

Regulation of the Vasopressin V2 Receptor by Vasopressin in Polarized Renal Collecting Duct Cells

J.H. Robben,* N.V.A.M. Knoers,[†] and P.M.T. Deen*[‡]

*Department of Physiology, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands; and [†]Department of Human Genetics, University Medical Center Nijmegen, 6500 HB Nijmegen, The Netherlands

Submitted April 23, 2004; Revised September 21, 2004; Accepted September 23, 2004
Monitoring Editor: Keith Mostov

Binding of arginine-vasopressin (AVP) to its V2 receptor (V2R) in the basolateral membrane of principal cells induces Aquaporin-2–mediated water reabsorption in the kidney. To study the regulation of the V2R by dDAVP in a proper model, a polarized renal cell line stably-expressing V2R-GFP was generated. Labeled AVP-binding studies revealed an equal basolateral vs. apical membrane distribution for V2R-GFP and endogenous V2R. In these cells, GFP-V2R was expressed in its mature form and localized for 75% in the basolateral membrane and for 25% to late endosomes/lysosomes. dDAVP caused a dose- and time-dependent internalization of V2R-GFP, which was completed within 1 h with 100 nM dDAVP, was prevented by coinubation with a V2R antagonist, and which reduced its half-life from 11.5 to 2.8 h. Semiquantification of the V2R-GFP colocalization with E-cadherin (basolateral membrane), early endosomal antigen-1 (EEA-1) and lysosome-associated membrane protein-2 (LAMP-2) in time revealed that most dDAVP-bound V2R was internalized via early endosomes to late endosomes/lysosomes, where it was degraded. The dDAVP-internalized V2R did not recycle to the basolateral membrane. In conclusion, we established the itinerary of the V2R in a polarized cell model that likely resembles the in vivo V2R localization and regulation by AVP to a great extent.

INTRODUCTION

The vasopressin V2 receptor (V2R) is a member of the 7 transmembrane domain 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. The major role of this receptor is the regulation of the body water homeostasis by determining the level of reabsorption of water from prourine through Aquaporin-2 (AQP2) water channels. On 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, among 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 (Bichet *et al.*, 1998; Birnbaumer, 1999; Knoers and Deen, 2001). 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, preeclampsia and the syndrome of inappropriate release of AVP (SIADH; Schrier *et al.*, 2001; Nielsen *et*

al., 2002). 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, most studies on the regulation of V2R trafficking and its mutants in NDI were done in nonpolarized cell models. However, it has recently been recognized that the regulation of proteins may differ between polarized and nonpolarized cell types (Tsao and von Zastrow, 2001). Indeed, in transiently transfected cells, the V2R is mainly expressed in an immature form and localizes to intracellular compartments (Schulein *et al.*, 1998a; Sadeghi and Birnbaumer, 1999), whereas in vivo, the V2R mainly localizes in the basolateral membrane (Noguchi *et al.*, 1995). Therefore, to study the regulation of the V2R, 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, [Adamantaneacetyl¹, O-Et-D-Tyr², Val⁴, Aminobutyryl⁶, 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; Schulein *et al.*, 1998a) was kindly provided by Dr. Alexander Oksche (FMP, Berlin, Germany)

Culture of MDCK Cells

MDCK type I and II cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowittaker, Verviers, Belgium) supplemented with 5%

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-04-0337. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-04-0337.

[‡] Corresponding author. E-mail address: p.deen@ncmls.ru.nl.

fetal bovine serum (PAA Laboratories, Karlsruhe, Germany), gentamicin, L-glutamine, sodium carbonate, and 1% nonessential amino acids. Transfection of these cells with 25 μg of the V2R-GFP expression construct was performed using the calcium phosphate method as described (Deen *et al.*, 2002).

Immunocytochemistry

For immunocytochemistry, the cells were seeded on Costar filters at a density of 3×10^5 cells/cm² and grown for 3 d. Immunocytochemistry and confocal laser scanning microscopy (CLSM) was performed as described (Deen *et al.*, 2002). 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 Transduction Laboratories, San Diego, CA) or 1:200-diluted mouse antilyso-some-associated membrane protein 2 (LAMP-2) antibodies (Nabi *et al.*, 1991); 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 colocalization, individual pictures of 15–20 cells were contrast-stretched for the green and the red signal, after which the percentage of colocalization was calculated using Metamorph software (Universal Imaging, Downingtown, PA). Averaged data obtained from three independent pictures was used to determine the extent of colocalization.

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, Bedford, MA) as described (Deen *et al.*, 2002). For detection of V2R-GFP, 1:5000 diluted rabbit anti-GFP antiserum (Cuppen *et al.*, 2000) was used (kindly provided by Dr. B. Wieringa, UMC Nijmegen). As secondary antibodies, goat anti-rabbit IgGs (Sigma) were used at a 1:5000 dilution.

[³H]AVP Binding Assay

Mock-transfected or V2R-GFP expressing MDCK cells were seeded at a density of 1.5×10^5 cells/cm² in 12 multiwells plates and grown to confluence. Cells were washed twice with ice-cold PBS-CM and incubated with 100 nM [³H]AVP (Perkin Elmer-Cetus, Boston, MA) in PBS-CM at 4°C for 1 h, which was added to the apical, basolateral, or both sides. Subsequently, the filters were excised, added to Opti-Fluor scintillation fluid (Perkin Elmer-Cetus) and counted in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard Bioscience, Boston, MA). Averaged data of at least three independent experiments were used. In internalization assays, cells were treated with 100 nM dDAVP for indicated periods of time, after which cells were treated as above. Cells treated with 100 nM dDAVP but kept on ice were taken as controls. In recycling assays, cells were treated with 100 nM dDAVP for 2 h, extensively washed, incubated in dDAVP-free medium for 4 h, and analyzed as above.

RESULTS

A changed distribution of a protein is the resultant of changes in endocytosis, exocytosis, or both. Our data below give time-fixed steady state localizations and therefore do not provide information on changes in endo- or exocytosis. For clarity, therefore, it needs to be noted that in this manuscript "internalization" is used when the steady state localization of the V2R is changed from the plasma membrane to intracellular organelles, whereas with "recycling" the reappearance of V2R-GFP on the plasma membrane after being internalized is meant.

Localization of the V2R Stably Expressed in Polarized MDCK Cells

Madin-Darby canine kidney (MDCK) cells have been shown to be a good cell model for the regulation of the collecting duct AQP2 water channel (Deen *et al.*, 2002). 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. After selection for G418 resistance, clones were analyzed for protein expression using immunoblotting. Numerous positive clones were isolated from both

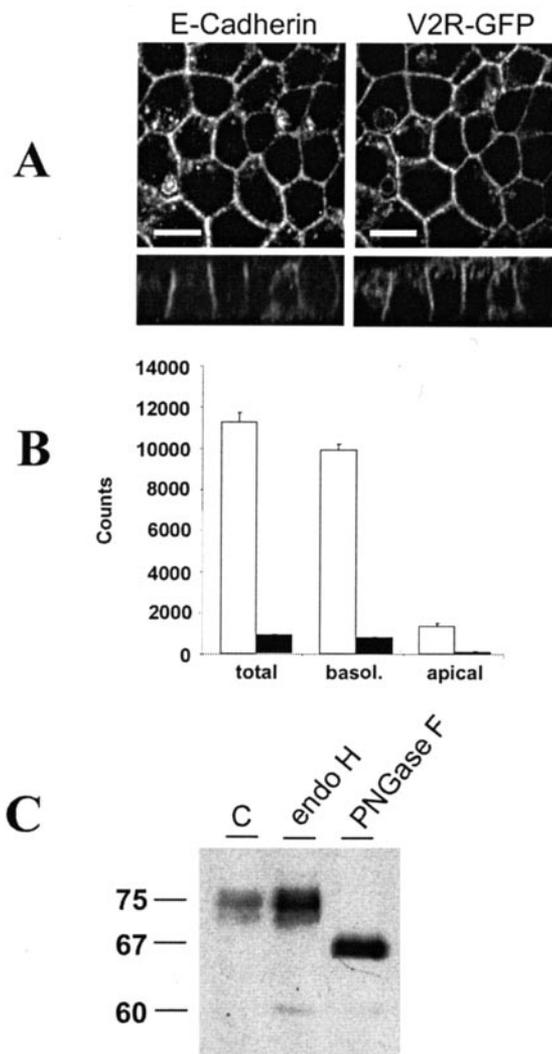


Figure 1. Localization and glycosylation of V2R-GFP in polarized MDCK cells. (A) MDCK type I cells stably transfected with a V2R-GFP expression construct were grown to confluence, fixed, and subjected to immunocytochemistry with rat anti-E-cadherin antibodies and Alexa 594-conjugated goat anti-rat antibodies. CLSM analysis revealed a large portion of V2R-GFP colocalized with the E-cadherin. Bar, 10 μm . (B) V2R-GFP expressing (□) or Mock-transfected cells (■) were seeded on filters and grown to confluence. Next, cells were labeled with 100 nM [³H]AVP at 4°C for 1 h on either the apical, basolateral (basol.) or both sides (total), after which the filters were excised, added to scintillation fluid, and counted for radioligand-bound signal. Bars represent the results of three independent experiments. (C) Confluent MDCK-V2R-GFP cells were lysed in Laemmli buffer and directly loaded (control: c) on a 10% SDS-PAAG or pretreated with either Endo H or PNGase F (indicated). V2R proteins were detected by immunoblotting using rabbit anti-GFP antibodies. The mass of these proteins, as deduced from those of marker proteins, is indicated in kDa.

cell lines. CLSM of six of these clones demonstrated that in both cell types V2R-GFP was predominantly expressed in the basolateral membrane, where it colocalized with the basolateral marker protein E-cadherin (Figure 1A). Besides, V2R-GFP was detected intracellularly (see below). To establish the apical vs. basolateral localization of V2R-GFP biochemically, [³H]AVP-binding experiments were done. Scintillation counting confirmed that the majority of V2R-GFP

was located in the basolateral membrane, because of the labeled agonist, $88.9 \pm 2.8\%$ bound to the basolateral membrane, whereas only $11.1 \pm 1.3\%$ bound to the apical membrane (Figure 1B). Mock-transfected MDCK cells, which express V2R endogenously (Deen *et al.*, 1997), bound ~ 13 -fold less of the labeled AVP, but the ratio of basolateral ($91.9 \pm 2.9\%$) vs. apical ($8.1 \pm 2.1\%$) labeling was similar (Figure 1B), which indicated that the distribution of V2R-GFP was similar to that of the endogenous V2R. 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 nonpolarized cells, high-mannose sugar moieties are added to Asn22 of the V2R in the endoplasmic reticulum (Innamorati *et al.*, 1996), which can be specifically cleaved off by Endo H. On its further transport to the membrane, these sugar groups will be modified to 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 (Sadeghi and Birnbaumer, 1999), 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 (Figure 1C). In the control sample, V2R-GFP was detected as a set of bands between 70 and 80 kDa. On treatment with Endo H, these 70–80-kDa bands remained, but a weak band of ~ 60 kDa appeared. The size of this band is consistent with that of nonglycosylated V2R-GFP. Treatment with PNGase F resulted in a shift of the 70–80-kDa bands to a band of ~ 67 kDa. Because this shift was not observed with Endo H, this indicated that most of V2R-GFP in MDCK cells is complex-glycosylated. The band at 67 kDa possibly represents O-glycosylated V2R-GFP, as this form of glycosylation is insensitive to Endo H and PNGase F (Sadeghi and Birnbaumer, 1999). 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 GPCRs 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 to lysosomes (Tsao and von Zastrow, 2000).

To investigate whether V2R-GFP 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 h, subjected to immunocytochemistry for E-cadherin, and analyzed for its colocalization with V2R-GFP by confocal microscopy (Figure 2, A–D). Subsequent semiquantification revealed that in control cells $74.7 \pm 4.5\%$ of V2R-GFP colocalized with E-cadherin, whereas 1, 10, and 100 nM dDAVP treatment resulted in 41.5 ± 3.3 , 25.4 ± 5.4 , and $16.6 \pm 4.3\%$ colocalization, respectively. These data showed that the level of internalization was dependent on the dose of the agonist.

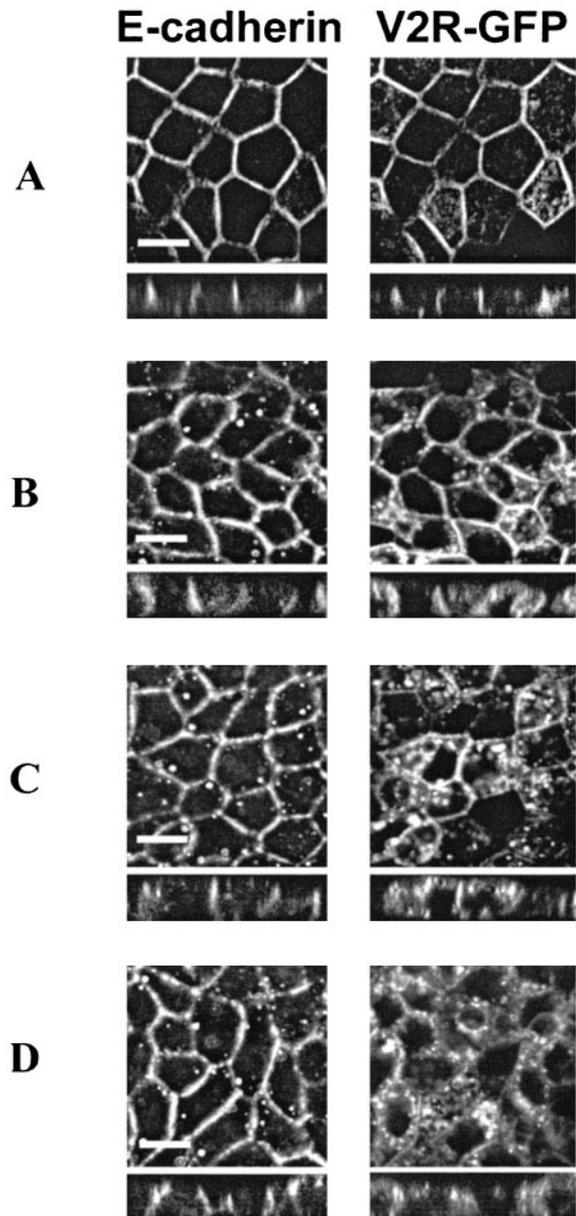


Figure 2. Dose-dependent internalization of V2R-GFP with dDAVP. Confluent MDCK-V2R-GFP cells were incubated at the basolateral side with normal medium (A), or medium containing 1 (B), 10 (C), or 100 nM (D) dDAVP for 1 h. Then, the cells were fixed and subjected to immunocytochemistry as described in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

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 semiquantification of the signals revealed that in control cells $75.9 \pm 4.5\%$ of the V2R was localized in the lateral membrane, whereas the level of colocalization with E-cadherin decreased to 31.2 ± 6.5 , 10.0 ± 3.5 , and $9.2 \pm 4.4\%$ at 30, 60, and 120 min after addition of dDAVP, respectively. 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 min of dDAVP treatment. To determine the remaining plasma membrane amounts of V2R biochemically, radioligand binding experiments at the basolateral membrane were done, in which cells were pretreated for 0, 30, or 60 min with dDAVP, followed by extensive washing and subsequent labeling of the remaining receptors on the cell surface. The available AVP-binding sites decreased to 31.5 ± 4.1 and $13.7 \pm 2.3\%$ for the 30- and 60-min pretreated samples compared with the control situation (unpublished data), which is similar to our immunocytochemical quantification data. As a control for the washing procedure, cells were incubated with 100 nM dDAVP for 1 h at 4°C to prevent internalization, followed by extensive washing and labeling as described above. Compared with MDCK-V2R cells that were only incubated with radioligand ($100 \pm 11.2\%$), this control showed $81.8 \pm 7.1\%$ binding, which was not significantly different ($p = 0.07$).

Time-resolved Localization of the V2R in Intracellular Organelles 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 colocalization of the V2R with EEA-1 was indeed transient. Semiquantification of the level of colocalization revealed no significant level of colocalization in untreated cells ($3.1 \pm 2.0\%$). At 15 or 30 min (Figure 3A) after administration of dDAVP, the level of colocalization increased to 15.0 ± 3.0 and $20.4 \pm 2.9\%$, respectively, whereas at 60 min, the rate of colocalization decreased to $8.5 \pm 1.7\%$.

To determine the level of localization of V2R-GFP in late endosomes/lysosomes in time, colocalization studies were done as described above, but now with the lysosomal-associated membrane protein-2 (LAMP-2) as a marker. In control cells, a considerable fraction ($26.8 \pm 3.8\%$) of V2R-GFP already colocalized with LAMP-2 (Figure 3B). Administration of 100 nM dDAVP for 30 min and 1 h increased the level of colocalization with LAMP-2 to 48.6 ± 5.5 and $80.2 \pm 5.8\%$, respectively (Figure 3C). This was not further increased with longer periods of dDAVP treatment (unpublished data). Incubation of MDCK-V2R cells with 100 nM dDAVP combined with 1 μ M of the V2R antagonist prevented the accumulation of V2R-GFP in late endosomes/lysosomes ($28.1 \pm 6.2\%$ colocalization; Figure 3D), which indicated that the late endosomal/lysosomal localization of V2R-GFP is increased 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 dissociate from their agonist and recycle back to the plasma membrane (Bonifacino and Traub, 2003; Hicke and Dunn, 2003). To determine the stability of unbound or dDAVP-bound V2R in time, we blocked protein synthesis in polarized MDCK-V2R cells with 50 μ M cycloheximide, immunoblotted the V2R cell samples, and relatively-quantified the amount of V2R-GFP in time (Figure 4A, left panel). Without dDAVP, 12.3 ± 4.8 , 27.9 ± 7.3 , and $34.3 \pm 6.4\%$ decreases in V2R-GFP expression were observed for 2-, 4-, and 8-h incubation, respectively.

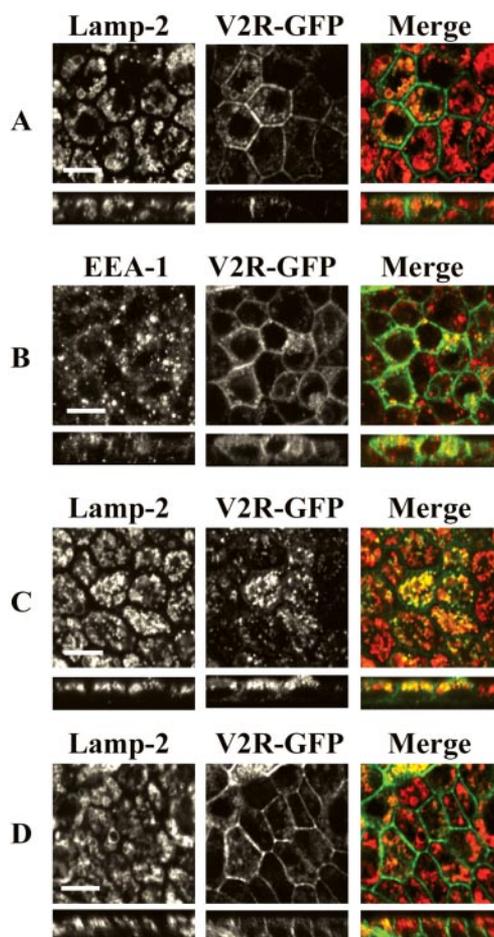


Figure 3. Time-dependent internalization of V2R-GFP with dDAVP. Confluent MDCK-V2R-GFP monolayers were incubated at the basolateral side with normal medium (B), medium containing 100 nM dDAVP for 30 min (A) or 1 h (C), or with medium containing a mixture of 100 nM dDAVP and 1 μ M V2R antagonist (D).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

Incubation of the cells with cycloheximide in the presence of a cell-permeable antagonist for 8 h did not significantly increase receptor stability ($30.9 \pm 5.9\%$ degradation; unpublished data). Treatment of V2R-GFP-expressing cells with dDAVP (Figure 4A, right panel) induced a much faster degradation of V2R, because at 2-, 4-, and 8-h dDAVP treatment, its expression was reduced 47.9 ± 6.8 , 64.9 ± 7.2 , and $82.3 \pm 4.6\%$, respectively. From these data it could be deduced that 100 nM dDAVP reduced the half-life of V2R from 11.52 ± 2.8 to 2.77 ± 0.41 h ($n = 3$). Coincubation with 100 μ M chloroquine for 8 h, which blocks lysosomal degradation, only resulted in $11.2 \pm 4.3\%$ degradation, which was not further decreased upon coincubation with the proteasomal inhibitor MG132 ($p = 0.14$; Figure 4A, utmost right lane). In addition, no differences in V2R-GFP amounts were observed when cells were treated with cycloheximide and dDAVP in either the presence or absence of MG-132 (unpublished data). This indicated that the majority of dDAVP-induced degradation of the V2R was through the lysosomal pathway. Although chloroquine treatment induced LAMP-2-positive vesicles to cluster and form large complexes,

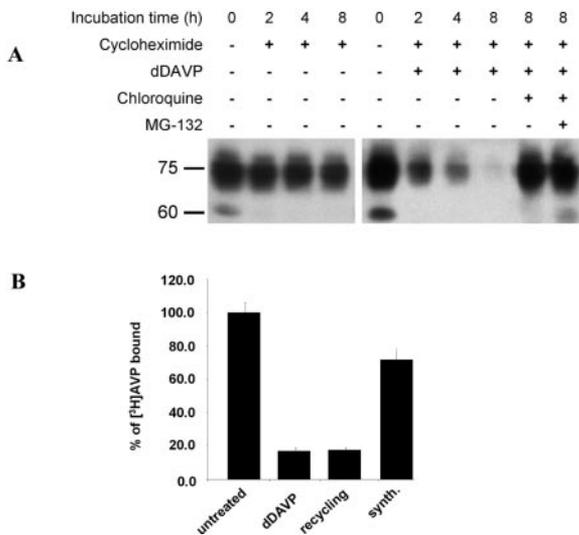


Figure 4. Degradation and recycling of V2R-GFP upon agonist treatment. (A) Degradation of V2R-GFP. MDCK-V2R-GFP cells that were grown to confluence on filters were incubated for the indicated time (in h) with or without 100 nM dDAVP, 50 μ M cycloheximide, 100 μ M chloroquine, and/or 20 μ M MG-132 (indicated). After these treatments, the cells were lysed in Laemml 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. (B) Recycling of V2R-GFP. MDCK-V2R-GFP cells were seeded on filters and grown to confluence, followed by basolateral administration of 100 nM dDAVP for 2 h at 37°C to induce receptor internalization (dDAVP) in the presence of cycloheximide. Subsequently, dDAVP was extensively washed away, followed by a 4-h incubation period at 37°C in culture medium with cycloheximide to allow recycling (recycling). Subsequently, the availability of ligand-binding sites on the basolateral membrane of the cells was determined as described in the legend of Figure 1. [³H]AVP binding to cells treated with dDAVP for 2 h and recovered for 4 h in the absence of cycloheximide (synth.) reveals the level of newly synthesized V2R-GFP appearing at the plasma membrane within 4 h. V2R-GFP expressing cells not pretreated with dDAVP or cycloheximide (untreated) were used as a reference for binding. Each bar represents three independent experiments.

CLSM analysis of the cells treated with either cycloheximide, MG-132, or chloroquine treatment for 8 h revealed no difference the level of V2R expression in the basolateral plasma membrane compared with nontreated V2R-GFP cells (unpublished data).

After agonist-induced internalization, the receptor might recycle to the plasma membrane after dissociation from the ligand. If recycling occurs, as has been shown for the vasopressin V1 (Terrillon *et al.*, 2004) and the β 2-adrenergic receptor (Tsao and von Zastrow, 2000), this is usually detected within 4 h after removal of the agonist. Therefore, to test whether the V2R is able to recycle to the basolateral membrane after dDAVP treatment, MDCK-V2R cells were incubated in medium lacking dDAVP for 4 h, after a dDAVP treatment for 2 h. These incubations were done in the presence of cycloheximide to prevent the appearance of newly synthesized V2R-GFP on the membrane. Subsequent immunocytochemistry for E-cadherin, CLSM analysis, and semiquantification revealed a $15.4 \pm 5.2\%$ of colocalization with V2R-GFP.

To biochemically investigate whether the V2R recycles, MDCK-V2R cells were treated as above, but subsequently subjected to a [³H]AVP-binding assay (Figure 4B). Two hours of treatment with dDAVP reduced the amount of

basolateral AVP binding to $15.4 \pm 2.0\%$ of the untreated situation, which did not significantly increase after removal of the ligand and the recovery incubation period of 4 h in the absence of dDAVP ($17.0 \pm 2.4\%$). Without cycloheximide, a 4-h recovery period after 2-h dDAVP treatment resulted in a $68.3 \pm 4.1\%$ of [³H]AVP binding. The 51% of difference in binding compared with the level bound at 2 h of dDAVP treatment is indicative for newly synthesized V2R-GFP appearing on the plasma membrane within this time frame.

DISCUSSION

MDCK Type I Cells Expressing V2R-GFP Are a Proper Model for V2R Regulation in Renal Collecting Duct Cells

Studying the localization and translocation of the V2R in the polarized principal cells of the renal collecting duct is difficult, because its expression level is low. Therefore, to identify pathways involved in the molecular regulation of the human V2R, a polarized epithelial cell model was generated of MDCK type I cells, which resemble renal collecting duct cells, and type II cells, which resemble proximal tubule cells (Richardson *et al.*, 1981), by transfection with a human V2R-GFP expression construct. As seen in vivo (Nonoguchi *et al.*, 1995), both cell lines showed a strong expression of V2R-GFP in the basolateral membrane, whereas it also localized to intracellular compartments. This intracellular localization of V2R-GFP in MDCK cells mainly represents late endosomes or lysosomes, because without stimulation around 75% of the V2R localized in the basolateral membrane, whereas $\sim 26\%$ colocalized with LAMP2. 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 (Seifert and Wenzel-Seifert, 2003).

The MDCK type I cells showed a more distinct plasma membrane staining for V2R-GFP than type II cells and were therefore selected for further studies. These differences in lateral localization between both MDCK cell lines may be due to the sparse lateral cell space with type I cells compared with type II cells (Richardson *et al.*, 1981). Using the same construct, Schülein *et al.* (1998b) reported a similar localization of V2R-GFP in another MDCK cell type, which indicates that this localization is common for MDCK cells.

Biochemical analysis further revealed that in the selected MDCK cells, the vast majority of the V2R-GFP has a molecular mass of 70–80 kDa. On Endo H treatment, a minor amount of unglycosylated 60-kDa V2R-GFP appeared, which indicated that some is present in the high mannose glycosylated form. Although we cannot exclude that some V2R-GFP is retained in the endoplasmic reticulum, it is more likely that this represents V2R-GFP on its biosynthetic itinerary to the Golgi complex.

On PNGase F digestion, the 70–80-kDa band shifted to a 67-kDa band, indicating that the vast majority of V2R-GFP is expressed in the mature, complex glycosylated form. The identity of the 67-kDa band, however, remains unclear. Because V2R has been reported to be phosphorylated at several sites (Innamorati *et al.*, 1997), the 67-kDa band could represent multiphosphorylated V2R-GFP. However, this is rather unlikely, because V2R phosphorylation has been reported to precede internalization after agonist binding and the 67-kDa signal of PNGase F-treated V2R-GFP did not increase at different time points after dDAVP administration to our V2R-GFP cells (unpublished data). Alternatively, the 67-kDa protein could be O-glycosylated V2R-GFP, because sialidase treatment of V2R obtained from transfected COS cells re-

duced the mass of the receptor (Sadeghi and Birnbaumer, 1999). In our V2R-GFP cell sample, however, the mass of PNGaseF-treated V2R-GFP was not changed upon cotreatment with sialidase (unpublished data), which indicated that either the 67-kDa band does not represent O-glycosylated V2R-GFP or that V2R-GFP in MDCK cells undergoes sialidase-insensitive O-glycosylation. However, because complex glycosylation (and O-glycosylation) only take place in the Golgi complex on properly folded V2R, these data reveal that the V2R folds and matures properly in MDCK type I cells. The localization and high level of maturation of V2R-GFP in our MDCK cells indicated that 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 vitro, basolateral stimulation of the V2R with AVP or dDAVP leads to translocation of AQP2 from intracellular vesicles to the apical membrane (Deen *et al.*, 2000; Jeon *et al.*, 2003), but 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. Although V2R-GFP sequestration already occurs at a physiological concentration of 1 nM dDAVP, the level of internalization was increased with higher dDAVP concentrations, which is most likely due to increased receptor occupation by the hormone.

dDAVP Induces the Internalization of V2R via Early Endosomes to Late Endosomes/Lysosomes

The internalization of V2R-GFP was also time dependent. To determine the path and time frame of V2R internalization after binding of dDAVP, time-resolved colocalization experiments were performed between V2R-GFP and E-cadherin, EEA-1 and LAMP2, which mark the basolateral membrane, early endosomes and late endosomes/lysosomes, respectively. 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. On incubation with 100 nM dDAVP, colocalization studies with E-cadherin revealed that half of V2R-GFP was internalized within 30 min, whereas the maximal level was obtained within 1 h. During the first half an hour, most of the V2R seemed to pass the EEA1-positive early endosomes, because their colocalization increased from 3 to 15% within 15 min after dDAVP treatment, which was sustained until 30 min, after which it reduced again to 8% at 1 h after the start of the dDAVP treatment. Interestingly, as the combined colocalization of V2R-GFP with the markers E-cadherin, EEA-1, and LAMP-2 is ~100% at several time points tested ($t = 0$ min: 75, 0, and 26%; $t = 30$ min: 31, 20, and 48%; $t = 60$ min: 10, 9, and 80%, respectively), it is likely that most, if not all, V2R-GFP traffics from the plasma membrane via EEA-1-positive vesicles to the late endosomes and lysosomes. Although we cannot exclude that a minor fraction of V2R-GFP transits via other vesicles to its final location, this is consistent with the notion of Oakley *et al.* (1999) that both recycling and nonrecycling receptors travel to the early endosomes upon agonist stimulation.

After its passage through early endosomes, agonist-bound V2R-GFP accumulates in late endosomes/lysosomes as the level of colocalization of the V2R with LAMP2 increased from 27% at $t = 0$ min to 58, 80, and 83% at $t = 30, 60,$ and 120 min, respectively, after the start of the dDAVP treatment. Because the level of V2R internalization is dose de-

pendent, it is anticipated that the time frame of V2R-GFP sequestration is increased with lower doses of dDAVP.

Innamorati *et al.* (2001) reported that in transiently transfected HEK293 cells, dDAVP treatment resulted in the accumulation of HA-tagged V2 receptors in a rab11-positive perinuclear recycling compartment. In our cells, rab11-positive signals looked similar to those reported (Casanova *et al.*, 1999), but we did not find any colocalization of it with the fully internalized receptor (unpublished data). The cause of this discrepancy is unclear, but may be due to a difference in V2R localization in polarized vs. of nonpolarized cells.

dDAVP-induced Sequestered V2R Is Targeted for Lysosomal Degradation

From the early endosomes, the internalized receptor can either be recycled to the plasma membrane or degraded in lysosomes after being released from its agonist in late endosomes (Tsao and von Zastrow, 2000). All our data indicate that the V2R is a member of the latter group. After stimulation with 100 nM dDAVP, the level of V2R-GFP localization in late endosomes/lysosomes increased from 27 to 80% in 1 h. Also, during this first hour and beyond, the majority of these dDAVP-bound receptors were degraded, which could be prevented to a large extent with chloroquine, but not with MG132. Quantification revealed that dDAVP treatment reduced the half-life of V2R-GFP from 11.5 to 2.8 h. En gross, this is in line with the results of Martin *et al.* (2003), who also observed a decreased half-life for V2R-GFP in transiently transfected COS cells. However, their half-lives for unstimulated (~3.5 h) and AVP-stimulated receptor (~1 h) were considerably lower than the half-lives we found. These differences in stability may be due to the cell type used, transient vs. stable expression systems, differences in the level of V2R maturation between the different cells, and/or due to the usually high expression of exogenous proteins in transiently transfected cells.

In recycling experiments similar to the one we performed for the V2R, Innamorati *et al.* (2001) found that the vasopressin V1R reappeared in the plasma membrane within a 2-h recovery period from stimulation with AVP, from which the authors concluded that the V1R recycles to the plasma membrane. In our study, however, immunocytochemical colocalization studies with E-cadherin and [³H]AVP binding after dDAVP pretreatment and a 4-h recovery period, all in the presence of cycloheximide, revealed no difference in the number of available V2R compared with the situation before the recovery period. This finding indicates that the V2R does not recycle to the plasma membrane during the first 4 h after agonist-induced internalization.

It is unlikely that a lack of exocytotic proteins due to cycloheximide explains the absence of V2R recycling in our cells. At first because, as most membrane proteins, the V2R is likely to be continuously endo- and exocytosed. Second, because an 8-h incubation of the cells with only cycloheximide did not affect the steady state level of expression of V2R-GFP in the basolateral membrane (unpublished data), which suggest that recycling (i.e., exocytosis from an endosomal compartment) is unaffected by cycloheximide, because if cycloheximide blocked exocytosis from an endosomal compartment, one would expect an accumulation of V2R in endosomes.

For the V2R, this is in contrast to a study by Jans *et al.* (1991), who reported V2R recycling in LLC-PK1 cells. They also used [³H]AVP as a readout for receptor localization and recycling. However, instead of pretreating their cells with a V2R-specific agonist, such as dDAVP, they used AVP, which also binds V1 receptors. As LLC-PK1 cells endogenously express V1 receptors (Burnatowska-Hledin and Spielman,

1987; Weinberg *et al.*, 1989; Dibas *et al.*, 1997) and these receptors have been reported to recycle (Terrillon *et al.*, 2004), the recycling data reported by Jans *et al.* likely reflect recycling of V1Rs instead of V2Rs. Alternatively, it might be due to cellular differences, because there are subclasses of sorting machinery that regulate the trafficking of receptors differently along the same pathway, and MDCK and LLC-PK1 cells might express a different repertoire of such proteins.

Our data thus indicate that the agonist-bound V2R does not recycle and is extensively degraded. An extensive degradation appears quite common for nonrecycling GPCRs, as the nonrecycling δ -opioid receptor ($\geq 50\%$ after 3-h agonist incubation) and type 1-, 2-, and 5- sphingosine 1-phosphate receptors are also extensively down-regulated by their agonist (Tsao and von Zastrow, 2000; Graler and Goetzl, 2004). In contrast, the stability of the rapidly-recycling $\beta 2$ -adrenergic receptor was high ($< 10\%$ degradation after 3 h) and unaffected by stimulation with its agonist.

In conclusion, we have set up and characterized a polarized renal cell model that shows V2R-GFP maturation, localization and dDAVP-induced internalization and degradation as is anticipated to occur 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 NDI.

ACKNOWLEDGMENTS

We thank Dr. A. Oksche (FMP, Berlin, Germany) for providing the V2R-GFP expression construct, Dr. B. Wieringa (Cell Biology, UMC Nijmegen, The Netherlands) for the rabbit anti-GFP antiserum, and Dr. A. Le Bivic (Marseille, France) for the mouse anti-LAMP-2 antibodies. This project is supported by a grant from the Dutch Kidney Foundation (PC 104) to P.M.T.D. and N.V.A.M.K. and from the European Union (QLK3-CT-2001-00987) to P.M.T.D.

REFERENCES

Bichet, D.G., Turner, M., and Morin, D. (1998). Vasopressin receptor mutations causing nephrogenic diabetes insipidus. *Proc. Assoc. Am. Physicians* 110, 387-394.

Birnbaumer, M. (1999). Vasopressin receptor mutations and nephrogenic diabetes insipidus. *Arch. Med. Res.* 30, 465-474.

Bonifacino, J.S., and Traub, L.M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biotech.* 22, 395-447.

Burnatowska-Hledin, M. A. and Spielman, W. S. (1987) Vasopressin increases cytosolic free calcium in LLC-PK1 cells through a V1-receptor. *Am. J. Physiol.* 253(2 Pt. 2), F328-F332.

Casanova, J.E., Wang, X., Kumar, R., Bhartur, S.G., Navarre, J., Woodrum, J.E., Altschuler, Y., Ray, G.S., and Goldenring, J.R. (1999). Association of Rab25 and Rab11a with the apical recycling system of polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell* 10, 47-61.

Cuppen, E., van Ham, M., Wansink, D.G., de Leeuw, A., Wieringa, B., and Hendriks, W. (2000). The zyxin-related protein TRIP6 interacts with PDZ motifs in the adaptor protein RIL and the protein tyrosine phosphatase PTP-BL. *Eur. J. Cell Biol.* 79, 283-293.

Deen, P.M.T., Rijss, J.P.L., Mulders, S.M., Errington, R.J., VanBaal, J., and Vanos, C.H. (1997). Aquaporin-2 transfection of Madin-Darby canine kidney cells reconstitutes vasopressin-regulated transcellular osmotic water transport. *J. Am. Soc. Nephrol.* 8, 1493-1501.

Deen, P.M.T., Van Balkom, B.W.M., and Kamsteeg, E.J. (2000). Routing of the aquaporin-2 water channel in health and disease. *Eur. J. Cell Biol.* 79, 523-530.

Deen, P.M.T., Van Balkom, B.W.M., Savelkoul, P.J.M., Kamsteeg, E.J., van Raak, M., Jennings, M.L., Muth, T.R., Rajendran, V., and Caplan, M.J. (2002). Aquaporin-2, COOH terminus is necessary but not sufficient for routing to the apical membrane. *Am. J. Physiol.* 282, F330-F340.

Dibas, A.I., Rezazadeh, S.M., Vassan, R., Mia, A.J., and Yorio, T. (1997). Mechanism of vasopressin-induced increase in intracellular Ca²⁺ in LLC-PK1 porcine kidney cells. *Am. J. Physiol.* 41, C810-C817.

Graler, M.H., and Goetzl, E.J. (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* 18, 551-553.

Hicke, L., and Dunn, R. (2003). Regulation for membrane protein transport by ubiquitin and ubiquitin binding proteins. *Annu. Rev. Cell Dev. Biol.* 19, 141-172.

Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001). The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. *J. Biol. Chem.* 276, 13096-13103.

Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1996). A fully active non-glycosylated V2 vasopressin receptor. *Mol. Pharmacol.* 50, 467-473.

Innamorati, G., Sadeghi, H., Eberle, A.N., and Birnbaumer, M. (1997). Phosphorylation of the V2 vasopressin receptor. *J. Biol. Chem.* 272, 2486-2492.

Jans, D.A., Jans, P., Luzius, H., and Fahrenholz, F. (1991). Monensin-resistant Llc-Pk1 cell mutants are affected in recycling of the adenylate cyclase-stimulating vasopressin V2-receptor. *Mol. Cell. Endocrinol.* 81, 165-174.

Jeon, U.S. *et al.* (2003). Oxytocin induces apical and basolateral redistribution of aquaporin-2 in rat kidney. *Nephron Exp. Nephrol.* 93, e36-e45.

Knoers, N.V., and Deen, P.M. (2001). Molecular and cellular defects in nephrogenic diabetes insipidus. *Pediatr. Nephrol.* 16, 1146-1152.

Martin, N.P., Lefkowitz, R.J., and Shenoy, S.K. (2003). Regulation of V2 vasopressin receptor degradation by agonist-promoted ubiquitination. *J. Biol. Chem.* 278, 45954-45959.

Nabi, I.R., Le Bivic, A., Fambrough, D., and Rodriguez-Boulant, E. (1991). An endogenous MDCK lysosomal membrane glycoprotein is targeted basolaterally before delivery to lysosomes. *J. Cell Biol.* 115, 1573-1584.

Nielsen, S., Frokiaer, J., Marples, D., Kwon, T.H., Agre, P., and Knepper, M.A. (2002). Aquaporins in the kidney: from molecules to medicine. *Physiol. Rev.* 82, 205-244.

Nonoguchi, H. *et al.* (1995). Immunohistochemical localization of V2 vasopressin receptor along the nephron and functional-role of luminal V2 receptor in terminal inner medullary collecting ducts. *J. Clin. Invest.* 96, 1768-1778.

Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S., and Caron, M.G. (1999). Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* 274, 32248-32257.

Richardson, J.C.W., Scalera, V., and Simmons, N.L. (1981). Identification of 2 strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta* 673, 26-36.

Sadeghi, H., and Birnbaumer, M. (1999). O-Glycosylation of the V2 vasopressin receptor. *Glycobiology* 9, 731-737.

Schrier, R.W., Cadnapaphornchai, M.A., and Ohara, M. (2001). Water retention and aquaporins in heart failure, liver disease and pregnancy. *J. R. Soc. Med.* 94, 265-269.

Schulein, R., Hermosilla, R., Oksche, A., Dehe, M., Wiesner, B., Krause, G., and Rosenthal, W. (1998a). A dileucine sequence and an upstream glutamate residue in the intracellular carboxyl terminus of the vasopressin V2 receptor are essential for cell surface transport in COS.M6 cells. *Mol. Pharmacol.* 54, 525-535.

Schulein, R., Lorenz, D., Oksche, A., Wiesner, B., Hermosilla, R., Ebert, J., and Rosenthal, W. (1998b). Polarized cell surface expression of the green fluorescent protein-tagged vasopressin V2 receptor in Madin Darby canine kidney cells. *FEBS Lett.* 441, 170-176.

Seifert, R., and Wenzel-Seifert, K. (2003). The human formyl peptide receptor as model system for constitutively active G-protein-coupled receptors. *Life Sci.* 73, 2263-2280.

Terrillon, S., Barberis, C., and Bouvier, M. (2004). Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with β -arrestin and their trafficking. *Proc. Natl. Acad. Sci. USA* 101, 1548-1553.

Tsao, P.I., and von Zastrow, M. (2001). Diversity and specificity in the regulated endocytic membrane trafficking of G-protein-coupled receptors. *Pharmacol. Ther.* 89, 139-147.

Tsao, P.I., and von Zastrow, M. (2000). Type-specific sorting of G protein-coupled receptors after endocytosis. *J. Biol. Chem.* 275, 11130-11140.

Weinberg, J.M., Davis, J.A., Shayman, J.A., and Knight, P.R. (1989). Alterations of cytosolic calcium in Llc-Pk1 cells induced by vasopressin and exogenous purines. *Am. J. Physiol.* 256, C967-C976.