P cells. When treated with the non-peptide agonists FE88, FE89 and OPC51, increased signals for AQP2 were observed for both cell lines, indicating that the cAMP response evoked by these non-peptide agonists is strong enough to induce AQP2 translocation to the plasma membrane.

Discussion

Non-peptide agonsists activate intracellular V2R mutants

Similar to the δ-opioid receptor (DOR), which expression and maturation was improved upon treatment with cell-permeable agonists (21), we hypothesized that V2R agonists would activate V2R mutants at the plasma membrane after rescue of their cell surface expression. Our data, however, clearly indicate that three cell permeable agonists do not facilitate maturation of V2R mutants, but activate these mutants intracellularly. This conclusion is supported by several data:

First, in our stably-transfected MDCK cells, all V2R mutants show a strong overlap with the ER marker protein PDI (Fig. 2), and appear on blot as immature receptors, indicating that these mutants are misfolded and ER retained. When forced to the plasma membrane in an overexpression system, all V2R mutants tested, except for the non-functional V2R-S167L, responded to treatment with the cell-permeable agonists and the non cell-permeable dDAVP (Fig. 1). However, in stably-transfected MDCK cells, where V2R mutants are absent from the plasma membrane (chapter 6), the cell-permeable agonists, but not dDAVP, activate the ER-retained V2R mutants (Fig. 3 and 7B). In contrast, wtV2R was activated by the non-peptide agonists and dDAVP at the plasma membrane. It is highly unlikely that the observed cAMP responses shown in figure 4 are due to trafficking of V2R mutants to the plasma membrane, as it takes 4-8 hours for V2R mutants be inserted in the plasma membrane and function there (chapter 6 and (33)).

Second, immunocytochemistry revealed no shift in localization of the V2R mutants upon incubation with the non-peptide agonists (Fig. 4), and consistently, no maturation was observed (Fig. 5). In contrast, as shown in figure 5 and in chapter 6,

the cell permeable V2R antagonist SR1 induced maturation and translocation of the mutants to the basolateral membrane. Recently, we (chapters 5 and 6) and others (3; 6; 18; 29) have shown that cell-permeable antagonists can promote the plasma/basolateral membrane localization and maturation of ER-retained V2R mutants. As shown in figures 4 and 5, however, cell-permeable agonists do not allow ER exit and subsequent maturation of ER-retained mutant V2R. This may be explained by the different effects antagonists and agonists have on the conformation of the receptor. Whereas binding of an antagonist (or inverse agonist) promotes a structure that prevents the receptor to attain the active conformation, and thus a favorable low-energy state of the receptor, agonists induce a conformational change, promoting the active state (14; 17). Apparently, this agonist-bound state is not recognized by the quality control mechanism as a conformation suitable for ER exit, and therefore, the mutant receptor remains ER retained.

Third, the ER-localized GPR30 was the first GPCR shown to be able to induce rapid signalling upon activation by its endogenous agonist estrogen (13; 23). Although the exact cellular mechanism underlying intracellular activation of ER-retained V2R mutants or GPR30 has not been described in detail yet, these signal transduction pathways would require at least the presence of functional stimulatory G proteins, if not also adenylate cyclase, in the ER.

Non-peptide agonists differentially affect the expression of V2R mutants

For the DOR, several non-peptide agonists were able to stabilize the expression and maturation the ER-retained DOR-D95A (21). Our data reveal that non-peptide agonists variably affect the expression of V2R mutants (Fig. 5). The expression level of V2R-Y128S increased with all non-peptide agonists, while FE88 and OPC51 decreased the expression of all other V2R mutants, and FE89 did not affect their expression levels. However, the compounds did not affect the post-translation stability of any of the V2R mutants tested, as co-incubation of cycloheximide with the non-peptide agonists did not affect expression levels compared to cells treated with cycloheximide alone. As anticipated for agonists (chapter 3), the non-peptide agonists and dDAVP induced internalization of wtV2R (Fig. 3) to late endosomes-lysosomes (data not shown). This increased the degradation of wtV2R, which was visualized by the appearance of the 47 kDa breakdown product (Fig. 5 and 6). As suggested by Bouley et al., who reported the appearance of a similar band in LLC-PK1 cells expressing V2R-GFP, this band most likely represents a non-glycosylated degradation product (9). Interestingly, in MDCK type I cells, this degradation product was not observed (chapter 3), suggesting that these cells lack the specific protease responsible for the agonist-induced digestion of V2R.

Likely, the V2R mutants are not increasingly targeted for lysosomal or proteasomal degradation upon agonist stimulation. However, the pathways that are responsible for the discrepancy between the effects of non-peptide agonists on expression levels and post-translation stability remain to be established.

Cell permeable agonists as a future treatment for congenital NDI

The data in figure 7 clearly demonstrate that the cAMP response generated as a result of incubation of ER-retained V2R mutants is sufficiently strong to allow PKA activation and subsequent trafficking of AQP2 to the apical and basolateral membrane. The increased plasma membrane localization of AQP2 observed after treatment with

the non-peptide agonists was similar to the increase observed upon forskolin stimulation (Fig. 7B). Although sufficiently efficient for PKA activation, intracellular activation of V2R mutants yielded lower levels of cAMP (Fig. 3). This may be due to less efficient signalling from the ER vs. the plasma membrane, lower expression levels of the mutant proteins vs. the wild-type, decreased affinities of the agonists for the mutants, or a combination of these factors.

The fact that a minor amount of AQP2 traffics to the basolateral membrane is likely an effect specific to MDCKII cells, similar to what is observed by others in LLC-PK1 cells (8). Thus, the ability of non-peptide agonists to induce AQP2 plasma membrane trafficking clearly indicates that these compounds may be highly valuable to relieve NDI in patients harbouring functional ER retained V2R mutants. As the expression of AQP2 is regulated by a cAMP-responsive promoter, also the expression of AQP2 is regulated by cAMP. Therefore, patients harbouring functional V2R mutations may also benefit form treatment with non-peptide agonists by increased expression levels of AQP2, as these are normally reduced in NDI patients (16).

In recent years, pharmaceutical companies have put great effort in the generation of non-peptide ligands, antagonists as well as agonists, as these compounds can be designed to target specific receptor subtypes within a larger family of GPCRs. In addition, such non-peptide ligands can be orally administered, and are better absorbed compared to peptidic ligands. Currently, OPC51(803) is phase II clinical trials (http://www.japancorp.net/Article.Asp?Art_ID=9474), and may thus become available for clinical application in the near future. Thus, OPC51803, FE999088 and FE999089 represent highly promising candidate to relieve NDI in patients. In addition, the increasing availability of non-peptide agonists may offer new possibilities for treatment of other disorders involving ER-retained G protein-coupled receptors.

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Chapter 8

Cell biological analysis of non-sense V2 receptor mutants in Nephrogenic Diabetes Insipidus

Abstract

The presence of a nonsense mutation in a gene will lead to expression of truncated proteins due to the introduction of a premature stop codon, and is one of the causes of inheritable disorders like cystic fibrosis, Duchenne muscle dystrophy, or nephrogenic diabetes insipidus (NDI). The latter disorder can be caused by nonsense mutations in the gene encoding the vasopressin V2 receptor (V2R). Premature stop codons may be susceptible for read-through upon treatment with aminoglycoside antibiotics may thus allow expression of the full-length receptor and restore its function. To this extent, we generated Madin-Darby canine kidney cell lines stably transfected with expression constructs for FLAG-V2R-GFP, or the mutants FLAG-V2R-Q119X, -W293X, –R337X and -C358X. Whereas fully matured FLAG-V2R-GFP was expressed in the basolateral membrane of these cells, none of the mutants could be detected by immunoblot or immunohistochemistry in G418 resistent clones, suggesting protein instability. To obtain higher expression levels, we transiently expressed the FLAG-V2R-GFP and the mutants in COS cells. FLAG-V2R-Q119X was expressed as an unglycosylated protein of 17 kDa, whereas –W293X, –R337X and -C358X were present as unglycosylated and high-mannose glycosylated proteins of 32-35 kDa, 34-37 kDa and 35-38 kDa, respectively. Treatment with the aminoglycosides gentamycin, geneticin, amikacin or tobramycin did not lead to detectable signals of full-length proteins for any of the mutants on western blot. The sequences of the mutations are sub-optimal for read-through, which may, in combination with the already low expression levels, be responsible for the absence of rescued full-length V2R. As the mutants are ER retained, we tested whether they could be activated by cell-permeable agonists. Whereas V2R-Q119X, -W293X and -R337X did not respond, stimulation of V2R-C358X by three cell-permeable agonists resulted in a significant cAMP response. Thus, also patients harboring functional V2R nonsense mutations may benefit from treatment with cell-permeable agonists.

Introduction

In the collecting duct of the kidney, the aquaporin-2 mediated water reabsorption is regulated by the antidiuretic hormone arginine-vasopressin (AVP). By binding to its vasopressin V2 receptor (V2R), it activates a cAMP cascade that will eventually lead to insertion of aquaporin-2 containing vesicles in the apical membrane of the principle cells, which allows water reabsorption from the tubular fluid to the interstitium. The X-linked congenital form of Nephrogenic Diabetes Insipidus (NDI) is caused by mutations in the AVPR2 gene that encodes the V2R. The most common type of mutation causes substitution or deletion of one or a few amino acids that will subsequently lead to misfolding and retention of the mutant V2R in the endoplasmic reticulum (class II mutations)(1). In addition, mutations that introduce a premature stop codon in the coding region of the AVPR2 gene have been described in several patients (class I mutations)(2). Due to the introduction of such a premature stop codon, truncated V2R will be expressed, that may be instable, intracellularly retained, deficient in AVP or G protein binding, or a combination of these factors.

A group of antibiotics known as aminoglycosides is known to promote read-through on susceptible stop codons, and may thus restore the expression of full length proteins. The efficiency of read-through at a premature stop codon is not only determined

by the stop codon itself, but also by the nucleotide sequence up- and downstream, and may thus vary between different mutations (3). Recent studies on non-sense mutants of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel in cystic fibrosis (4), the dystrophin protein in Duchenne muscle dystrophy (5; 6), and the V2R in NDI (2; 7) clearly demonstrated increased functionality of the mutant receptors and channels upon aminoglycoside treatment in vitro and in vivo.

In chapter 7, we showed that non-peptide agonists are able to restore the function of functional ER retained missense mutants of the V2R in NDI. As some nonsense V2R mutations in NDI are present in the extreme C-terminus, and are therefore almost full-length, agonist binding and/or G protein binding may not be severely disturbed. As truncated proteins are often ER retained, the function of such nonsense mutants may also be restored upon treatment with cell permeable agonists.

Here, we characterize the expression of four non-sense V2R mutants in NDI, V2R-Q119X, -W293X, –R337X, and -C358X and analyze their susceptibility to read-through by aminoglycosides, and their ability to be activated by cell permeable agonists.

Materials and methods

Expression constructs

The pEGFP-N1-V2R construct was a kind gift of Dr. Alexander Oksche (FMP, Berlin, Germany). To allow N-terminal detection of the V2R, a FLAG-tag was fused to the N-terminus using the polymerase chain reaction with the sense primers 5'-ATGGACTACAAGGATGACGATGACAAG ACCATGCTCATGGCGTCCACCAC3-' (FLAG-encoding sequence in bold), and the anti-sense primer 5'-GCTGAGAAGGAGCGAGAAG-3'. In a second PCR reaction, using the same anti-sense primer and the sense primer 5'-GTCTACTCG AGCCACCATGGACTACAAG-3', an XhoI site and the Kozak-sequence (indicated in bold) were added to the 5' end of the first PCR product. The resulting 529 bp PCR product was cut with XhoI and PstI, and the cloned into the XhoI/PstI digested pEGFP-N1-V2R construct to yield pEGFP-N1-FLAG-V2R. Site directed mutagenesis was performed on pEGFP-N1-wtV2R as described (8) using the sense primers 5'-GTGAAGTATCTGTAGATGGTGGGCATG-3' (Q119X; stop codon indicated in bold), 5'-GTGCAGCTGTGAGCCGCGTGGGAC-3' (W293X) and 5'-CCTCAGAGCTGTGAAGCTTGCTC-3' (R337X), 5'-CAAGATGAGTCCTG AACCACCGCCAGCTC-3' (C358X) and their complementary anti-sense primers. After digesting correct clones with XhoI and HindIII (Q119X, W293X, R337X), or HindIII and BamHI (C358X), the mutation containing fragments were isolated and cloned into the corresponding sites of the pEGFP-N1-FLAG-V2R. Sequence analysis of selected clones confirmed that only the desired mutations were introduced.

Cell culture, transfection, and aminoglycoside treatment

Maintenance of MDCK type I cells and generation of stable clones was performed as described (9). COS-M6 cells were maintained and transfected with 1 µg expression construct (unless indicated otherwise) as described (8). COS-cells transiently expressing FLAG-wtV2R-GFP or the mutants FLAG-V2R-Q119X, -W293X, –R337X or -C358X were seeded in 12 multiwell plates (Costar), and grown for two

days. Subsequently, cells were grown 16 hours in culture medium supplemented with 0.5 mg/ml gentamycin, 0.4 mg/ml geneticin, 0.5 mg/ml tobramycin, or 0.5 mg/ml amikacin (Sigma, St. Louis, MO), or in culture medium without aminoglycoside antibiotics. Subsequently, cells were lysed in Laemmli sample buffer. Digestion of protein samples with endoglycosidase H (Endo H) or protein N-glycosidase F (PNGase F) was performed as described (10).

Immunoblotting and immunocytochemistry

Poly-acryl-amide gel electrophoresis and immunoblotting was performed as described (9). Immunodetection was performed using 1:5000 diluted peroxidase-conjugated mouse anti-FLAG antibodies (Sigma, St. Louis, MO). Immunocytochemistry and confocal laser scanning microscopy (CLSM) were performed as described (9). As primary antibodies, 1:100 diluted mouse anti-FLAG antibodies (Sigma, St. Louis, MO) were used. As secondary antibodies, 1:100-diluted goat anti-mouse coupled to Alexa-594 (Molecular Probes, Leiden, The Netherlands) were used.

Results

Stable expression in MDCK cells

As shown in chapter 3, MDCK type I cells are an accurate model for the collecting duct, and the wtV2R-GFP is localized and regulated as can be expected in vivo. Therefore, we transfected expression constructs encoding FLAG-wtV2R-GFP and the mutants FLAG-V2R-Q119X, -W293X, –R337X and –C358X into these cells. For each transfection, 24 clones were isolated and analyzed. Immunoblotting of clones transfected for FLAG-wtV2R-GFP revealed that more than 60% of these clones were positive. As shown in figure 1, FLAG-V2R-GFP was predominantly expressed as fully

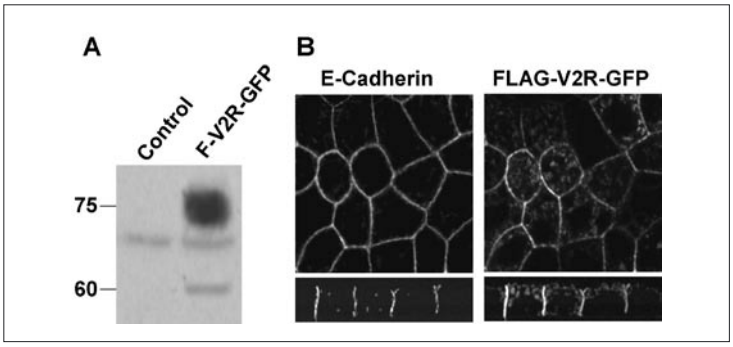

Figure 8-1 **Expression and localization of FLAG-V2R-GFP.**
A. Madin-Darby canine kidney (MDCK) type I cells stably expressing FLAG-V2R-GFP were grown to confluence, subsequently lysed, and subjected to SDS-PAGE. Subsequently, they were analyzed by immunoblotting using anti-Flag antibodies. An equivalent sample of untransfected cells is shown as a negative control.
B. MDCK-FLAG-V2R-GFP cells were grown to confluence and subjected to immunocytochemistry using anti-FLAG antibodies to detect FLAG-V2R-GFP and anti-E-Cadherin antibodies to stain the basolateral membrane. Cells were subsequently analyzed by CLSM.

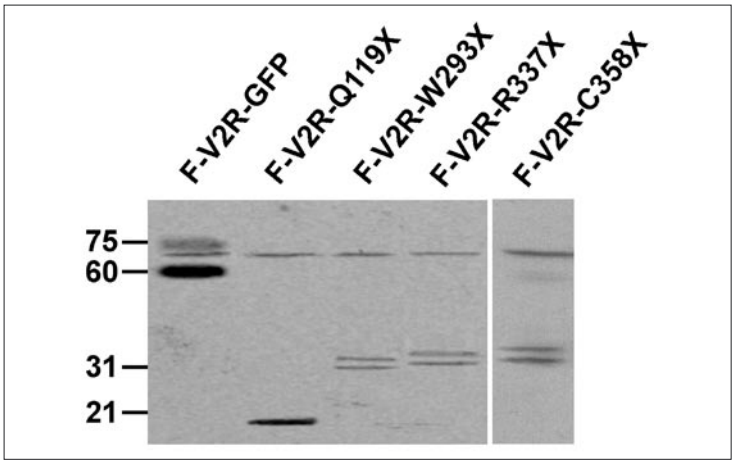

Figure 8-2 **Expression of FLAG-V2R-GFP and V2R non-sense mutants**
COS cells transiently expressing FLAG-V2R-GFP or the mutants FLAG-V2R-Q119X, -W293X, –R337X or –C358X (indicated) were grown for two days, followed by lysis in Laemmli sample buffer, separation by SDS-PAGE followed by immunoblot analysis as described in the legend of figure 1.

mature proteins of approx. 75 kDa, whereas only a weak band was observed of approx. 60 kDa, representing the non-glycosylated or high-mannose glycosylated forms. Subsequent confocal laser scanning microscopy showed that FLAG-V2R-GFP was predominantly localized in the basolateral plasma membrane similar to what we have previously shown for V2R-GFP (Fig. 1).

Immunoblot analysis of the numerous G418-resistent clones obtained from the transfection of the V2R mutants, however, did not show any V2R-specific signals. Since this may be due to a low percentage of cells expressing the mutants, six randomly chosen clones per mutant were subjected to immunocytochemistry followed by CLSM analysis. However, no specific signals were detected.

Transient expression in COS-M6 cells

The absence of detectable protein levels of the mutants in MDCK cells suggested that these mutants may be highly unstable. Therefore, COS-M6 cells were transfected with the above-mentioned expression constructs, as higher expression levels can be achieved in these cells compared to stably transfected MDCK cells. In COS cells, FLAG-V2R-GFP was expressed predominately in its immature 60-63 kDa form, and only weakly as mature, 75 kDa proteins (Fig. 2). In line with the anticipated length of the abrogated V2R proteins, FLAG-V2R-Q119X was detected as a single band of approx. 17 kDa, whereas -W293X, –R337X and –C358X were expressed double bands of 32-35, 34-37 and 35-38 kDa, respectively.

Next, we analyzed the glycosylation states of FLAG-V2R-GFP and the non-sense mutants by digesting total cell lysates of COS cells expressing these proteins

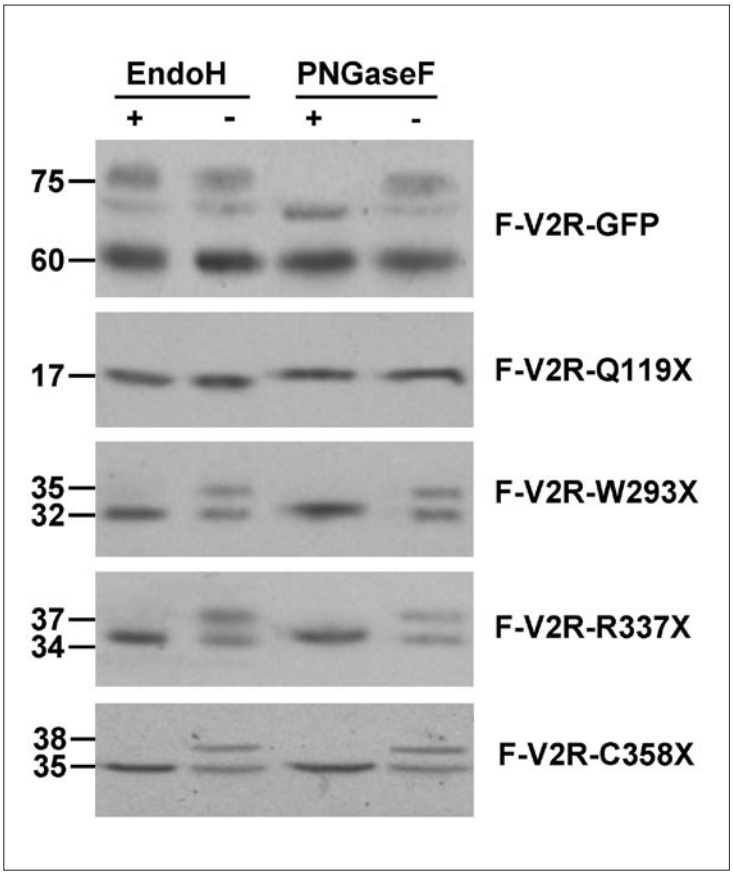

Figure 8-3 **Maturation of FLAG-V2R-GFP and V2R non-sense mutants**
Protein samples were generated as described in the legend of figure 2. The samples were incubated for 1 hour in the presence (+) or absence (-) of Endo H or PNGase F, and subsequently analyzed by immunoblotting as described in the legend of figure 1.

with Endo H and PNGase F, which may offer insight into their maturation state. As shown in figure 3, Flag-V2R-GFP is not sensitive to Endo H, but upon digestion with PNGase F, the 75 kDa signal shifts to a band of approx. 67-68 kDa. This indicates that FLAG-V2R-GFP is partially complex glycosylated in COS cells. The 17 kDa band observed for FLAG-V2R-Q119X was not affected by treatment by Endo H or PNGase F, suggesting this mutant was not subject to N-glycosylation at all. The top bands found for FLAG-V2R-W293X, –R337X and -C358X were sensitive to Endo H and PNGase F, since top bands disappeared upon digestion. This was accompanied with an increased signal of the 32, 34 and 35 kDa signals for FLAG-V2R-W293X, –R337X and –C358X, respectively, and indicates that the latter mutants are high-mannose glycosylated. Immunoblot analysis for GFP did not give any specific signals (data not shown).

Aminoglycoside treatment

To detect read-through of the nonsense mutants upon treatment with aminoglycosides, we made use of the GFP-coding region cloned in frame to the V2R-coding sequence in the expression constructs. When read-through occurs, GFP will be translated and fluorescent signals should be detectable. However, treatment of COS cells transiently expressing the non-sense mutants with gentamycin, geneticin, tobramycin or amikacin for two days, did not yield any GFP signal on immunoblot, or any detectable GFP fluorescence as detected by CLSM. In contrast, GFP form FLAG-V2R-GFP was still detected (data not shown), indicating that the used drugs were not toxic to the cells.

Treatment with cell permeable agonists

As shown in chapter 7, non-peptide agonists can rescue the function of ER retained missense V2R mutants by intracellular receptor activation. Therefore, we tested whether these agonists may restore signaling of nonsense V2R mutants. Treatment of

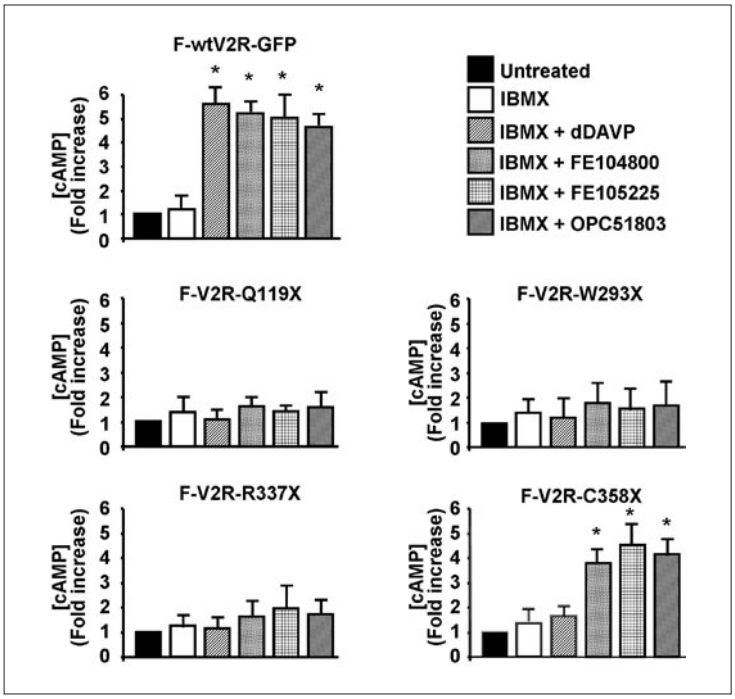

Figure 8-4 **Effect of non-peptide agonists treatment on V2R non-sense mutants**
COS cells transiently expressing FLAG-V2R-GFP or the mutants FLAG-V2R-Q119X, -W293X, -R337X or -C358X were left untreated, treated with IBMX, or a combination of IBMX with FE999088, FE999089 or OPC51803 (indicated) for 10 minutes. Subsequently, cells were lysed in 0.1 M HCl, and cAMP was measured using a fluorescent assay kit. cAMP levels of the untreated samples was set to 1, and relative changes are shown. Triplicate samples were measured, and experiments were performed in threefold.

FLAG-wtV2R-GFP with the non-permeable peptide agonist dDAVP, and the non-peptide agonists FE999088, FE999089 and OPC51803 resulted in a profound increase of intracellular cAMP (Fig. 4). COS cells expressing FLAG-V2R-Q119X, -W293X and -R337X did not show any significantly increased cAMP levels upon treatment with any of the compounds. The almost full-length mutant FLAG-V2R-C358X, however, responded to treatment with any of the three non-peptide agonists by 4-5 fold increased cAMP levels, whereas dDAVP did not affect cAMP levels in these cells.

Discussion

Approximately 9 % of the mutations in the gene encoding the V2R results in NDI because of the introduction of a premature stop codon (http://www.medcon.mcgill.ca/nephros/). Expression in a physiologically relevant cell model, like MDCK cells, did not yield any positive clones for the three mutants tested, despite the clones' resistance to the antibiotic marker present in the transfected expression construct. Since the FLAG-tagged wild-type V2R-GFP was expressed similar as V2R-GFP without the N-terminal tag, misfolding and/or intracellular retention due to the presence of this tag was excluded. More likely, the introduction of the mutations in the V2R caused expression levels that were below the detection limit using western blotting when these mutants were stably expressed in MDCK cells. Transient expression in COS cells, in which much higher expression levels can be achieved, showed receptor expression of the truncated receptors, although weaker than found for wild-type V2R (Fig.2).

Receptor maturation

In line with previous results (10; 11), the wild-type receptor is complex- and O-glycosylated (Fig. 3), from which it can be concluded that the N-terminal FLAG tag does not alter the receptor's glycosylation pattern. Transient expression of the FLAG-V2R-GFP in COS cells, however, drastically reduces the rate of complex glycosylated vs. high-mannose glycosylated and unglycosylated signals compared to signals found for stable expression in MDCK cells. Likely, the high expression level of FLAG-V2R-GFP in COS cells prevents a large pool of receptors to be properly folded, due to overloading of the ER folding machinery. Remarkably, the FLAG-V2R-Q119X showed no N-linked glycosylation, as it was insensitive to treatment with Endo H and PNGase F, despite the presence of the N-glycosylation consensus site in the V2R N-terminus (12).

The mutants FLAG-V2R-W293X, –R337X, and –C358X were sensitive to treatment with Endo H and PNGase F, which suggests that these receptors are high-mannose glycosylated on amino acid N22. The presence of high-mannose glycosylation indicates that these receptors are, besides being not fully translated, also intracellularly retained by the quality control mechanism of the endoplasmic reticulum (13).

Aminoglycoside treatment

The efficiency of termination of translation and the level of read-through upon aminoglycoside treatment are determined by the sequence of the premature stop codon itself, and the sequence neighboring it. Howard et al. showed that the TGA stop codon is most sensitive to read-through, and allowed a maximum read-through of 15% for the sequence TGAT (5). Based on their data, the maximum read-through for the

mutants V2R-Q119X (CAGA→TAGA), -W293X (TGGG→TGAG) and –R337X (CGAA→TGAA) would be 2, 3, and 6% respectively. This low efficiency, in combination with the observed low expression levels of the mutants, is a likely explanation for our inability to detect full-length proteins after aminoglycoside treatment.

However, Schulz et al. were able to show significantly increased levels of full length V2R upon treatment of the V2R-R337X with geneticin using highly sensitive techniques as cAMP measurement and sandwich ELISA (2). Thus, western blotting may not be sensitive enough to detect low amounts of full length proteins.

Functional rescue of V2R-C358X by cell permeable agonists.

We reported in chapter 7 that cell permeable agonists can rescue the function of functional ER retained mutants of the V2R by inducing a signaling cascade from the inside of the cell. The mutant V2R-C358X, which lacks only the final 14 amino acids compared to the wild-type receptor, was not expressed at the cell surface, but was retained inside the cell when transiently expressed in COS cells (chapter 9). In addition, we show in figure 4, that this mutant increases intracellular cAMP in response to non-peptide agonists, whereas cAMP levels are unaffected by treatment with the non-cell permeable agonist dDAVP. It is therefore likely, that restored function of V2R-C358X can be attributed to intracellular activation by non-peptide agonists.

In conclusion, we found that the three V2R non-sense mutants in NDI are not (MDCK) or weakly (COS) expressed, which is likely due to their high instability, and that this expression could not be restored upon treatment with several aminoglycosides. Therefore, besides the issue of toxicity for the kidney (14), the use of aminoglycosides as a treatment for nonsense V2R mutants in NDI is anticipated to be very limited. Finally, we show for the first time that cell permeable agonists can restore the function of functional nonsense V2R mutants, and these compounds are therefore promising candidates to cure NDI in patients encoding the V2R-C358X mutation.

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submitted

Chapter 9

Relief of nocturnal enuresis by desmopressin is vasopressin type-2 receptor independent

Introduction

Primary Nocturnal Enuresis (PNE) is defined as the persis-tence of nightly bedwetting after the fifth year of age, and is one of the most frequent complaints in pediatric and urologic practice. Despite a maturation rate of 15% per year, 0.5% of all cases remain in adulthood. It has been proposed that patients with PNE have insufficient nightly increase of the endogenous pituitary hormone arginine vasopressin (AVP) leading to the production of large amounts of dilute urine, thereby surpassing the bladder capacity (8). Successful application of a synthetic analogue of AVP, desmopressin (dDAVP), a vasopressin type-2 receptor (V2R) agonist seemed to confirm this hypothesis.

However, one of the major criticisms on this concept is that patients with PNE do not wake up before, during or after voiding, which can not be explained by an increased urine production. We have recently shown that desmopressin is successful in patients with PNE combined with congenital nephrogenic diabetes insipidus (NDI), due to an inactivating mutation in the Aquaporin-2 water channel (AQP2; (7)). Since functional AQP2 is essential for urine concentration (3) and desmopressin is generally considered to be V2R-specific (11), AQP2 mutated-NDI patients are unable to concentrate their urine in response to desmopressin. The fact that there was a beneficial effect of desmopressin in patients with PNE combined with NDI indicates that PNE is kidney independent and possibly acts via non-renal V2R, e.g. in tissues such as cerebellum (6). However, a recent study indicated that a renal V2R effect of desmopressin can not be totally excluded in our patients above, because dDAVP still increases principal cell sodium and urea uptake and thus the interstitial tonicity in NDI patients lacking

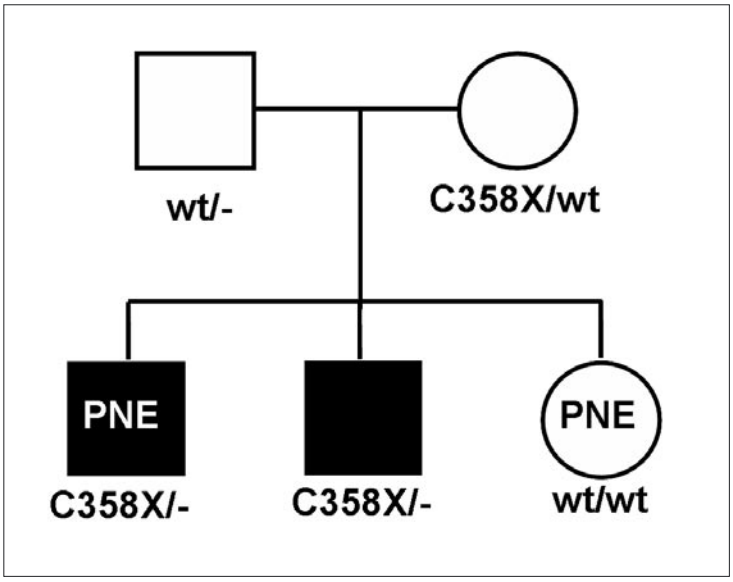

Figure 9-1 **Pedigree of the family**
Black: Patients with NDI. PNE: primary nocturnal enuresis. Wt: wildtype. V2R is located on the X-chromosome, therefore male family members have only one copy of the gene.

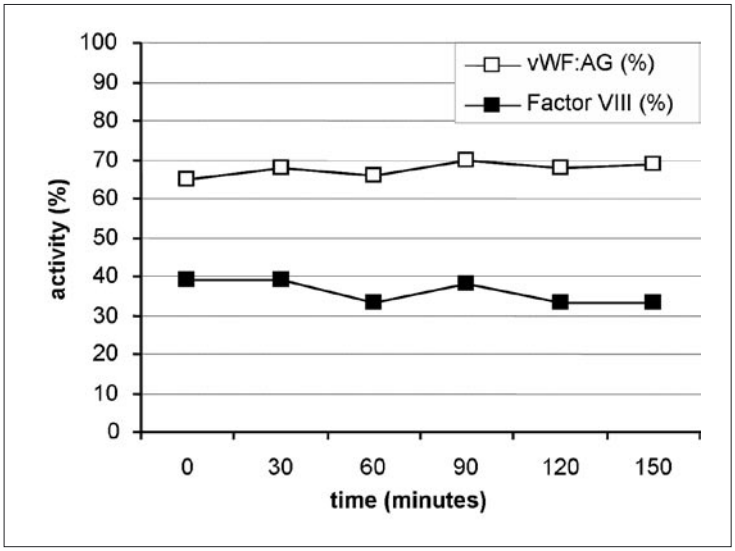

Figure 9-2 **Activity of vWF:Ag and FVIII (%) after dDAVP infusion (0.3 µg/kg) in our PNE-NDI patient.** Method after Bichet et al.(2)

AQP2, but not those lacking V2R (1; 12). The interstitial tonicity is the driving force for water reabsorption.

Here, we identified a family with two brothers with NDI due to a mutation in the AVPR2 gene (Xq28) leading to a premature stop codon (C358X) in the V2R. Besides NDI, one patient also suffered from PNE. The administration of desmopressin and the use of alarm treatment relieved PNE completely, while NDI remained unaffected. Cell biological characterization of this novel mutation revealed extreme instability of the mutant V2R protein, thereby explaining underlying NDI. These findings not only exclude a functioning urinary concentration system as a prerequisite for the beneficial action of desmopressin in PNE, but also indicate that this effect is V2R independent.

Patients and Methods

The oldest son of a Caucasian, non-consanguineous family was born in the 34th week of gestation (Figure 1). Polyhydramnion was noted during pregnancy. After birth, the child developed poorly with vomiting and repeated episodes of hyperpyrexia. Measurements of serum and urinary electrolyte levels and osmolality pointed at a renal concentration defect (serum Na^+ 158 mmol/l). A thirst trial with additional infusion of desmopressin (0.3 µg/kg body weight) revealed an unchanged urinary osmolality (before: 235 mosmol/kg H2O, after: 229 mosmol/kg H2O), thereby establishing the diagnosis of NDI. Indomethacin and hydrochlorothiazide treatment

was started, which reduced urinary volumes by one third. Infusion of desmopressin (0.3 μg/kg body weight) followed by measurement of von Willebrand factor and factor VIII responses, revealed no changes (Figure 2), indicating that a V2R defect is the molecular cause of NDI.

Sequence analysis of the AVPR2 gene of the patient revealed a mutation at nucleotide position 1074 (NM_000054), substituting a cytosine for an adenine (NM_000054). This substitution leads to the introduction of a premature stop codon (C358X) in the extreme C-terminus of the V2R. Ever since birth, the patient wetted his bed at night without waking up. At the age of 10 years, desmopressin administration (20 μg before going to bed) was initiated. After 3 days, the child did not wet his bed anymore, but woke up and went to the toilet instead. Withdrawal of the medication resulted in reoccurrence of nocturnal enuresis, whereas re-introduction again led to disappearance of PNE. The treatment left the urinary volume unchanged (3.8 ml/kg bodyweight/h).

A brother of this patient, also suffering from NDI, did not show PNE after the 4th year of age, but went every night 3-5 times to the toilet for voiding. A sister suffered from nocturnal enuresis but had no signs for impaired renal concentration ability on a concentration test. Sequence analysis did not reveal a mutation in the sister. Initiation of desmopressin administration resolved all symptoms of PNE completely.

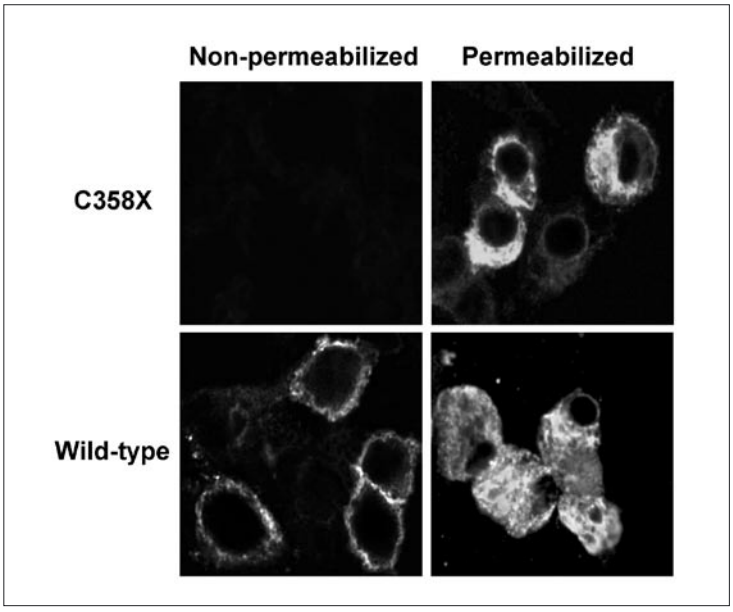

Figure 9-3 **V2R-C358X is not expressed in the plasma membrane of COS cells**
Non-permeabilized or permeabilized (indicated) COS cells transiently expressing FLAG-V2R-C358X or FLAG-V2R-GFP were subjected to immunocytochemistry using anti-FLAG antibodies, followed by confocal laser scanning microscopy.

Cell biological analysis

To determine the molecular cause of NDI, Madin-Darby Canine kidney (MDCK) cells, which are an appropriate model for renal principal cells (9), were stably-transfected with constructs encoding wild-type V2R or V2R–C358X. Both were N-terminally fused to a FLAG (F) epitope tag to allow detection (from the extracellular side). Under conditions that would allow detection throughout the cells, F-V2R expression was easily detected in the basolateral membrane of many clones, whereas F-V2R-C358X expression was not observed (data not shown). This suggested that V2R-C358X is unstable. To investigate this further, COS cells were transiently-transfected with expression constructs encoding F-V2R-GFP and F-V2R-C358X. Here, the green fluorescent protein (GFP) cDNA was cloned in frame at the C-terminus of F-V2R and F-V2R-C358X, which, due to the stopcodon in F-V2R-C358X, would yield expression of green F-V2R only. Two days after transfection, the cells were incubated with anti-FLAG antibodies in ice-cold phosphate-buffered saline for 1 hour to label V2R at the cell surface and fixed. Parallel-transfected cells were fixed, permeabilized and incubated with anti-FLAG antibodies to stain V2R at any location. Subsequently all cells were incubated with secondary antibodies and subjected to immunocytochemistry. Confocal laser scanning microscopy (CLSM) analysis revealed that F-V2R-C358X was not expressed at the cell surface, whereas the F-V2R showed clear membrane localization (Figure 3, left panel). Analysis of permeabilized cells, however, showed expression of F-V2R and F-V2R-C358X, indicating that V2R-C358X was retained in the cell and that the lack of plasma membrane expression of V2R-C358X was not due to the absence of expression (Figure 3, right panel). This explains the NDI phenotype in the patients, since intracellularly-retained receptors are not accessible for AVP or dDAVP.

To determine semi-quantitatively whether V2R-C358X is unstable, we analyzed the expression of F-V2R-GFP and F-V2R-C358X, normalized for their amounts of mRNA. For this, we transiently-transfected COS cells with different amounts of F-V2R-GFP or F-V2R-C358X expression constructs. After 2 days cells were lysed for immunoblotting or total RNA was isolated and subjected to northern blotting. V2R mRNA was detected using a [^{32}P]-labeled cDNA probe of 0.65 kb corresponding to the V2R coding sequence. As shown in figure 4, for similar or somewhat higher levels of F-V2R-C358X mRNA compared to that of F-V2R-GFP (top panel), the expression levels of F-V2R-C358X (35 kDa) were clearly lower than of V2R-GFP (60, 75 kDa; bottom panel). In fact, the expression of F-V2R-GFP stayed relatively constant with decreasing amounts of mRNA, whereas the F-V2R-C358X levels rapidly decreased, which underscores the instability of V2R-R358X compared to wild-type V2R. Please note the a-specific band of 70 kDa. As found by others, the 120 kDa band likely represents a dimer of V2R.

Discussion

Despite extensive research, the etiology of PNE still remains unknown. Moreover, three different therapeutic options (alarm treatment, tricyclic antidepressants and desmopressin) have been demonstrated in prospective controlled trials as being highly efficient (5). However, their different modes of action have not led to one unifying concept on the etiology of PNE. Desmopressin has been used successfully since the 1970's for the treatment of PNE. The hypothesis of increased tubular water reabsorp-

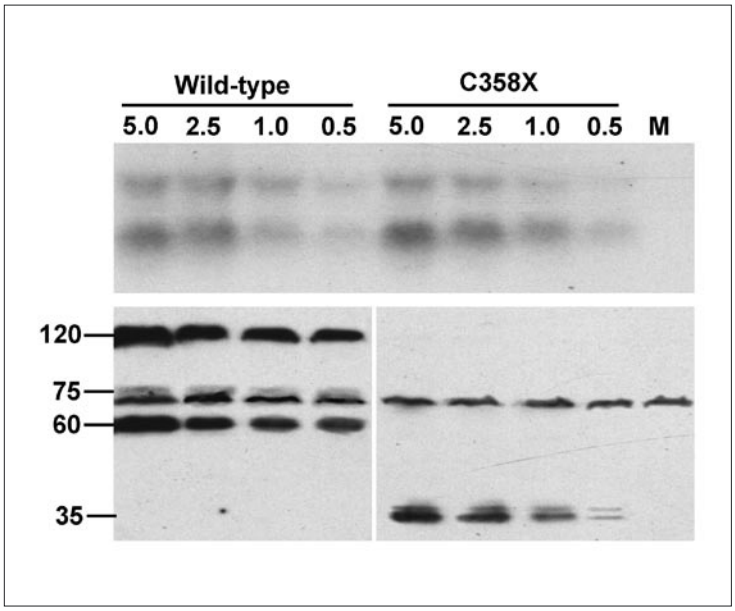

Figure 9-4 **FLAG-V2R-C358X is an unstable protein.**
COS cells were mock (M) transfected, or transiently transfected with decreasing amounts of expression constructs for FLAG-V2R-GFP or FLAG-V2R-C358X (indicated). Subsequently, mRNA was isolated and subjected to electrophoresis followed by northern blotting. V2R mRNA was detected using a radioactively labeled V2R probe (top panel). Of cells transfected in parallel, lysates were generated and analyzed by poly-acrylamide gel electrophoresis followed by western blotting. FLAG-V2R-GFP and FLAG-V2R-C358X were detected using anti-FLAG antibodies. Approximate masses (in kDa) of the observed bands are indicated on the left.

tion and a consequent reduced nightly urinary volume has later been fueled by a study demonstrating an insufficient nightly increase in AVP (8). However, this concept does not explain the success of the other treatments; neither does it explain the substantial difference between PNE and incontinence, i.e. the inability to arouse.

Recently we have shown that patients with co-segregating NDI, due to a mutation in the AQP2 gene, and PNE respond to the treatment with desmopressin (7). Although these patients still have large nightly urine volumes, with desmopressin treatment they arouse on the sensation of a full bladder and go to the bathroom. In this way, desmopressin has converted PNE into nocturia. These patients have an impaired renal urine concentrating mechanism, which indicated that desmopressin likely exerts its therapeutic effect through the V2R in a non renal target. However, recent studies indicated that a role for renal V2R could not be completely ruled out (1; 12).

Here, we have identified a novel V2R mutation as the underlying cause of NDI in a family in which one of the affected boys also suffered from PNE, as dehydration, or desmopressin administration, did not reduce their urine volume or increase their blood clotting factors. Moreover, the molecular characterization of the mutant V2R demonstrated that the encoded protein is unstable and does not reach the plasma membrane, where it is normally bound by AVP or desmopressin to induce the intracellular cAMP signaling cascade. Considering the non-functional V2R, the replacement of PNE by nocturia after initiation of desmopressin treatment therefore indicates that the V2R is not essential for the action of desmopressin in the treatment of PNE. Although in non-NDI PNE patients, desmopressin likely reduces nocturia by decreasing the nightly urine volume, with our previous findings, the present data reveal that the success of desmopressin in PNE does not involve the renal concentrating mechanism and is V2R-independent. Moreover, together with the beneficial action of central nervous targeted treatments, like the alarm treatment and tricyclic antidepressants, our data underscore the likeliness that the action of desmopressin in PNE mainly concerns an increase of arousability in our patients by acting on the central nervous system (4). One of the best candidates for this is the human V1b receptor, as this receptor is localized in brain and has a similar affinity for desmopressin as the human V2R (10). Determination of an involvement of the V1b receptor in PNE has to await FDA-approved specific agonists for this receptor.

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Chapter 10

General Discussion

Mutations in genes encoding G protein-coupled receptors (GPCRs) are the molecular cause of many inheritable disorders, such as mental retardation (60), retinitis pigmentosa (26), hypogonadotropic hypogonadism (16), ovarian dysplasia (1), obesity (56), hypothyroidism (9), and nephrogenic diabetes insipidus (NDI) (49; 57). Researchers and clinicians are becoming increasingly aware that for many genetic disorders, identification of the specific cellular or molecular defect underlying the disorder may allow the development of tailored therapies for individual patients. The goal of this thesis was to identify cellular defects in mutants of the vasopressin V2 receptor (V2R) in X-linked NDI, and to develop strategies to overcome these defects.

Classification of V2R mutants

According to the classification system for GPCR mutants (chapter 1), all missense mutants described in this thesis (table 1) are in class II, as they are ER retained. However, also the four nonsense mutants (class I) described in chapters 7 and 8 are retained in the endoplasmic reticulum (ER). This however, does not automatically render them class II mutants, as the main reason of their dysfunction is not ER retention, but the presence of a premature stopcodon that leads to protein truncation and thereby often a nonfunctional protein. Thus, the strategy to overcome the molecular defects of premature stop mutants will primarily be aimed at restoring full translation of the protein, whereas for ER-retained mutants, restoring their plasma membrane localization or induce intracellular signaling via nonpeptide agonists is the primary goal. Although the classification system described in chapter 1 is still highly useful, the notion that nonpeptide agonists can activate functional ER retained receptors inside the ER (chapters 6 and 7) makes it worthwhile to re-classify certain mutants according their pharmacological, rather than their cell biological profile. As such, the V206D mutation (chapter 3 and 4), which induces ER retention of the receptor, but also interferes with AVP binding would fall in class IV, as its primary defect is agonist binding, and not ER retention. A second example is V2R-C358X, which is truncated in its extreme C-terminus. As the truncation does not interfere with the receptor's function, but the receptor's ER retention does (chapter 8), this mutant would better fit in class II than in class I. Thus, classification of mutants is a useful tool to allow development of tailored therapies, but will require thorough cell biological analysis of each individual receptor mutant.

Receptor functionality

As shown in table 1, out of twelve missense mutants in V2R described in this study, nine are functional of the molecular level, and may thus benefit from treatment with cell-permeable antagonists or agonists. This suggests that approximately 75% of the patients carrying missense mutations may benefit from treatment with these compounds. This percentage, however, is an overestimation, as the mutants studied in this thesis have been selected on their likelihood to be functional based on studies by others (2; 3; 28; 39; 45; 50; 59; 63). Although we can rescue the function of ER-retained mutants with pharmacological chaperones and/or nonpeptide agonists, several V2R mutants are nonfunctional on the molecular level, i.e. they are unable to bind AVP and/or activate Gs (Table 1). V2R mutants that are unable to bind AVP because the mutation disturbs only the AVP binding pocket, but not so much the overall receptor structure, may respond to nonpeptide agonists, as these agonists may bind to different amino acids of the receptor than AVP. For patients encoding severely misfolded V2R mutants, strategies are being developed that circumvent the use of the V2R. For example, the use of the phophodiesterase inhibitor sildenafil increases the intracellular concentration of cGMP, which mimics

the action of cAMP in the collecting duct, resulting in translocation of aquaporin-2 to the apical membrane (13).

Model system

In search for a cure to relieve NDI, the model system of choice should optimally mimic the situation in patients. For many genetic disorders, animal models in which a specific gene is knocked out are the ideal system, as they highly resemble the disease phenotype. However, they are not suitable for testing the effects of compounds that require direct interaction with the target protein to relieve the disorderm as a V2R knockout animal would completely lack the AVPR2 gene. Alternatively, a knock-in model for X-linked NDI could be generated, in which a mutation is introduced into the AVPR2 gene. The only in vivo model for X-linked NDI thus far, is a mouse expressing the premature stop mutant V2R-E242X (68). Its usefulness was clearly demonstrated by the successful in vivo application of aminoglycosides for read-through on the premature stopcodon, which relieved NDI to some extent in this model (52). However, approximately 200 different mutations are known to cause NDI (http://www.medicine.mcgill.ca/nephros), which have different cellular defects (for examples see chapters 1, 3 and 7). Therefore, the generation of a knock-in animal for each type of cellular defect caused by these mutations would be required. Although highly physiologically relevant, the generation of such animal models would be laborious, time consuming and costly.

We chose a polarized cell model mimicking collecting tubule cells for our experiments. Stably transfected with either the wild-type (wt) V2R, or several V2R mutants known to cause NDI, our studies show that these cells are highly useful to study the cell-biological effects of various compounds on the trafficking and signalling of V2R mutants. Particularly, these cells are highly efficient in processing wtV2R, which is most obvious from the high rate of mature vs. immature receptors in MDCK cells, whereas, even in stably transfected non-polarized cells, this rate is much lower (Chapter 2) (25). In chapters 2, 3 and 4, type I MDCK cells were used, which express low levels of endogenous V2R. This cell line was selected because of certain practical advantages over type II cells, as type I cells polarize better when grown in a confluent monolayer, and they do not grow on top of each other. For functional testing, however, type II cells are more suitable, as they lack expression of endogenous V2R. As such, these cells will show less background signals in functional assays like radioligand binding or cAMP accumulation experiments. Although we have not tested it, MDCK type I cells may thus also be suitable for functional studies, as the expression of ER retained V2R mutants may cause retention of the endogenous wild-type V2R due to the formation of heterodimers or –oligomers with the mutant V2R (69).

Mechanisms of rescue

The work described in this thesis (chapters 4 and 5) and by others (6; 27; 39; 43; 55) has established the major determinants for the efficiency by which cell permeable antagonists restore the plasma membrane localization of V2R mutants and other ER-retained GPCRs. The antagonist's affinity for a receptor and the concentration in which it is used, largely determine the compound's ability to induce receptor translocation to the plasma membrane. This is confirmed by studies on the gonadotropin releasing hormone receptor (27). However, not all missense V2R mutants can be rescued. For example, Wüller et al. have described a subset of mutations in the cytosolic domain of the V2R that are not susceptible to localizational rescue, including the V2R-S167L,

which structure is severely distorted by the introduction of a bulky leucine residue (64). Although mutation studies and in silico docking models provide useful information (34) on this topic, definitive answers likely will have to await the crystal structure of the V2R in its native conformation, or bound by antagonists.

The determinants for displacement of the pharmacological chaperones by an agonist after plasma membrane rescue are largely determined by the affinity of the pharmacological chaperone, and the ratio between antagonist and agonist. However, from the data in chapter 6 it is clear that these are not the sole determinants. Differences in the nature and amount of the contact residues to which the pharmacological chaperone binds, and the extent by which these differ with the contact residues of the agonist that is to displace the pharmacological chaperone, also likely influence the extent of functional rescue.

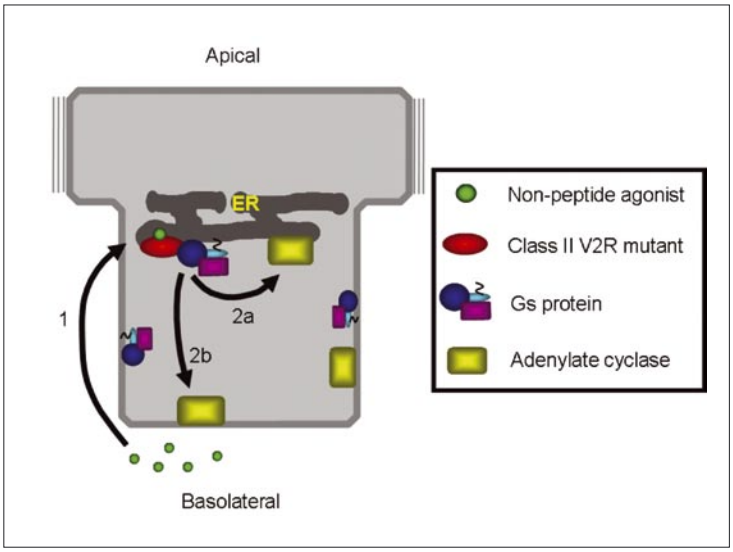

Figure 10-1 **Possible mechanisms for V2R signalling from the ER**
(1) The non-peptide agonist enters the cell and binds to the ER retained V2R mutant, activating the Gs protein. (2a) The Gαs subunit will then dissociate from Gβγ and activate adenylate cyclase that is present in the ER membrane. (2b) Alternatively, Gαs may become a cytosolic protein after dissociation from Gβγ, and activate adenylate cyclase at the plasma membrane.

Intracellular signalling by cell permeable agonists

The cellular mechanism by which nonpeptide agonists exert their action is highly novel, and remains to be elucidated. In order for V2R to signal from the ER, functional Gs proteins have to be present at the ER that are able to the V2R mutants. Although trimerization between the Gαs, a Gβ and a Gγ subunit occurs at the ER level (37) Gs is predominantly localized at the plasma membrane in a steady-state situation (54), and is anchored to this membrane with two palmitoyl groups. One palmitoyl residue is irreversibly attached to Gαs' N-terminal glycine residue (31), whereas a second

palmitoyl residue is reversibly fused to the neighbouring cysteine (31; 40). At present, it remains unknown at which biosynthetic step these palmitoyl groups are added (i.e. at the ER level, or post-ER). From these two options, two signalling pathways can be postulated:

1. Entry of the non-peptide agonist into the lumen of the ER and its binding to a mutant receptor allows the activation Gs proteins that are present in the ER (Fig. 1, step 1). If Gαs is N-terminally palmitoylated, it will be anchored to the ER membrane, and can thus only induce a cAMP cascade if also adenylate cyclase is present in the ER membrane (Fig. 1, step 2a), and thus requires the presence of complete signalling mechanism for cAMP in the ER.

2. If Gαs is not, or reversibly, palmitoylated in the ER, it may become a cytosolic protein by dissociating from the βγ subunit. It may then interact with adenylate cyclase at the plasma membrane to induce a cAMP response (Fig. 1, step 2b).

GPCR signalling from the ER has thus far only been described for the ER-localized GPR30, which responds to the cell permeable ligand estrogen. Also for this receptor, the exact signalling pathway involved remains to be elucidated (47). In contrast, maturation of the inefficiently folded, and therefore partially ER-retained, wild-type δ-opioid receptor (DOR) or the fully ER-retained mutant DOR-D95A, was increased upon incubation with nonpeptide agonists (43; 44), suggesting that these receptors leave the ER to signal at the plasma membrane.

Cellular implications of intracellular activation of ER-retained V2R

The different modes of action by which cell permeable antagonists and agonists mediate rescue of ER-retained V2R mutants may be explained by their allosteric effects on the V2R. Receptor activation by an agonist leads to a conformational change from the inactive (R) to the active state (R*), whereas binding of an antagonist fixes the receptor in its R state (11). Possibly, the R* state of V2R mutants are not recognized by the ER's quality control mechanism as a stable properly folded protein, and does not allow mutant V2R to leave the ER. In contrast, binding of nonpeptide agonists to ER retained DOR will change the receptor's conformation the R* state, but may in addition contribute to stabilization and folding of the receptor, and thus induce maturation and plasma membrane trafficking of these receptors. The different modes of action of cell-permeable agonists on ER-retained DOR and mutant V2R may be receptor- or mutation-specific. Alternatively, compound-intrinsic properties of the agonists such as full or partial agonism, or their affinities for their respective receptors may be determinant.

GPCR signaling from the inside of the cell may also contribute to the action of constitutively active (mutants of) GPCRs. Either due to intrinsic receptor properties, or due to an altered receptor conformation as a result of mutation, these do not require the presence of an agonist in order to be able to signal (38; 53). Recently, two mutations were identified that lead to constitutive activation of the V2R (R137L and R137C), and thereby to hyponatreamia in patients despite undetectable plasma levels of AVP (18). Assuming these mutants share the partial ER retained and partial late endosomal/lysosomal subcellular localization with the (constitutively) internalized mutant V2R-R137H (5; 6), the ER retained pool of V2R-R137L and R137C may signal directly from the ER, and would not require to traffic to the plasma membrane in order to signal.

Intracellular activation of V2R may also have consequences for signalling. Receptor activation at the plasma membrane, but also treatment with inverse agonists like SR121463B (4), increases V2R phosphorylation by GPCR kinases or protein kinase C (46), which triggers the recruit β-arrestin to the receptor. In recent years, the awareness has risen that GPCRs do not only signal via G proteins, but also via β-arrestin (32; 33; 36), which is directly involved in the activation of the mitogen-activated protein kinase pathway, and several other signalling pathways, and may thus be necessary for proper cell function (33). Therefore, it would be interesting to study whether ER retained V2R are phosphorylated and recruit β-arrestin after receptor stimulation, and what the cellular consequences of a lack of β-arrestin-mediated signalling are.

Involvement of the V2R in PNE

As shown in chapter 9, binding of dDAVP to the renal or extra-renal V2R is not a prerequisite to relieve Primary Nocturnal Enuresis (PNE) in a combined NDI/PNE patient. dDAVP was long considered to be a V2R specific agonist, but after cloning of the human vasopressin V3 receptor (V3R, previously known as V1bR), the latter receptor was shown to have a similar affinity for dDAVP as the V2R(51). The V3R is predominantly expressed in the pituitary, where it is involved in the secretion of adrenocorticotropic hormone (10). In addition, the V3R is likely involved in the increased arousability of PNE patients after administration of dDAVP to these patients (17). Likely, in patients that suffer from PNE, but not from NDI, dDAVP will also increase the renal urine concentrating ability, thereby reducing the pressure on the bladder and aiding in the relieve of PNE (23). Administration of V2R- and V3R-specific agonists to PNE patients may answer the question whether the renal concentrating ability or the arousal response is the most important factor in relieving PNE.

Clinical application of nonpeptide antagonists and agonists

Recently, the first clinical trial using the pharmacological chape-rone SR49059 in NDI patients was published, demonstrating the proof of principle that nonpeptide antagonists can successfully be applied in patients (7). However, the effect of this compound was relatively weak in patients harbouring full class II mutations. In this thesis five V2R-targeted compounds are described that are promising candidates to relieve NDI in patients. As both nonpeptide antagonists and agonists act on a broad range series of mutants (Table 1), the lack of general applicability is not a criterion to preclude the application of antagonists or agonists in the clinic. Based on the cell biological data in chapters 6, the two nonpeptide antagonists OPC31260 and OPC41061 are more effective to functionally rescue full class II V2R mutants compared to SR49059 at clinically feasible concentrations (chapter 6). In addition, the three non-peptide agonists FE999088, FE999089 and OPC51803 are highly promising candidates to relieve NDI in patients, as these do not require endogenous AVP, or supplementation thereof, for receptor activation (chapter 7).

Administration of such compounds to patients may not yield an immediate increase of the patients' urine concentration ability, as cAMP regulates not only the shuttling of aquaporin-2 to the apical membrane, but also AQP2 expression levels. As the AQP2 gene promoter contains a cAMP responsive element binding domain that is necessary for gene transcription (35; 67), patients suffering from X-linked NDI usually have very low expression levels of AQP2 (29). Thus, the principal cells may require 1-2 days to increase the AQP2 expression, and only then may an increased urine concentration

capacity become apparent. Further development of the above-mentioned highly-promising approaches and, especially, clinical testing of these separate or combined strategies in patients with X-linked NDI is anticipated to bring us an exciting scientific and clinical future, as it will tell us whether we can relief or cure the major form of congenital NDI. Besides in the kidney, V2R is also expressed in endothelial cells, where it regulates the excretion of the clotting factors von Willebrand Factor and Factor VIII (30). Although blood clotting is normal in NDI patients despite a blunted response of these factors to administration of dDAVP (8), blood levels of these factors should be closely monitored when non-peptide agonists are administered to patients. In addition, urine and blood electrolyte levels should be closely monitored.

Besides for NDI, the increasing availability of cell-permeable antagonists and agonists may also offer opportunities for treatment of other genetic disorders involving

Mutation	Localization	Functional	BM rescue	Functional Rescue	Reference (Chapter)
L44P	ER	Yes	CPAn	CPAn, CPA	4-7
del62-64	ER	G	CPAn	No	4-6
R113W	ER/BM	Yes	CPAn	ND	4-6
Q119X	ER	No	ND	ND	8
Y128S	ER	Yes	CPAn	CPA[1]	7
I130F	ER	Yes	CPAn	CPAn, CPA	4-7
S167T	ER	Yes	CPAn	CPAn, CPA	4-7
S167L	ER	AVP/G	No	No	4-7
G201D	ER/BM	Yes	CPAn	ND	4-6
T204N	ER/BM	Yes	CPAn	ND	4-6
V206D	ER	AVP	CC, CPAn	ND	4-6
Y280C	ER	Yes	CPAn	CPA[1]	7
W293X	ER	No	ND	No	8
P322S	ER	Yes	CPAn	CPA[1]	7
R337X	ER	No	ND	No	8
C358X	ER	Yes	ND	CPA[1]	8,9

Table 10-1 **Overview of V2R mutants and their rescue**

Abbreviations: ER, endoplasmic reticulum; BM, basolateral membrane; G, G protein binding disturbed; AVP, AVP binding disturbed; CPAn, cell permeable antagonist; ND, not determined; CPA, cell permeable agonist; CC, chemical chaperone. [1]Functional rescue after CPAn treatment has not been tested.

GPCR	Disorder	(Putative) Pharm. Chap.	Reference
AT2R	mental retardation	Agonists and antagonists	(57; 58)
DOR		Agonists and antagonists	(23)
ETBR	Hirschprung's disease	Antagonists	(59; 60)
FSHR	Ovarian displasia, amenorrhea	Antagonists	(61)
GNRHR	Hypogonadotropic hypoganadism	Antagonists	(25)
MC4R	Obesity	Agonists and antagonists	(62)
Rhodopsin	Retinitis pigmentosa	Agonists	(63)
TRHR	Isolated central hypothyroidism	Antagonists	(64)
V1R		Antagonists	(65)

Table 10-2 **Involvement of GPCRs in genetic**
disorders and (putative) pharmacological chaperones
Indicated are the abbreviated names of GPCRs, the loss-of-function disorders they are involved in, whether (ant)agonists are available that (may) function as pharmacological chaperones, and the references to these compounds.
Abbreviations: AT2R, angtiotensin type 2 receptor; DOR, δ-opioid receptor; ETBR, endothelin B receptor; FSHR, follicle stimulating hormone receptor; GNRHR, gonadotropin releasing hormone receptor; MC4R, melanocortin type 4 receptor; TRHR, thyrotropin releasing hormone receptor; V1R, vasopressin receptor type 1.

misfolded GPCRs, as indicated in table 2. In addition to GPCRs, also misfolding and ER retention of channel proteins such as the cystic fibrosis transmembrane conductance regulator ΔF508 mutant (62), or mutants of the HERG potassium channel (19; 21) can be overcome by pharmacological chaperones, and may thus allow the development of therapies for disorders such as cystic fibrosis and long QT syndrome, respectively.

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Summary
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Summary

The physiology of water homeostasis

The action of the antidiuretic hormone arginine-vasopressin (AVP) on the collecting duct of the kidney is essential for the human body to maintain a proper water balance. An increased blood osmolality, or a decreased blood volume will trigger the pituitary to secrete AVP. Via the blood, AVP is transported to the kidney, where it will bind to the vasopressin V2 receptor (V2R) on the basolateral (interstitial) side of the principal cells of the collecting duct. Via the stimulatory G protein (Gs) adenylate cyclase is activated, which results in a rise of the intracellular cAMP level. As a result, protein kinase A phosphorylates aquaporin-2 (AQP2) water channels, which leads to their insertion in the apical membrane (luminal side) of the cell, rendering this membrane highly permeable to water. Following an osmotic gradient, water is then absorbed from the pro-urine to the interstitium, thereby concentrating the urine. When the water balance is back to normal, AVP levels will decrease, which will result in the removal of AQP2 from the apical membrane, restoring its impermeability to water.

Congenital Nephrogenic Diabetes Insipidus

In congenital Nephrogenic Diabetes Insipidus (NDI), the AVP-mediated water reabsorption in the kidney is disturbed. As a result, the kidney is not able to actively concentrate the pro-urine, which may lead to dehydration a disturbed electrolyte balance, especially in young infants. Congenital NDI is a heterogenous disorder, which has three modes of inheritance that can be traced back to mutations in two genes. In the majority of patients, the disorder is X-linked (MIM 304800) and is caused by mutations in the AVPR2 gene, which encodes the V2R. In the remainder of the patients inheritance is autosomal recessive (9%; MIM 222000) or dominant (1%; MIM 125800) as a result of mutations in the gene encoding the AQP2 water channel.

The AVPR2 gene is located on chromosome Xq28 and codes for a member of the family of G protein-coupled receptors. Until now, over 200 unique mutations have been identified in the AVPR2 gene, many of which are summarized at http://www.medicine.mcgill.ca/nephros/. According to the type of mutation, and cellular consequences thereof, V2R mutants can be classified, as described in the introduction (chapter 1).

The goal of this thesis was to describe the molecular and cell biological causes of X-linked NDI, to identify means to restore these defects in order to develop new therapies that may relieve this disorder.

MDCK cells as a model for renal tubular cells

In chapter 2, we show that Madin-Darby canine kidney (MDCK) cells mimic the principal cells of the collecting duct to a high extent and thus represent a more suitable cell model compared to non-polarized cell systems. In chapter 2, and in more detail in chapter 3, we demonstrate that stable transfection of wild-type (wt) V2R, fused at its C-terminus to a green fluorescent protein (GFP), in MDCK cells leads to expression of a fully mature receptor that is localized predominantly in the basolateral membrane, as is the case in the kidney collecting duct. Activation of V2R with AVP, or its synthetic analogue dDAVP, induces internalization of the receptor into late endosomes and lysosomes, where the receptor is degraded.

In chapter 4, we describe the generation and analysis of MDCK cells stably expressing nine V2R mutants in NDI. In contrast to wtV2R, all mutants are fully (V2R-L44P, del62-64, -I130F, -S167T, -S167L en V206D) or partially (V2R-R113W, -G201D en –T204N) retained in the endoplasmic reticulum (ER) and are expressed as immature proteins. As such, they are class II mutants. In general, these mutants appear less stable compared to the wt receptor. With overexpression in COS cells, we analyzed the functionality of these V2R mutants. Three mutants are non-functional, as they are unable to bind AVP and/or activate the Gs protein. The mutants V2R-L44P, -R113W, -I130F, -S167T, -G201D en –T204N, however, are able to induce a cAMP response upon stimulation with dDAVP. Their functionality allows the development of a tailored therapy that targets these mutant receptors, as the cellular defect of NDI is thus the intracellular retention of the receptor, which prevents the binding of AVP. Therefore, we set out to rescue the function of V2R missense mutants using different approaches, which are described in chapters 5-7.

Functional rescue of class II mutations in V2R

In chapter 5, we report the effect of a subset of 'chemical chaperones' that are known to affect the localization of ER retained proteins and induce their translocation to the plasma membrane. Although not effective on eight out of nine mutants, incubation with the osmolytes glycerol and DMSO, the sarco- and endoplasmic reticulum calcium pump-inhibitors thapsigargin and curcumin, and the calcium ionophore ionomycin restores the plasma membrane expression of V2R-V206D. Furthermore, we showed that thapsigargin and curcumin induce rescue by increasing cytosolic calcium levels, rather than allowing the depletion of ER calcium. The molecular mechanism underlying rescue by glycerol and DMSO remains unknown; changes in the expression levels of the molecular chaperones heat shock protein 70 and 90, calnexin and protein disulfide isomerase are ruled out. Unfortunately, the V206D mutation interferes with vasopressin binding, which does not allow functional restoration of this mutant.

In chapter 5, and in more detail in chapter 6, we describe the action of cell permeable receptor antagonists on the ER-retained V2R mutants. These antagonists can enter the cell and, by binding to the mutant V2R, directly aid in receptor folding. Because of their pharmacological applicability, these compounds are termed 'pharmacological chaperones'. We compare the characteristics of four different pharmacological chaperones on nine V2R mutants, and find that all compounds increase the plasma membrane localization of the V2R mutants, except for V2R-S167L. The efficiency by which the plasma membrane localization is restored largely correlates with the affinities of the pharmacological chaperones for V2R and the concentration in which these compounds are used. For restoration of receptor function, however, the pharmacochaperone needs to be displaced by an agonist at the plasma membrane. We find that the efficiency of functional rescue is a balance between an antagonist's ability to induce plasma membrane localization and its ability to subsequently be displaced by agonist. At high concentrations, the V1R antagonist SR49059, which acts as weak antagonist on V2R, combines these characteristics best, whereas it is hardly efficient at lower, clinically-feasible concentrations. In contrast, the high-affinity antagonist SR121463B induced plasma membrane expression, but could not be displaced with agonist, and may thus not be suitable to relieve NDI in patients. The medium- and high-affinity antagonists OCP31260 and OPC41061, respectively, showed optimal rescue when used at clinically-feasible concentrations, although relatively high concentrations of agonist were needed to displace

these antagonists. As these latter two compounds are safe for use in humans, they are promising candidates to relieve NDI in patients.

In chapter 7, we show the effect of cell permeable agonists on class II mutants. If agonists induce ER exit of V2R mutants, they will immediately activate the receptor upon arrival at the plasma membrane. However, the three non-peptide agonists FE999088, FE999089 and OPC51803 are unable to induce plasma membrane translocation or maturation of six ER retained V2R mutants. Remarkably, all three compounds induce a cAMP response, whereas the non-cell permeable agonist dDAVP does not. Combined with their persistent ER localization, this indicates that V2R mutants can be activated in the ER. The generated cAMP response was sufficiently strong to induce translocation of AQP2 to the plasma membrane, thus restoring the healthy phenotype. Also, this represents the first example of a synthetic hormone to be used for the restoration of the function of an otherwise non-functional receptor.

Restoration of Class I mutations in NDI

Besides Class II mutations, also Class I mutations in V2R can cause NDI, by introducing a premature stop codon in the V2R coding sequence. In chapter 8, the expression of the nonsense mutants V2R-Q119X, -W293X, -R337X and –C358X in NDI, and the in vitro effect of aminoglycosides on the expression these mutants are described. Stable expression of these mutants in MDCK cells does not lead to detectable receptor proteins, suggesting that these receptor mutants are highly unstable. Transient expression in COS cells, however, resulted in expression of low levels of truncated proteins, that were high-mannose glycosylated and therefore likely ER retained. Treatment with the aminoglycosides gentamycin, geneticin, amikacin or tobramycin did not lead to detection of full-length proteins for any of the mutants. The nucleotide sequences on and around the mutations are sub-optimal for read-through, which may, in combination with the already low expression levels, be responsible for the absence of rescued full-length V2R. As the mutants V2R-R337X and –C358X are truncated in their C-termini, these mutations may not severely interfere with receptor function. Since these receptors are likely ER retained, we tested their ability to be activated by cell permeable agonists. Whereas V2R-R337X could not be activated, V2R-C358X induced a clear cAMP response upon stimulation with cell permeable agonists. Thus, also patients harboring functional nonsense mutations may benefit from treatment with cell permeable agonists.

The patient harboring the C358X mutation suffers, besides from NDI, also from primary nocturnal enuresis (PNE; bedwetting). A common treatment for PNE is the intranasal administration of the synthetic AVP analogue dDAVP, which is postulated to increase water reabsorption and thereby reduce the urine volume and the pressure on the bladder. However, as described in chapter 8, V2R-C358X is ER retained, thus explaining NDI in this patient. In chapter 9, we demonstrate that upon administration of dDAVP to the patient, PNE is completely resolved, whereas all symptoms of NDI persisted, suggesting an extrarenal effect of dDAVP in PNE. In the NDI/PNE patient, however, no V2R-mediated extrarenal response is detected as measured by the plasma levels of the blood clotting factors von Willebrand Factor and Factor VIII. Cell biological experiments confirm that V2R-C358X is absent from the plasma membrane, and is less stable compared to the wild-type V2R (chapter 9). Thus, our data indicate that it is highly unlikely that dDAVP acts on V2R to relieve of PNE.

In conclusion, we have described the cellular fate of class I and II V2R mutants in NDI and we studied how chemical chaperones and cell-permeable V2R antagonists and agonists can restore these cellular defects. As several of these compounds are FDA approved, or close to approval, they are highly promising candidates to relieve NDI in patients. In addition, the increased understanding of the rescue of misfolded proteins may aid in identifying cures for other conformational diseases.

Samenvatting

De fysiologie van de waterhomeostase

De werking van vasopressine op de verzamelbuis van de nier is van groot belang voor het in stand houden van een goede vochtbalans in het menselijk lichaam. Verhoging van de bloedosmolariteit of verlaging van het bloedvolume leidt tot afgifte van het antidiuretisch hormoon arginine-vasopressine (AVP) door de hypofyse. In de verzamelbuis van het nefron bindt AVP aan de type 2 vasopressine receptor (V2R), die zich bevindt aan de basolaterale (interstitiële) zijde van de hoofdcellen. Via het stimulerende Gs eiwit wordt vervolgens adenylaat cyclase geactiveerd wat resulteert in een tijdelijke toename van de intracellulaire cAMP concentratie. Als gevolg hiervan zal proteïne kinase A aquaporine-2 (AQP2) waterkanalen fosforyleren, wat leidt tot insertie van deze kanalen in de apicale membraan (luminale zijde) van de cel, waardoor deze membraan permeabel wordt voor water. Gedreven door een osmotische gradiënt, zal dit leiden tot resorptie van water uit de voorurine naar het interstitium, waardoor uiteindelijk de urine geconcentreerd wordt. Zodra de waterbalans is hersteld, zal de afgifte van AVP stoppen en AQP2 uit de apicale membraan verwijderd worden, waardoor deze weer impermeabel wordt voor water en er geen verdere resorptie van water plaatsvindt.

Congenitale Nefrogene Diabetes Insipidus.

In congenitale Nefrogene Diabetes Insipidus (NDI) is de hierboven beschreven AVP-gereguleerde resorptie van water in de nier verstoord. Als gevolg hiervan is de nier niet in staat de voorurine te concentreren, wat kan leiden tot ernstige uitdroging en/of een verstoorde electrolyten-balans. Congenitale NDI is een heterogene aandoening, die op drie verschillende manieren kan overerven, en waarbij de oorzaken zijn terug te voeren is naar mutaties in twee genen. In de meeste gevallen (90%) erft NDI X-chromosomaal over (MIM 304800) en wordt veroozaakt door mutaties in het gen dat codeert voor de V2R. Een kleinere groep van de patiënten vertoont een autosmaal recessieve (9%; MIM 222000) of dominante (1%; MIM 125800) overerving als gevolg van mutaties in het gen dat codeert voor het waterkanaal AQP2.

Het V2R gen bevindt zich op chromosoom Xq28 en codeert voor een lid van de familie van G eiwit-gekoppelde receptoren (Birnbaumer et al., 1992;Lolait et al., 1992). Tot op heden zijn meer dan 200 mutaties in V2R beschreven, waarvan de meeste zijn samengevat op http://www.medicine.mcgill.ca/nephros/. Naar gelang het type mutatie en de cellulaire consequenties die deze mutatie heeft kunnen V2R mutanten ingedeeld worden in verschillende klasses. Deze zijn, samen met een meer gedetailleerde beschrijving van congenitale NDI, weergegeven in de inleiding (hoofdstuk 1). Het doel van dit proefschrift was het identificeren van de moleculaire en celbiologische oorzaken van de X-chromosomaal overervende vorm van NDI, om te komen tot een of meerdere nieuwe therapievormen voor deze ziekte.

MDCK cellen als model voor renale tubuluscellen

Voor dit doeleinde hebben we gebruik gemaakt van gepolariseerde niercellen, aangezien deze net als de hoofdcellen in de nier een afzonderlijk apicale en basolaterale membraan bezitten (hoofdstuk 2). Hierdoor zijn deze cellen een beter celmodel voor niertubulus cellen in vergelijking met niet-gepolariseerde celsystemen. In hoofdstuk 2, en in meer detail in hoofdstuk 3, wordt de generatie en karakterisatie van een gepolariseerde MDCK cellijn, die de wild-type V2R stabiel tot expressie brengt,

beschreven. Aan de carboxy-terminus van deze V2R is een groen fluorescerend eiwit (GFP) gekoppeld, waardoor V2R gemakkelijk te detecteren is. Het V2R-GFP eiwit bevindt zich in ongestimuleerde toestand hoofdzakelijk in de basolaterale membraan van de MDCK cellen, net zoals in de verzamelbuis van de nier. Na stimulatie met AVP, of diens synthetische analoog dDAVP, wordt de receptor geïnternaliseerd en versneld afgebroken in de lysosomen, hetgeen een veel voorkomende vorm van negatieve terugkoppeling is voor de hormonale regulatie van het watertransport.

Vervolgens werden ook negen V2R mutanten, die in patiënten NDI veroorzaken, tot expressie gebracht in deze cellen, hetgeen beschreven staat in hoofdstuk 4. In tegenstelling tot de wild-type receptor, worden deze NDI mutanten geheel (V2R-L44P, del62-64, -I130F, -S167T, -S167L en V206D) of gedeeltelijk (V2R-R113W, -G201D en –T204N) vastgehouden in het endoplasmatisch reticulum (ER) door het kwaliteitscontrole mechanisme van de cel. Hierdoor worden ze ingedeeld in klasse II. Deze ER retentie gaat samen met een hogere mate van proteasomale afbraak waardoor mutanten overwegend instabiel zijn in vergelijking met de wild-type receptor. Met behulp van overexpressie-studies wordt de functionaliteit van V2R mutanten in NDI bestudeerd. Door zeer hoge expressie in COS cellen kunnen normaliter ER geretardeerde eiwitten toch de plasmamembraan bereiken. Drie V2R mutanten waren niet functioneel vanwege hun onvermogen om AVP te binden en/of het Gs eiwit te activeren. Echter, de mutanten V2R-L44P, R113W, -I130F, -S167T, -G201D en -T204N waren in staat een cAMP cascade te initiëren na stimulatie met dDAVP. Hun functionaliteit biedt de mogelijkheid voor het ontwikkelen van een op de receptor gerichte therapie. De moleculaire oorzaak van NDI in patienten met dergelijke functionele mutaties is namelijk de intracellulaire localisatie van de mutante receptor, waardoor deze receptoren niet bereikbaar zijn voor AVP.

Functioneel herstel van klasse II mutaties in V2R

In hoofdstuk 5-7 is beschreven hoe de BM localisatie en functie van intracellulair geretardeerde receptor mutanten hersteld kunnen worden om zo dit moleculair defect te overkomen. In hoofdstuk 5 wordt het effect beschreven van een aantal 'chemische chaperones' waarvan bekend is dat ze de localisatie van ER geretardeerde eiwitten kunnen beïnvloeden en translocatie naar de plasmamembraan kunnen induceren. Hoewel ze geen effect hadden op acht van de negen bovengenoemde V2R mutanten, leidde incubatie met de osmolieten glycerol en DMSO, de sarco- en endoplasmatisch reticulum pomp remmers thapsigargine en kurkuma, of de calcium ionofoor ionomycine, tot een toename van V2R-V206D in de BM. Thapsigargine en kurkuma veroorzaakten een relatieve daling van de calciumconcentratie in het ER, omdat de calciumconcentratie in het cytosol juist steeg. Onze data tonen aan dat, in tegenstelling tot wat gedacht wordt voor andere eiwitten, juist deze stijging van het cytosolaire calciumniveau de oorzaak is van de herstelde membraanlokalisatie van V2R-V206D, terwijl veranderingen in ER calciumniveaus geen effect hebben op de lokalisatie van deze mutant. Het moleculaire mechanisme waarmee glycerol en DMSO de BM lokalisatie van V2R-V206D herstellen is niet bekend; een expressieverhoging van de moleculare chaperones, zoals vaak gesuggereerd, werd voor heat shock protein 70 en 90, calnexine, en proteine disulfide isomerase uitgesloten. Functioneel herstel van V2R-V206D is niet mogelijk, aangezien de V206D mutatie de binding van AVP verhindert.

In hoofdstuk 5 en, in meer detail in hoofdstuk 6 wordt het effect beschreven van celpermeabele antagonisten op ER geretardeerde V2R mutanten. Als deze antagonisten de cel binnendringen en aan een mutante V2R binden, kunnen ze diens verstoorde structuur helpen herstellen. Vanwege hun mogelijke farmacologische toepasbaarheid en specificiteit, worden ze farmacologische chaparones genoemd. Alle vier celpermeabele antagonisten beschreven in hoofdstuk 6 bleken in staat om de BM lokalisatie en maturatie te herstellen van acht van de negen geteste V2R mutanten, waarbij alleen V2R-S167L mutant niet reageerde op behandeling. De mate van herstel van de plasmamembraan-lokalisatie en de daaraan gekoppelde eiwit-maturatie correlleerde in grote lijnen met de affiniteit van de gebruikte stoffen voor de V2R, en hun gebruiksconcentratie. Teneinde naast lokalisatie en maturatie ook de functie van mutante V2R te herstellen, is het noodzakelijk dat de V2R-gebonden antagonist aan de plasmamembraan verdrongen wordt door een agonist, om zo de signaleringscascade in werking te stellen. Recent onderzoek door Bernier et al. toonde aan dat de V1R antagonist SR49059, die ook werkt als een zwakke antagonist op V2R, in staat is om in patiënten met een partiëel ER geretardeerde V2R het urine-volume te verlagen. Echter, in patiënten met zuiver ER geretardeerde mutaties werd nauwelijks een effect waargenomen. De data in hoofdstuk 6 tonen aan dat bij hoge concentraties SR49059 de beste farmacologische chaperone is, omdat deze stof, bij de beperkte mate van maturatie en plasmamembraan lokalisatie en een volledige vervanging door dDAVP voor een grote cAMP respons zorgde. Echter, deze stof werkt niet meer bij klinisch haalbare concentraties. De V2R antagonist SR121463B, met een hoge affiniteit voor V2R, was daarentegen zeer goed in staat translocatie van V2R naar de BM te induceren, maar kon vervolgens niet vervangen worden door een agonist, waardoor geen functioneel herstel optrad. OPC31260 en OPC41061, die respectievelijk een middelmatige en hoge affineit hebben voor V2R, waren bij gebruik in hoge concentraties in staat een ruime mate van translocatie en maturatie te veroorzaken, en waren beperkt vervangbaar door een agonist. Echter, ook bij klinisch toepasbare concentraties waren deze beide stoffen nog in staat om een functioneel herstel te bewerkstelligen. Functioneel herstel door farmacologische chaperones is dus een balans tussen de mogelijkheid van een antagonist om de plasmamembraan-lokalisatie van een mutant te induceren, en de efficiëntie waarmee hij vervolgens vervangen kan worden door een agonist. Aangezien OPC31260 en OPC41061 goedgekeurd zijn voor gebruik in de kliniek, en ze bovenstaande criteria optimaal combineren in klinisch toepasbare concentraties zijn ze veelbelovende kandidaten om de symptomen van NDI in patiënten te verlichten.

In hoofdstuk 7 beschrijven we het effect van celpermeable agonisten op klasse II mutanten. Agonisten hoeven na het induceren van receptoren translokatie niet vervangen te worden zoals een antagonist, maar zullen direct leiden tot receptor activatie. De drie V2R agonisten FE999088, FE999089 en OPC51803 blijken geen invloed te hebben op de maturatie of subcellulaire localisatie van zes ER-geretardeerde V2R mutanten. Desondanks resulteerde een korte incubatie met elk van de drie celpermeabele agonisten tot een verhoging van het cAMP niveau in de cellen, terwijl incubatie met het niet-celpermeabele dDAVP geen effect had. Deze data, in combinatie met de onveranderde ER localisatie van de mutanten, leidde tot de conclusie dat ER-geretardeerde V2R mutanten intracellulair geactiveerd kunnen worden, hetgeen deze studie tot het eerste voorbeeld maakt van hoe synthetische agonisten gebruikt kunnen worden voor het functioneel herstel van een normaliter niet-functionerende receptor. Tevens was de gegenereerde cAMP respons sterk genoeg om translocatie van AQP2 naar de PM te induceren.

Herstel van klasse I mutaties in NDI

Naast klasse II mutanten, kunnen ook klasse I mutanten NDI veroorzaken. Meestal handelt het zich hierbij om de introductie van een prematuur stopcodon in de sequentie die codeert voor de V2R. Hoofdstuk 8 beschrijft de expressie de stopmutanten V2R-Q119X, -W293X, -R337X en –C358X in NDI, en het effect van aminoglycosides op deze mutanten. De stopmutanten kunnen niet stabiel tot expressie gebracht worden in de MDCK cellen, hetgeen suggereert dat deze mutanten instabiel zijn. Echter, in transiënt getransfecteerde COS cellen komen deze receptoren tot expressie in hun hoog-mannose geglycosyleerde vorm, hetgeen duidt op ER retentie. Premature stopcodons zijn mogelijk gevoelig voor behandeling met aminoglycosides, hetgeen er toe kan leiden dat de ribosomen door het premature stopcodon heen lezen, hetgeen kan leiden tot herstel van de functie en/of de localisatie van de mutante receptor. Behandeling met de aminoglycosides gentamycine, geneticine, amikacine of tobramicine resulteerde niet tot expressie van volledig getransleerde receptoren voor elk van de mutanten. De sub-optimale sequenties van de premature stopcodons zelf, en de hen omliggende sequenties zijn, in combinatie met de lage expressieniveaus van de mutanten, een mogelijke verklaring voor het niet optreden van functioneel herstel van deze stopmutanten. Aangezien bij de mutanten V2R-R337X en –C358X slechts hun C-termini getrunceerd zijn, zijn ze mogelijk functioneel, maar ER geretardeerd. Behandeling van deze mutanten met celpermeable agonisten leidde niet tot receptoractivatie voor V2R-R337X, maar -C358X laat een duidelijke verhoging van het intracellulaire cAMP niveau zien. Dit toont aan dat ook ER geretardeerde, maar functionele, V2R stopmutanten geactiveerd kunnen worden door celpermeabele agonisten.

De patient die de voorgenoemde V2R-C358X heeft lijdt, naast aan NDI, ook aan een congenitale vorm van primaire nocturele enurese (PNE; oftewel bedplassen). Een veelgebruikte behandeling voor PNE is intranasale toediening van dDAVP, teneinde een verhoogde waterresorptie in de nier te realiseren, waardoor de druk op de blaas af zou nemen en de patiënt niet meer in bed zou plassen. Gebaseerd op de data in hoofdstuk 8 echter, wordt V2R-C358X in het ER vastgehouden, en is zodoende niet in staat om dDAVP te binden aan de plasmamembraan. Zoals beschreven in hoofdstuk 9, leidt toediening van dDAVP tot het volledig verdwijnen van PNE in de patiënt, maar de symptomen van NDI bleven onveranderd, hetgeen suggereert dat het effect van dDAVP in PNE extrarenaal is. Overeenkomstig met een niet functionle V2R werd in de PNE/NDI patiënt ook geen extrarenale respons waargenomen van de verhoogde afgifte van de stollingsfactoren von Willebrand Factor en Factor VIII. Celbiologisch onderzoek bevestigde dat V2R-C358X afwezig was van de PM, en tot expressie kwam als een intracellulair geretardeerd, instabiel eiwit. Onze data suggereren daarmee dat de V2R niet betrokken is bij het verminderen van PNE bij patiënten met behulp van dDAVP.

Concluderend, werd het cellulaire lot van klasse I en II V2R mutanten in NDI beschreven, en werd bestudeerd hoe chemische chaperones en cel-permeabele V2R antagonisten en agonisten deze cellulaire defecten kunnen herstellen. Mede aangezien een aantal van deze stoffen (bijna) goedgekeurd zijn door de FDA, zijn ze veelbelovende kandidaten voor het verlichten van NDI in patiënten. Tevens kan het toegenomen inzicht in het mechanisme van functioneel herstel van mutante eiwitten bijdragen aan het ontwikkelen van therapiëen voor ziektes veroorzaakt door misvouwing van eiwitten.

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Curriculum Vitae

Joris Hubertus Robben werd geboren op 14 juni 1978 te Weert. In juni 1996 behaalde hij zijn diploma voortgezet wetenschappelijk onderwijs aan het Bisschoppelijk College te Weert. In september van dat jaar begon hij met een studie Biologie en Medisch Laboratoriumonderzoek aan Fontys Hogescholen in Eindhoven. Zijn eerste wetenschappelijke stage tijdens deze opleiding werd uitgevoerd binnen de afdeling Celfysiologie van het UMC St. Radboud onder begeleiding van Dr. Erik-Jan Kamsteeg, gevolgd door een afstudeerstage bij de afdeling Advanced Biotechnologies van Janssen Pharmaceutica (Beerse, B) onder begeleiding van Dr. Arjan Buist. Met de laatstgenoemde stage werd tevens deze opleiding afgesloten, waarvoor hij in juni 2000 zijn diploma ontving.

Joris accepteerde vervolgens een positie als onderzoeksanalist op zijn afstudeerplek, waar hij werkte onder begeleiding van Dr. Eckhard Bender, die hem aanmoedigde een part-time Master of Science opleiding in Moleculaire Biologie en Biotechnologie te volgen aan de Vrije Universiteit van Brussel. Gefinancierd door Janssen Pharmaceutica, werd in september 2000 met deze opleiding aangevangen, en in september 2001 met onderscheiding afgesloten.

Inmiddels was Joris begonnen aan zijn promotie-onderzoek bij de sectie Celfysiolgie van de afdeling Fysiologie op een project van de Nierstichting Nederland, onder begeleiding van Dr. Peter Deen en mw. Prof. Dr. Nine Knoers, hetgeen geleid heeft tot dit proefschrift. Momenteel is hij werkzaam bij het Institute for Biochemistry and Life Sciences van de University of Glasgow (Glasgow, UK) onder supervisie van Prof. dr. Graeme Milligan, gefinancierd door een Rubicon-subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek.

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1. Robben JH, Knoers NVAM, Deen PMT
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