Pharmacocochaperones Post-translationally Enhance Cell Surface Expression by Increasing Conformational Stability of Wild-type and Mutant Vasopressin V_2 Receptors*§

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Some membrane-permeable antagonists restore cell surface expression of misfolded receptors retained in the endoplasmic reticulum (ER) and are therefore termed pharmacocochaperones. Whether pharmacocochaperones increase protein stability, thereby preventing rapid degradation, or assist folding via direct receptor interactions or interfere with quality control components remains elusive. We now show that the cell surface expression and function (binding of the agonist) of the mainly ER-retained wild-type murine vasopressin V_2 receptor (mV_2R) is restored by the vasopressin receptor antagonists SR49059 and SR121463B with EC_{50} values similar to their K_{D} values. This effect was preserved when protein synthesis was abolished. In addition, SR121463B rescued eight mutant human V_2Rs (hV_2Rs, three are responsible for nephrogenic diabetes insipidus) characterized by amino acid exchanges at the C-terminal end of transmembrane helix TM I and TM VII. In contrast, mutants with amino acid exchanges at the interface of TM II and IV were not rescued by either antagonist. The mechanisms involved in successful rescue of cell surface delivery are explained in a three-dimensional homology model of the antagonist-bound hV_2R.

Water homeostasis in mammals is regulated through arginine-vasopressin (AVP), acting through the vasopressin V_2 receptor (V_2R) expressed in the renal collecting duct (1). In X-linked nephrogenic diabetes insipidus (NDI), mutations in the vasopressin V_2 receptor (V_2R) gene (2). More than 150 different mutations have been described (for review, see Ref. 3), 50% of which are missense mutations resulting in the substitution of a single amino acid. Most of the hV_2R mutants with a single amino acid exchange are retained within the ER and not transported to the cell surface (for review, see Ref. 3). Most likely, the amino acid exchanges result in improper folding of the mutant hV_2Rs and subsequently prolonged association with molecular chaperones. For example, for the NDI mutant hR337X, a prolonged association with the ER-chaperone calnexin has been observed (4). Chaperone association prevents the aggregation of misfolded proteins, but also inhibits the exit of improperly folded proteins from the ER until correct folding is established.

Recently, it has been found that membrane-permeable antagonists not only inhibit receptor activation, but also promote cell surface expression of misfolded, ER-retained G protein-coupled receptors (GPCRs). This concept represents an intriguing new approach for the therapy of congenital diseases caused by mutations in genes encoding GPCRs. For the ER-retained rhodopsin mutant P23H (a frequent cause of autosomal-dominant retinitis pigmentosa), it has been shown in vitro that the inverse agonist 9-cis-retinal or the non-hydrolyzable inverse agonist 11-cis-7-ring-retinal promoted cell surface expression (5,6). Restoration of cell surface expression by antagonists or inverse agonists has also been found for various mutants of the hV_2R (7) and the gonadotropin releasing hormone receptor (8).

The molecular mechanisms by which antagonists or inverse agonists promote cell surface delivery remain elusive. Antagonists may act on misfolded proteins by increasing protein stability, e.g., inhibiting their rapid degradation or by preventing misfolding and aggregation of the nascent proteins. Alternatively, although less likely, hydrophobic pharmacocochaperones could interfere with components of the quality control system. To explore the mechanisms by which antagonists rescue intracellularly retained GPCRs, we used the wild-type murine V_2R (mV_2R), which is predominantly retained within the ER as an immature protein (9). In contrast, the hV_2R is mainly located within the plasma membrane as a complex glycosylated protein. These differences are caused by a variant amino acid at the junction of the second transmembrane domain and the first extracellular loop (lysine 100 in hV_2R, aspartate 100 in mV_2R, Ref. 9). We show here that antagonists increase the conformational stability of the mV_2R at a post-translational level via direct interactions. Antagonist-mediated cell surface delivery was also found for a subset of mutant hV_2Rs, which showed amino acid exchanges at the C-terminal end of transmembrane.
regions TM I and TM VII. In contrast, mutant hV2Rs with amino acid exchanges at the interface of TM II and TM IV did not show antagonist-mediated cell surface delivery, most likely because of more severe folding defects. The mechanisms involved in successful antagonist-mediated delivery of cell surface delivery are explained in a three-dimensional homology model of the antagonist-bound hV2R.

EXPERIMENTAL PROCEDURES

Materials—Trypsin, cycloheximide, and LipofectAMINE were from Invitrogen (Leek, The Netherlands), puromycin, BQ788, BQ123, and G418 were from Calbiochem-Novabiochem (Bad Soden, Germany), BQ123 was from Alexis (Laufelfingen, Switzerland), trypan blue from Seromed (Berlin, Germany), EZ-Link TM Sulfo-NHS Biotin and NeutrAvidin beads from Pierce, the QuikChange mutagenesis kit from Stratagene (Heidelberg, Germany), and FuGENE 6 from Roche Applied Science (Mannheim, Germany). Fetal calf serum was from Biochrom (Heidelberg, Germany), and FuGENE 6 from Roche Applied Science (Mannheim, Germany). Fetal calf serum was from Biochrom (Heidelberg, Germany). All other reagents were from Sigma. [3H]AVP (68.5 Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany). The vasopressin V2 and V1 receptor-selective antagonists Ci/mmol) was purchased from PerkinElmer Life Sciences (Rodgau, Germany).

SR121463B and SR49059 were kindly provided by Dr. C. Serradeil-LeGal (Sanofi Synthelabo, Montpellier, France). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany). Polyclonal antibodies to rabbit V2R were from Biotrend (Cologne, Germany).

PLASMIDS—Plasmids encoding the wild-type human vasopressin V2 receptor (hV2R) and its mutants were made by subcloning the CDNA from the pMVA7 plasmid (6) into the mV2R and hV2R fusion proteins are derivatives of the plasmid mV2R. The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9). The CDNA encoding the wild-type mature human vasopressin V2 receptor was amplified by PCR and cloned into the multiple cloning site of pCDNA3 plasmid (9).}

LSTM Analysis. LSTM-FRET Imaging, and Fluorescence Recovery after Photobleaching—Coverslips with HEK293 cells transiently expressing the different GFP fusion proteins were mounted in a temperature insert (Zeiss, Jena Germany) and analyzed with an LSM 510 META system using an Axiovert 135 microscope equipped with an Plan-Apochromat 63/1.4 PlanNeofluar 100/1.3 objectives (all from Zeiss). GFP and YFP were excited with 488 nm and 530 nm light, respectively. The plasma membrane was visualized after addition of 20 μl of trypan blue (0.05% in PBS) at λex = 543 nm and λem > 590 nm. The thickness of optical sections was between 0.3 and 0.6 μm. For quantitative analysis of antagonist-mediated cell surface expression, the plasma membrane identified in the trypan blue image was marked as region of interest (ROI) and subsequently transferred to the GFP image. In the GFP fluorescence channel the ROI representing the plasma membrane was defined. The average fluorescence intensities in the plasma membrane and the cell interior were determined. For each single cell, the ratio of fluorescence in the plasma membrane and the cell interior was calculated.

For FRET analyses, HEK293 cells grown on coverslips were transiently co-transfected with mV2RF and mV2RF. LSM-FRET imaging and fluorescence recovery after photobleaching were performed as described in detail previously (11,14). LSM-FRET imaging was performed with the LSM510 META and for fluorescence recovery after photobleaching we used an inverted microscope (Axiovert 100; Zeiss) equipped with a Plan-Apochromat 63×/1.4 objective, a monochromator (Polychrome II; TILL Photonics, Gräfelfing, Germany) and a cooled CCD camera (Imago, TILL Photonics). For excitation a dual reflectivity dichroic filter (530 nm; Chroma Technol., Rockingham, VT) and for emission band pass filters of 460–500 nm (CFP) or 535–580 nm (YFP) were used.

Molecular Modeling of Antagonist-bound hV2R—The initial three-dimensional structure of the transmembrane helices of the hV2R was established on the basis of the three-dimensional structure of bovine rhodopsin (15). The construction of the complete hV2R model has been described previously (9,16). The energetically preferred conformations for SR49059 and SR121463 were selected from searches of their conformational spaces by the random search module in the Sybyl 6.9 package (TRIPOS Inc., St. Louis, MO). The starting orientation for docking of the V1R-specific antagonist SR49059 to the hV2R model was compared to the reported SR49059-bound V1R model (17). In the case of SR121463, the initial orientation was derived after superposition of the common ring systems of SR121463 and SR49059. After minimization of the starting complex using the AMBER 5.0 force field (18), molecular dynamics simulations were performed at 300 K for 500 ps, where only hydrogen bonds of the TM backbones maintaining the TM helices were restrained. Low energy conformations of the last 50 ps were compared.

RESULTS

Investigated Wild-type and Mutant hV2Rs and V1R-GFP Fusion Proteins—To clarify the mechanisms of antagonist-promoted restoration of cell surface expression, we investigated the predominantly ER-retained mV2R (9). In addition, eight NDI-causing hV2R mutants (Fig. 1, gray boxes) (19–23) and
several in vitro mutants (Fig. 1, white boxes) were included in this study. The mutants hW164R and hC319Y represent novel NDI-causing mutations. The mutant hW164R was identified in a patient with severe NDI, whereas patients with the mutant C319Y suffered from partial NDI, indicating that this mutant has retained residual activity. The in vitro mutant hD368K/S371X codes for a hV2R with an engineered dibasic ER-retrieval motif at the very C terminus of the hV2R. For all mutants with the exception of hD368K/S371X, plasmids were generated, which encode C-terminal GFP fusion proteins suitable for LSM of living cells and immunoblotting using GFP antibodies. In the case of the hD368K/S371X mutant, an N-terminally Myc epitope-tagged receptor was used (10).

Retention of V2R Mutants in the ER—HEK293 cells transiently expressing the wild-type mV2R or different mutant hV2Rs show little or no [3H]AVP binding (Fig. 2A). For mV2R, hK100D and hF328A binding of [3H]AVP ranged between 8 and 16% of that for the wild-type hV2R. For all other mutants, including the hD368K/S371X mutant, [3H]AVP binding was less than 3% of that of the wild-type hV2R. LSM of transiently transfected HEK293 cells revealed that wild-type mV2R-GFP and mutant hV2R-GFPs were predominantly located within the ER (Fig. 2B). The staining patterns were indistinguishable from that of an ER-targeted cyan fluorescent protein derivative (ER-CFP).

Membrane-permeable Receptor Antagonists Promote Cell Surface Delivery of the mV2R at a Post-translational Level—HEK293 cells transiently expressing the mV2R-GFP were treated with the non-peptide vasopressin V1 receptor (V1R)-antagonist SR49059 or the V1R-selective non-peptide antagonist SR121463B. Both antagonists (1 μM, 16 h) restored cell surface expression (Fig. 3A). In contrast, ETα and ETβ receptor-selective non-peptide antagonists BQ123 and BQ788 (both 10 μM for 16 h) had no effect on the subcellular distribution of the mV2R (Fig. 3A, panels d and e). Co-transfection of the mV2R-GFP with dominant-negative K44A dynamin did not increase the cell surface delivery of the mV2R-GFP, indicating that SR121463B- and SR49059-promoted cell surface expression was not caused by an inhibition of internalization. The antagonist effects were preserved when cells were preincubated with cycloheximide (20 μg/ml) for 30 min prior to the addition of antagonists or when puromycin (20 μg/ml) was used instead of cycloheximide (data not shown). These results were further confirmed in [3H]AVP binding experiments. Since only SR49059, but not SR121463B can be removed from the mV2R and hV2R by washing (see “Experimental Procedures”), the experiments were performed with SR49059. Cells were treated for up to 6 h with buffer, SR49059, cycloheximide or the combination of SR49059 and cycloheximide. Membranes of cells treated with cycloheximide alone or left untreated did not differ in [3H]AVP binding, whereas membranes of cells treated with SR49059 or with cycloheximide and SR49059 revealed a 3-fold increase in [3H]AVP binding (Fig. 3B). These results suggest that the ER-retained wild-type mV2R is not rapidly degraded, but remains in the ER for longer periods. Thus, the antagonists do not simply function by preventing degradation. Rather the antagonists promote the proper folding, e.g. increase conformational stability of the already synthesized mV2R retained in the ER.

To test whether SR49059 also promotes cell surface delivery in cells that express the V2R endogenously, we studied primary cultured rat IMCD (13). The V2Rs of rat and mouse are highly homologous and share the aspartate residue at position 100. Both receptors differ only by six amino acids (five in the extreme N terminus, one in the third intracellular loop). Treatment of rat IMCD cells with 1 μM SR49059 for 16 h increased specific [3H]AVP binding 2.5-fold (Fig. 3C), indicating that antagonists also promote cell surface expression of the endogenous rat V2R.

SR121463B Promotes Maturation of the Wild-type mV2R-GFP—In immunoblots with membrane preparations of HEK293 cells transiently expressing hV2R-GFP, bands at 55 and 70–75 kDa were observed (Fig. 4). The broad band at 70–75 kDa corresponds to the mature, complex glycosylated hV2R-GFP, whereas the band at 55 kDa represents the immature core-glycosylated hV2R-GFP (16). Like the hV2R-GFP, the mV2R-GFP yields bands at 75 and 55 kDa, representing the mature and immature receptor, respectively (see Fig. 4). The complex glycosylated mV2R-GFP (75-kDa band) was less abundant than the corresponding one of the hV2R-GFP. Precubation of cells with 1 μM SR121463B for 16 h prior to membrane preparation did not qualitatively change the hV2R-GFP and mV2R-GFP patterns; however, the intensity of the band representing the mature mV2R-GFP (75 kDa) was increased. These
results were confirmed in cell surface biotinylation assays. HEK293 cells transiently expressing wild-type and mutant V₂Rs were incubated with 10 nM [³H]AVP in the presence or absence of 10 μM AVP for 2 h at 4 °C. Specific binding was calculated and normalized to the binding of the wild-type hV₂R. The values are means ± S.D. of three independent experiments performed in triplicate. B, subcellular distribution of wild-type and mutant V₂R-GFP fusion proteins was analyzed by LSM. The figures are representative of at least five independently performed experiments, in which at least 30 different cells were analyzed, respectively. ER-CFP-ER-targeted CFP fusion protein.

**Quantitative LSM**—In displacement binding analysis with [³H]AVP we found that the affinity of the mV₂R for SR49059 was about 30-fold lower than for SR121463B (Kᵗ values of the mV₂R for SR49059 and SR121463B were 618 ± 317 nM and 17.2 ± 6.7 nM, respectively). In order to compare the potencies and time courses of both molecules to promote cell surface expression of the mV₂R, we analyzed transiently transfected HEK293 cells for plasma membrane delivery of the mV₂R-GFP in the presence of SR121463B or SR49059 for up to 16 h and calculated the ratio of specific binding. Both antagonists promoted cell surface delivery to an almost identical extent (6–7-fold), but revealed differences in their half-times (tᵢ₅₀) for maximal increase in cell surface delivery (tᵢ₅₀ of SR121463- and SR49059-promoted cell surface delivery were 4.95 ± 0.11 h and 6.48 ± 0.14 h, Fig. 5A). In concentration response analyses we found that the half-maximal concentrations (EC₅₀) required for SR121463B- and SR49059-promoted cell surface delivery of the mV₂R were 22.6 ± 6.4 nM and 382 ± 73 nM, respectively. It is of note that the EC₅₀ values were very similar to the Kᵗ values of both antagonists. To validate the data from quantitative LSM, we also performed [³H]AVP binding experiments. Incubation of HEK293 cells transiently expressing the mV₂R-GFP with SR49059 for up to 16 h resulted in a 10-fold increase in [³H]AVP binding sites (Fig. 5B). Interestingly, the half-time for SR49059-mediated increase in [³H]AVP binding was about 9.5 h, which was significantly slower than that observed in quantitative LSM (compare with Fig. 5A). The reason for this difference is not known. One explanation could be that LSM does not allow us to distinguish between mV₂R-containing vesicles in close proximity to the plasma membrane (~200 nm) and mV₂Rs in the plasma membrane. Alternatively, not all receptors inserted into the plasma membrane are fully functional, but could require other protein or lipid contacts, which are established more slowly.

**Oligomerization Is Not Sufficient for the Exit of the mV₂R from the ER**—The hV₂R and the hV₁aR form dimers/oligomers within the ER. It has been suggested that oligomerization of vasopressin receptors is essential for the transport to the Golgi apparatus (24). Because ER retention of the mV₂R could be caused by a lack of oligomerization, we analyzed the oligomeric state of mV₂Rs retained in the ER and expressed at the cell surface after treatment with SR121463B by FRET experiments. In the first series of experiments, we studied HEK293
Fig. 3. Antagonist-promoted restoration of cell surface expression of the mV2R-GFP occurs at a co- and post-translational level. A, LSM of HEK293 cells transiently expressing the mV2R-GFP (a–c) or co-expressing the mV2R-GFP and K44A.dynamin 1 (f) were incubated for 16 h with 1 μM SR121463B (a), 1 μM SR49059 (b), 10 μM BQ233 (c), or 10 μM BQ788 (d) or buffer (e). In g, cells were incubated with cycloheximide (20 μg/ml) for 30 min prior to the incubation with SR121463B (1 μM) for up to 6 h. The plasma membrane was visualized by trypan blue. The figures are representative of at least three independently performed experiments, in which at least 30 different cells were analyzed, respectively. B, HEK293 cells transiently expressing the mV2R-GFP were left untreated or treated with cycloheximide (20 μg/ml), SR49059 (1 μM), or the combination of both for 6 h. Cells were then washed and subjected to [3H]AVP binding analysis at 4 °C. Shown are means of duplicates, which differed by less than 5%. The results are representative of three independent experiments. C, confluent primary cultured rat inner medullary collecting duct (IMCD) cells were incubated with SR49059 or buffer for 16 h and finally subjected to membrane preparation. Membranes were then analyzed in [3H]AVP binding experiments. Shown are mean values ± S.D. of quadruplicates of specifically bound [3H]AVP. The results are representative of three independent experiments.

Fig. 4. SR121463B promotes complex glycosylation and cell surface delivery of the mV2R-GFP. HEK293 cells transiently expressing hV2R-GFP and mV2R-GFP were incubated in the absence or presence of SR121463B (1 μM) for 16 h. A, crude membrane preparations (60 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose filters and probed with polyclonal anti-GFP and alkaline phosphatase-conjugated anti-rabbit antibodies as primary and secondary antibodies. B, following treatment of intact cells with Sulfo-NHS-biotin, biotinylated membrane proteins were detected in immunoblot analysis (for details see “Experimental Procedures”). The immunoblots are representative of at least three independently performed experiments. #, mature receptor; *, immature receptor.

SR121463B restores cell surface expression of mutant hV2R-GFPs—In further experiments we tested several mutant hV2R-GFPs for their ability to undergo antagonist-promoted cell surface delivery. In transiently transfected HEK293 cells both antagonists restored cell surface delivery of the mutants hK100D and hC319Y (Fig. 7). In case of the mutants hΔLAR62–64, hD136A, hS167T, hP322S, hW323H, and hF328A, cell surface delivery was only restored by SR121463B but not by SR49059. Similar to the mV2R, restoration of cell surface delivery was not abolished by cycloheximide (data not shown). In case of the mutants hL62P, hH80R, hW164R, and hS167L neither antagonist was capable of restoring cell surface delivery (Fig. 7). The LSM results were confirmed in immunoblot and cell surface biotinylation experiments (Fig. 8).

In cell surface binding experiments with SR49059-treated HEK293 cells transiently expressing the hC319Y mutant we observed only a small increase in [3H]AVP binding when compared with untreated controls (Table I). This small increase in [3H]AVP binding was unexpected, since SR49059 promoted the cell surface delivery of hC319Y to a similar extent as for mV2R (Table I). The increase in [3H]AVP binding was comparable to that of the hS167T or hD136A mutants, which showed no significant SR49059-mediated restoration of cell surface delivery in LSM and biotinylation experiments. Thus, it is likely that treatment with SR49059 yields transport-competent, but binding-defective hC319Y receptors.

Transport-competent Folding of hV2R Mutants Is Induced by Receptor-Antagonist Interaction—Cell surface delivery of the...
To gain insight into the interactions of the hV2R with the two antagonists and to understand the potential mechanisms underlying antagonist-promoted folding, we established a computer-assisted homology model of the SR121463B- and SR49059-bound hV2R. The three-dimensional structure of rhodopsin was used as a template (15), which was refined by molecular dynamics simulations. Since SR49059 and SR121463B share a common structural core, consisting of an aryl-sulfonfyl-indoline ring-system (Fig. 10A), we started with an orientation of SR121463B that was similar to that of SR49059 bound to the human vasopressin V1A receptor (hV1AR, Ref. 17). The aromatic interactions of the core ring system with Phe225 (TM V), Tyr296, Trp304, Phe307, Phe308 (all TM VI) found for SR49059 in the hV1AR also apply for SR121463B and hV2R. Here, the aryl-sulfonfyl-indoline core of SR121463B interacts with the corresponding residues Phe314 (TM V) and Tyr326, Trp324, Phe287, and Phe288 (all TM VI) in the hV2R. The additional morpholinoethero-cyclohexane group of SR121463B most likely projects between TM II and TM VII and interacts mainly with the side chains of phenylalanine 307 (cyclohexane group) and valine 308 (morpholinoethero group) of TM VII (Fig. 10B and Supplement 1). These interactions could also contribute to the V2R-selectivity of SR121463B since the corresponding amino acids isoleucine 330 and threonine 331 in the hV2R can only weakly interact, if at all with the cyclohexane and morpholinoethero groups.

The hV2R mutants, which can be rescued by SR121463 only harbor amino acid replacements which cluster at the C-terminal part of both TM I and TM VII, whereas in the mutants lacking antagonist-mediated rescue, amino acid replacements are found mainly at the interface of TM II and TM IV (Fig. 10B and Supplement 1).

**DISCUSSION**

In this study we show that the wild-type mV2R, which is predominately retained in the ER, reveals an almost complete restoration of cell surface delivery in the presence of the antagonists SR121463B or SR49059. However, antagonist treatment fails to increase cell surface delivery of the hD368/S371X mutant, which is most likely properly folded but retained in the ER via a dibasic retention signal at the very C terminus (25). In a previous study we have shown that aspartate 100 in the mV2R confers a low conformational stability, whereas the exchange of aspartate by lysine, found at the corresponding position in the mainly cell surface expressed hV2R increases conformational stability. The role of lysine 100 for proper folding may be explained by its participation in a hydrogen bond network formed between the side chains of Lys100 (TM II), Gln40 (TM I), and Asp191 (2nd extracellular loop, Ref. 9). With an acidic aspartate at position 100 in the mV2R, this hydrogen bond network cannot be established. Instead a repulsion of Asp191, Gln40, and Gln100 (2nd extracellular loop, Ref. 9) is likely. As a result the free energy between the properly folded, native, and the improperly folded, non-native states could be at a similar level, so that the mV2R switches between the different states. Both, substitution of aspartate 100 by lysine (mD100K, Ref. 9) or binding of an antagonist (this work) may enhance conformational stability of the mV2R, resulting in the exit from the ER. The fact that the EC50 values for antagonist-promoted cell surface delivery of the mV2R were similar to their Kd values, is strong evidence for the notion that the antagonists interact directly with the ER-retained receptor via the binding cleft. In addition, the data obtained with cycloheximide-treated cells show that antagonists act on a post-translational level, suggesting that antagonists are unlikely to serve as folding templates for nascent proteins preventing misfolding.

For hV2R mutants, variable effects of antagonists on cell surface delivery were found. In the hV2R mutants studied, cell
surface delivery was restored with the antagonists SR49059 and SR121463B (hK100D, C319Y) or only with SR121463B (hH80R, LAR62–64, S167T, P322S, W323H, F328A). In addition, some hV2R mutants did not respond to either antagonist. In the hV2R mutants, which do not undergo antagonist-mediated cell surface expression (hH80R, W164R, S167L), the amino acid replacements affect residues, which are of major structural importance for receptor folding. For example, tryptophan 164 in TM IV is the most conserved residue among class A GPCR, and at position 167 in TM IV, only small amino acids, such as alanine, serine or cysteine are found (26). In agreement with these structural considerations, the introduction of a methyl group at position 167 (hS167T) causes only a slight distortion of the interaction with the neighboring valine 121 (TM III) and allow rescue, whereas the insertion of an isopropyl group (hS167L) results in a severe disturbance of receptor folding, and a rescue is not possible. Similarly, replacement of highly conserved tryptophan 164 may lead to a severe folding defect.

The mutant hV2R's which show cell surface delivery only in response to SR121463B (hΔLAR62–64, S167T, P322S, W323H, F328A), point to structural properties of the antagonists, which are of crucial importance for the rescue activity. These mutants form a class characterized by amino acid exchanges at the C-terminal ends of TM I and TM VII (Fig. 10B). The amino acid replacements most likely cause a slightly altered arrangement of the transmembrane helices, which results in ER retention. Although the substitutions are distant from the ligand binding site, SR121463B could promote folding by its extended morpholinoethoxy group. The latter most likely binds between TM II and TM VII of the hV2R and via side chains interactions with TM II and TM VII forces a proper rearrangement of the transmembrane helices either pushing or attracting TM I, TM II, and TM VII on the extracellular side. As a consequence, the transmembrane helices are forced together on the intracellular side and slightly disturbed helix-helix interactions are corrected. Consequently, SR49059, which lacks the extended morpholinoethoxy group, does not restore cell surface delivery of these mutants. It is unlikely, that the mutants lack affinity for SR49059, since the hF328A mutant, a small fraction of which is expressed at the cell surface in

**FIG. 6. FRET analysis of HEK293 cells transiently expressing mV2R-CFP and mV2R-YFP.** A, HEK293 cells transiently co-expressing mV2R-CFP and mV2R-YFP were incubated without or with 1 μM SR121463B for 16 h. Cells were then analyzed by combined excitation and emission fingerprinting (LSM510 META). Shown are images of CFP and YFP emissions and calculated FRET images. The figures are representative for at least five independently performed experiments, in which at least 20 different cells were analyzed, respectively. Bars, 5 μm. HEK293 cells transiently co-expressing mV2R-CFP and mV2R-YFP were incubated without (I) or with (II) 1 μM SR121463B for 16 h. Following a baseline recording of CFP (I) and YFP (II), YFP was selectively photobleached at 510 nm. IIIa, linear regression analysis of donor recovery (ΔFCFP) versus fractional acceptor (FYFP) photobleach. The respective calculated molar ratios of YFP:CFP were 0.95 in the absence and 1.1 in the presence of SR121463B. III b, Shown are means ± S.D. of FRET efficiencies determined in at least 35 individual cells. The difference between the FRET values in the absence (B) and presence of SR121463B (C) are significant as determined by Student’s t test (p < 0.01).
untreated cells, exhibits the same affinity to SR49059 as the wild-type hV₂R. Evidence that SR49059 interacts with the hF328A mutant even in its ER-retained form is derived from the fact that SR49059 promotes transport of the hF328A mutant from the ER to the ERGIC in bafilomycin A1-treated cells. Thus, the SR49059-bound hF328A mutant adopts a conformation, which can exit the ER, but underlies ER retrieval. The results also show that the ERGIC functions as a further site of...
quality control for proteins, which exit the ER, but have not established the native conformation.2

Analysis of functional activity following treatment with antagonists could only be performed for the mV2R and the hC319Y mutant, since only these V2Rs showed cell surface expression with SR49059, which in contrast to SR121463B can be removed by washing. While SR49059-promoted cell surface delivery of the mV2R was paralleled by a strong increase in [3H]AVP binding, this was not the case for the hC319Y mutant. Here, SR49059 yielded a mainly binding-defective receptor population. Thus, it is likely that some hV2Rs mutants not only display a reduced conformational stability, but also a functional impairment. Similar results have been described for the hV2R mutant hR202C. This mutant is delivered to the cell surface to the same extent as the wild-type hV2R, but only a small fraction of these receptors (~5%) revealed functional activity (27). In the case of the ER-retained hV2R mutant hΔV278, SR121463B restored cell surface transport. However, the SR121463B-bound hΔV278 does not show complex glycosylation and is targeted to the apical instead of the basolateral plasma membrane (28). Similar observations have also been made for other mutant proteins, for which small molecule-assisted folding was demonstrated. For example, small, membrane-permeable peptidic and non-peptidic ligands increased the conformational stability of several p53 mutants, finally resulting in a restoration of DNA binding activity (29,30). However, the stabilized mutant p53 proteins did not inhibit growth (as does the wild-type p53) and restored apoptosis only partially (30).

While the investigated antagonists can enhance conformational stability of mutant hV2Rs, thereby enabling their exit from the ER, they fail to restore complete functional activity. Thus, further studies have to focus on the identification of pharmacochaperones, which not only restore cell surface delivery, but also functional activity.

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Pharmacochaperones Post-translationally Enhance Cell Surface Expression by Increasing Conformational Stability of Wild-type and Mutant Vasopressin V$_2$ Receptors

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