Mechanisms of Cell-surface Rerouting of an Endoplasmic Reticulum-retained Mutant of the Vasopressin V1b/V3 Receptor by a Pharmacological Chaperone*

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Cell-surface expression and biological functions of several intracellular-retained G protein-coupled receptors are restored by membrane-permeable ligands called pharmacological chaperones. We have previously demonstrated that a mutation of the hydrophobic motif341FNXLLX350 in the C terminus of the human pituitary vasopressin V3 receptor (MUT V3R) led to it being retained in the endoplasmic reticulum (ER). Here, we establish the precise role of this motif and investigate whether SSR149415, a non-peptide V3R antagonist, behaves as a pharmacological chaperone for the ER-retained MUT V3R. The absence of the mutated receptor in the plasma membrane is linked to its prolonged association with the molecular chaperone calnexin in the ER and to its intensive degradation by the ubiquitin-proteasomal machinery. However, this is not because of a lack of oligomerization, as demonstrated by the presence of MUT V3R homodimers in the ER. Treatment with SSR149415 restores expression of the mutated receptor on the cell surface and its correct maturation, resulting into the functional recovery of its signaling properties. SSR149415 acts by stabilizing a native-like conformation of the V3R, reducing its association with calnexin and, thus, favoring a secretory pathway rather than the proteasomal degradation pathway. In conclusion, the FNXLL motif in the C terminus of the protein is an important motif for the V3R conformation, and the misfolding resulting from its mutation alters the receptor export but can be reverted by SSR149415.

The physiological actions of vasopressin (AVP) are mediated by three distinct seven-transmembrane domain G protein-coupled receptor (GPCR) subtypes, V1aR, V2R, and V1b/V3R (1–3). The V2 receptor has been extensively studied and mediates the antidiuretic effects of AVP on corticotropin-releasing hormone-induced adrenocorticotropic hormone secretion (9) and in the regulation of the stress response, as recently demonstrated by the receptor knock-out (10, 11). To date, no natural mutation of this receptor has been associated with disease, but its structure-function relationships have been investigated using site-directed mutagenesis (12, 13). The mutation of the hydrophobic motif341FNXLLX350 in the proximal C terminus of the human V3R prevents its export from the ER to the cell surface (13). However, the precise role of this motif in folding, oligomerization, or its direct interaction with the export machinery is unknown.

The transport from the ER of newly synthesized proteins is strictly regulated by a conformation-based process called quality control (QC) (14, 15). During QC, different molecular chaperones transiently interact with the nascent polypeptide chain, thus ensuring its proper conformation. Properly folded proteins are directed to the secretory pathway via the export machinery. Misfolded proteins remain associated with the ER, and are finally degraded by the proteasome after ubiquitination (16–18). This has been well documented for the δ opioid receptor (19). For example, calnexin, an ER transmembrane lectin chaperone, interacts with the cystic fibrosis transmembrane conductance regulator and assists in the maturation and folding of membrane-bound glycoproteins, including several GPCRs, such as V2R (20), thyrotropin receptor (21), luteinizing hormone receptor, and follicle-stimulating hormone receptor (22). Moreover, calnexin interacts for longer durations with mutants of the V2R, which leads to their retention in the ER (20).

Correcting these misfolding defects is a major therapeutic challenge in many genetic diseases that involve the ER retention of mutated proteins. A recent promising approach is the discovery of small membrane-permeable molecules called pharmacological chaperones that bind to the misfolded protein and allow it to be correctly addressed (23–25). For example, 11-cis-7-ring retinal, a rhodopsin inverse agonist, and SR121463A, a V2R antagonist, have been shown to restore the cell-surface expression and biological functions of the P23H-opsin mutant involved in retinitis pigmentosa and of several V2R mutants involved in NDI (26, 27). However, it is not known whether these pharmacological chaperones stabilize the newly synthesized protein, bypass the QC process, or prevent degradation of the protein. It is also possible that they promote GPCR oligomerization, which has been recently identified for the vasopressin receptors (28) and is thought to be important in GPCR export (29).

Here, we have examined the effects of SSR149415, the first, selective, non-peptide vasopressin V3R antagonist (30), on the previously described ER-retained V3R mutant (13). We show that SSR149415 behaves as a pharmacological chaperone and rescues maturation,
cell-surface expression, and the signaling functions of the mutant. This is due to shorter duration interactions with the QC chaperone calnexin and reduced proteasomal degradation without modifying oligomerization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture reagents were obtained from Sigma, BD Biosciences, Hyclone (Fetalclone III) or Invitrogen. Enzymes for molecular cloning were purchased from New England Biolabs. Radioactive vasopressin, [3H]AVP (60–70 Ci/mmol), was obtained from PerkinElmer Life Sciences, and Arp2-vasopressin (AVP) was obtained from Bachem. Myo-[2-3H]inositol with PT6–271 (16 Ci/mmol) and PRO-MIX™ L-35S in vitro cell labeling mix (1000 Ci/mmol) were obtained from Amersham Biosciences. TOPRO-3 iodide and Coelenterazine h substrate were purchased from Molecular Probes. We used the following antibodies: mouse anti-c-Myc (9E10), mouse anti-ubiquitin (P4D1) (Santa Cruz Biotechnology), mouse anti-adaptin-γ, phycoerythrin-conjugated anti-mouse IgG (BD Biosciences), rabbit anti-calnexin, peroxidase-conjugated anti-rabbit IgG, peroxidase-conjugated anti-mouse IgG (Sigma), Alexa Fluor 594-conjugated anti-mouse IgG, Alexa Fluor 546-conjugated anti-rabbit IgG, Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes), and rabbit anti-calnexin (StressGen).

**DNA Constructs**—Human V3 wild-type (WT) (341ENXLLXJ350) and mutated (MUT) (341TTLXJ350) receptor sequences were inserted into the pcDNA3 vector (Invitrogen) and the ps-6Myc vector anti-calnexin (StressGen).

**Fluor 488-conjugated anti-rabbit IgG (Molecular Probes), and rabbit dase-conjugated anti-mouse IgG (Sigma), Alexa Fluor 594-conjugated**

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**To construct the pV3-EGFP vector, the entire coding sequence of the wild-type V3R was amplified by PCR using 2 primers, which deleted the stop codon and introduced HindIII and KpnI sites at the 5’ and 3’ ends of the fragment. This HindIII/KpnI PCR fragment was subcloned into the polylinker of pEGFP-N1 (Clontech).**

**The expression vectors coding for WT V3R and MUT V3R with Myc epitopes at their N terminus and the EGFP tag at their C terminus were called ps-6Myc-WT-V3R-EGFP and ps-6Myc-MUT-V3R-EGFP. For their construction, the starting material was the previously described ps-6Myc-EGFP-WT-V3R (13), which was first deleted from the EGFP sequence using restriction enzymes and a linker. From this intermediate construction, a 1.1-kilobase NdeI/PstI fragment containing the cytomegalovirus promoter, the signal peptide (ps), the Myc epitopes (6Myc), and 350 bp of the V3R coding sequence, was used to substitute the homologous 730-bp NdeI/PstI fragment in the phV3-EGFP vector. The ps-6Myc-MUT-V3R-EGFP was obtained by substituting the 571 bp of EcoN1/SgrA1 wild-type V3R fragment by the mutated one in the ps-6Myc-WT-V3R-EGFP construct. **

**EYFP and RLuc tagged WT V3R constructions, WT-V3R-EYFP and WT-V3R-RLuc, were obtained after digestion of the phV3-EGFP vector with HindIII/KpnI or HindIII/BamHI and subcloning of the WT V3R insert into the polylinker of pEYFP-N1 (Clontech) or hpRLuc-N3 (Bio-signal Packard), respectively. From these two wild-type constructs, the corresponding mutant vectors MUT-V3R-EYFP and MUT-V3R-RLuc were obtained by substitution of a 1.7-kilobase NdeI/KpnI fragment containing the cytomegalovirus promoter and the entire V3R coding sequence by a corresponding 2-kilobase fragment of the ps-6Myc-MUT-V3R-EGFP construct. The ps-6Myc sequence was then deleted from these intermediate constructions using the QuikChange site-directed mutagenesis kit (Stratagene). The Myc-GABARR1-RLuc was a gift from Dr. Marullo and has been previously described (29).**

**Cell Culture and Transfection**—Human embryonic kidney HEK-293 cells (ATCC, F-14742, 1573-CRL) were cultured (37 °C, 5% CO2) in minimum Eagle’s medium supplemented with 7.5% fetal calf serum, 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mouse corticotropin AT20 cells (ATCC, CRL-1795) were cultured (37 °C, 5% CO2) in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 7.5% Fetalclone III, 7.5% Nu-serum, and 0.5 mM glutamine. HEK-293 cells were transiently transfected using the FuGENE reagent (Roche Applied Science) and the Effecten reagent (Invitrogen), and AtT20 cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The cells were pretreated with the non-peptide V3R antagonist SR149415 (a gift from Dr. Serradeil-Le Gal of Sanofi Research, 31036 Toulouse Cedex, France) by incubation for 24 h or as indicated in the figure legends. All analyses were carried out 48 h after transfection.

**Fluorescence-activated Cell Sorting (FACS)**—HEK-293 cells were transiently transfected with either the ps-6Myc-WT-V3R-EGFP or the ps-6Myc-MUT-V3R-EGFP constructs, incubated with primary monoclonal anti-c-Myc and secondary phycoerythrin-conjugated anti-mouse antibodies, and analyzed by FACS using a BD Biosciences FACScan flow cytometer, as previously described (13).

**Functional Characterization of V3 Receptor**—We carried out saturation binding experiments on transiently transfected HEK-293 cells using [3H]AVP as a ligand, as previously described (13). We measured vasopressin-induced inositol phosphates (IP) production in transiently transfected HEK-293 cells as previously described (31). Cells were incubated with AVP and 10 mM LiCl.

**Immunofluorescence Confocal Microscopy**—We carried out selective labeling of cell-surface receptors and internal compartment markers using the appropriate primary and secondary antibodies on fixed and transiently transfected HEK-293 and AtT20 cells as previously described (13). Fluorescence was detected using a LEICA TCS SP2 AOBS confocal microscope.

**Immunoprecipitation and Western Blot Analysis**—We carried out immunoprecipitation and Western blot analysis of the WT V3R and MUT V3R on transiently transfected HEK-293 cells as described previously (13). Western blots of calnexin and ubiquitin were carried out according to similar procedures using a polyclonal anti-calnexin antibody (1:2000 dilution) or a monoclonal anti-ubiquitin antibody (1:200 dilution) incubated at 4 °C overnight.

**Protein Metabolic Labeling and Immunoprecipitation**—HEK-293 cells were transfected in 60-mm dishes (Fig. 3) or 6-well dishes (Fig. 8). After 48 h, the transient transfectants were starved for 1 h in methionine- and cysteine-free Ham’s F-12 medium (Invitrogen) and then labeled with 50 μCi/ml (Fig. 3) or 100 μCi/ml (Fig. 8) of PRO-MIX™ L-35S in vitro cell labeling mix in the same medium. After pulse incubation at 37 °C for 30 min (Fig. 3) or 15 min (Fig. 8), the cells were washed twice in phosphate-buffered saline and chased for various periods of time in minimum Eagle’s medium supplemented with 7.5% fetal calf serum and 0.5 mM glutamine at 37 °C and then finally rinsed once in phosphate-buffered saline. The total pool of V3 receptors was immunoprecipitated as described above. V3 receptors interacting with calnexin were sequentially immunoprecipitated first by a polyclonal anti-calnexin antibody and protein G-Sepharose beads (12 h) and then by a monoclonal anti-c-Myc antibody and protein A-Sepharose beads (12 h). The immunopurified receptors were then resolved on 7.5% SDS-PAGE and exposed to Biomax film at −80 °C.

**Bioluminescence Resonance Energy Transfer (BRET) Assays**—HEK-293 cells were washed twice with phosphate-buffered saline and trypsinized 48 h after transfection in 6-well dishes. The cells were then centrifuged and resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and distributed in a 96-well microplate.
Coelenterazine h substrate (Molecular Probes) was added at a final concentration of 5 µM, and readings were taken 20 min later using a lumino/fluorometer (FusionTM, Packard Instrument Co.) that allows the luminescence and fluorescence signals to be detected and sequentially integrated using two filter settings (RLuc filter, 485 ± 10 nm; EYFP filter, 530 ± 12.5 nm). The BRET ratio was defined as the difference between the emission ratio at 530 and 485 nm of the co-transfected RLuc and EYFP fusion proteins and the emission ratio at 530 and 485 nm of the RLuc fusion protein expressed alone. The results were expressed in milliBRET units, 1 milliBRET corresponding to the BRET ratio values multiplied by 1000. We also determined total fluorescence and luminescence signals for all samples to assess the level of expression of the EYFP and RLuc fusion constructs and to ensure that the ratios of receptor-EYFP/receptor-RLuc remained constant. For BRET titration experiments, cells were transfected with different amounts of WT-V3R-EYFP for a given quantity of WT-V3R-RLuc or Myc-GABAB1-RLuc.

**Statistical Analysis**—Values are expressed as the means (±S.D.). We used analysis of variance to analyze differences between groups. When significant differences were detected by analysis of variance, a posteriori comparisons between means were conducted using the Fisher least significant difference (LSD) test (α = 0.05). Calculations were carried out using the StatView program. Curves were fitted with the Prism program (Graph Pad Software).

**RESULTS**

**Functional Characterization of the WT V3R and MUT V3R**—We carried out a comparative functional characterization of the WT V3 receptor and a mutated V3R in a proximal C-terminal hydrophobic motif (341FNQXXLXL350) replaced by 341TTX2TT2X350) in HEK-293 cells. This mutant (MUT V3R) was previously shown not to be targeted to the cell surface but to be retained in the ER (13). We introduced Myc epitopes at the N terminus of the two human V3 receptor coding sequences to allow us to detect and immunopurify the receptor proteins. The addition of an EGFP tag to the C terminus of the previous constructs helped us to quantify both receptor expression and cell-surface targeting by flow cytometry. The C-terminal position of the EGFP tag considerably improved the level of V3R cell-surface expression compared with the N-terminal position (13). As expected, the affinities of the untagged, Myc-tagged, and Myc-EGFP-tagged WT V3R for [3H]AVP and their level of expression at the plasma membrane were similar and tag-independent (Kd = 3.9 nM ± 0.57, 3.5 nM ± 0.62, and 3.8 nM ± 0.73, respectively) (Fig. 1A). All WT receptors equally stimulated IP production (Fig. 1B). We found that the mutant receptor was unable to bind AVP at the cell surface independent of the presence of the tag and that this agonist did not promote IP production (Fig. 1A and B). We used immunodetection to demonstrate the lack of cell-surface expression of the mutant MUT V3R and its sequestration in the endoplasmic reticulum in HEK-293 cells (Fig. 1C), thus confirming previous data (13).

**SSR149415 Treatment Promotes Cell-surface Targeting of the MUT V3R**—HEK-293 cells transiently expressing the double-Myc-EGFP-tagged-WT V3R or -MUT V3R were treated or not for 24 h with SSR149415, a selective, membrane-permeant V3R antagonist. We then quantified changes in cell-surface targeting by flow cytometry using the ratio of Myc fluorescence (cell-surface expression) to EGFP fluorescence (total expression). We observed that treatment with SSR149415 for 24 h restored the level of cell-surface expression of the MUT V3R to that observed for the WT V3R (Fig. 2A). The half-maximal effective concentration (EC50) of this effect was obtained for 1.2 × 10−6 M SSR149415 (Fig. 2B). Moreover, SSR149415 slightly increased the export of WT V3R (18.8%) compared with the corresponding untreated cells (Fig. 2A). We observed similar results in AtT20 corticotroph cells using immunofluorescence analysis. In the absence of SSR149415, the MUT V3R was totally retained in the ER and was absent from the trans-Golgi network (Fig. 2C, upper panels) and the plasma membrane (Fig. 2D, upper panels). In the presence of SSR149415, the intracellular-sequestrated MUT V3R was less retained in the ER, was processed through the secretory pathway, as shown by its presence in the trans-Golgi network (Fig. 2C, lower panels), and finally appeared at the cell surface (Fig. 2D, lower panels).

**SSR149415 Treatment Promotes the Correct Maturation of the MUT V3R**—We used pulse-chase metabolic labeling experiments in HEK-293 cells expressing the Myc-tagged WT V3R or MUT V3R to assess the role of SSR149415 on receptor processing and maturation. The WT V3R was synthesized as a 60-kDa receptor that was processed to form a
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FIGURE 3. SSR149415 promotes cell-surface targeting of the V3R. A, cell-surface receptor expression was measured by flow cytometry analysis of HEK-293 cells transiently expressing either Myc-EGFP-tagged-WT V3R (WT) or mutant Myc-EGFP-tagged-MUT V3R (MUT). Cells were treated or not with 10^{-5} M SSR149415 for 24 h. Total receptor levels were evaluated by measuring EGFP fluorescence, and surface receptor levels were evaluated indirectly by measuring immunofluorescence of the extracellular Myc epitopes. The ratio was expressed as arbitrary units. Values are the means (±S.D.) of three different experiments. ***, p < 0.0001 (LSD test). B, dose-dependent effects of a 24-h SSR149415 treatment on the expression of WT V3R (squares) and MUT V3R (circles) on the cell surface was measured as described in A, C, merged images of permeabilized AtT20 cells transiently expressing the mutant Myc-EGFP-tagged-MUT V3R (MUT) untreated (upper panels) or treated with 10^{-5} M SSR149415 for 24 h (lower panels) and the ER-specific marker, calnexin (left panels), and the trans-Golgi network marker, adaptin (right panels) (red fluorescence). D, EGFP fluorescence (left panels) and cell-surface staining with anti-c-Myc antibodies (right panels) of non-permeabilized AtT20 cells transiently expressing the mutant Myc-EGFP-tagged-MUT V3R (MUT) untreated (upper panels) or treated with 10^{-5} M SSR149415 for 24 h (lower panels).

70-kDa receptor (Fig. 3A). Previous experiments have shown that the fast migrating band is sensitive to both endoglycosidase H and peptide N-glycosidase F and, therefore, corresponds to an immature receptor, whereas the slower migrating band is resistant to endoglycosidase H and sensitive to peptide N-glycosidase F and, therefore, corresponds to a complex-glycosylated mature receptor (13). Treatment with SSR149415 enhanced the efficiency and the speed of the WT V3R processing, as shown by the faster accumulation of the mature form and the faster disappearance of the immature form for similar amounts of loaded proteins (Fig. 3B). In the absence of SSR149415, the MUT V3R revealed only a 60-kDa band corresponding to the immature protein and accumulated longer (t1/2 of 59 min) compared with the WT V3R (t1/2 of 27 min) (Fig. 3C). In the presence of SSR149415, the MUT V3R matured to reveal a 70-kDa band, with the band from the immature 60-kDa form disappearing at a rate similar to the untreated WT V3R (compare Fig. 3, A and D). Altogether, these results suggest that SSR149415 reroutes the MUT V3R from a degradative to a secretory pathway.

SSR149415 Treatment Restores the Signaling Properties of the MUT V3R—We then investigated whether SSR149415 also restores the signaling properties of the MUT V3R in addition to the rescue of its targeting to the plasma membrane. We pretreated HEK-293 cells transiently expressing the untagged-WT V3R or -MUT V3R with SSR149415 for 24 h. The cells were then washed to remove the antagonist and stimulated with AVP. AVP (1 μM) was able to stimulate the production of IP via the WT V3 receptor with the same efficacy in the presence or the absence of SSR149415 (Fig. 4A). However, the potency of AVP stimulation was greater in the absence of SSR149415 (EC50 = 1.5 × 10^{-9} M) than in its presence (EC50 = 4.9 × 10^{-8} M), indicating competition between AVP and the antagonist (Fig. 4C). This competition was confirmed in [3H]AVP binding assays (data not shown). AVP (1 μM) was able to stimulate IP production via the MUT V3R only after pretreatment with SSR149415 (Fig. 4B). In the presence of SSR149415, the potency of AVP for the IP response was the same for the MUT V3R (EC50 = 3.6 × 10^{-8} M) and WT V3R (Fig. 4C), indicating that SSR149415 fully restores the signaling properties of the mutant recep-
tor. The half maximal concentration of SSR149415 for this was 3.9 × 10^{-7} M (Fig. 4D), which was comparable with that necessary for cell-surface expression of the mutant (Fig. 2B).

Taken together, these data indicate that SSR149415 behaves as a pharmacological chaperone, restoring cell-surface expression and cor-
recting the maturation and signaling properties of the likely misfolded human MUT V3R. This suggests a conformational role for the FN(X)_2L(X)_L motif. We next analyzed whether SSR149415 either promotes the oligomerization, prevents the degradation, or stabilizes the mutant V3 receptor.

Constitutive Oligomerization of the V3R Is Unaffected by SSR149415 Treatment—Evidence suggests that GPCR oligomerization takes place in the ER and is a determinant process for targeting receptors to the cell surface (32). Constitutive homodimerization and heterodimerization has been demonstrated for the V1a and the V2 receptors (28) but not yet for the vasopressin V3 receptor. Therefore, we studied the possible oligomerization of V3R in living cells using the BRET assay. We observed a large BRET signal for the WT V3R, which we did not observe between V3R and an unrelated GPCR (GABABR1) (Fig. 5A). This specific signal demonstrates that V3R exists as homodimers. We also observed the same BRET signal for the MUT V3R, indicating that oligomerization of this receptor occurs in the ER and that the mutation does not cause an oligomerization defect (Fig. 5A). We also found that SSR149415 significantly increased the BRET signal of the WT V3R (×1.7) in a dose-dependent manner (EC_{50} = 1.6 × 10^{-7} m) (Fig. 5B). By contrast, SSR149415 did not modify the BRET signal for the MUT V3R except at the highest dose (10 μM), which means that its role at rescuing the mutant receptor targeting to the plasma membrane is not linked to oligomerization. We then produced BRET saturation curves to investigate whether SSR149415 modifies the affinity of one monomer to another and, therefore, the ratio between dimers and monomers or whether it modifies the conformation of preexisting dimers. The curves allowed us to determine the maximal BRET signal and the half-maximal BRET signal (BRET_{50}), which gives a measure of the affinity of the partners for each other. For the WT V3R, SSR149415 increased the maximal BRET signal without affecting the BRET_{50} signal (17.5 ± 1.3 without SSR149415 and 13.5 ± 0.8 with SSR149415) (Fig. 5C). We observed the same BRET_{50} signal for the MUT V3R (BRET_{50} = 19.0 ± 1.9 without SSR149415 and 12.0 ± 1.0 with SSR149415). This indicates that SSR149415 affects the dimer conformation rather than the oligomerization process itself.

SSR149415 Treatment Prevents Proteasomal Degradation of the V3R—After dissociation from the ER membrane, misfolded proteins are degraded by the ubiquitin-proteasomal system (18). Therefore, we studied whether this mechanism is involved in V3R maturation and is regulated by SSR149415. By measuring EGFP fluorescence using FACS, we found that SSR149415 treatment significantly increased the total expression of WT V3R and MUT V3R (49 and 41%, respectively) (Fig. 6A) in a dose-dependent manner (EC_{50} = 7.8 × 10^{-8} m for the WT V3R) (Fig. 6B). Because it is unlikely that SSR149415 acts as a transcriptional activator, we suggest that SSR149415-V3R complexes escape the degradation pathway and accumulate in the ER and at the plasma membrane, leading to an increase in level of the receptor. We next examined whether the WT V3R and MUT V3R are targeted to the ubiquitin-proteasomal system by estimating the incorporation of ubiquitin into immunoprecipitated Myc-tagged V3R. As expected, the WT V3R was extensively ubiquitinated, as demonstrated by the presence of a smear (Fig. 6C, left panel). We found that the MUT V3R was more strongly ubiquitinated, suggesting that it undergoes greater degradation. However, after a 24-h treatment with SSR149415, the ubiquitination of the WT V3R was completely suppressed. The same treatment also reduced the ubiquitination of the MUT V3R. These differences were not due to differences in the amounts of immunoprecipitated V3R, as seen in Fig. 6 (right panel). These data indicate that SSR149415 treatment prevents proteasomal degradation of the V3 receptor.

SSR149415 Treatment Reduces Interaction of the V3R with the Molecular Chaperone Calnexin—The intracellular retention of mutated proteins has often been linked to prolonged interaction of the mutant receptor with the molecular chaperones of the ER. Therefore, we immunoprecipitated Myc-tagged WT V3R and MUT V3R and detected the receptor-associated physiological chaperone calnexin with anti-calnexin antibodies to determine whether calnexin can interact with the...
FIGURE 5. SSR149415 effect on constitutive V3R oligomerization in living HEK-293 cells. A, BRET ratios were measured in HEK-293 cells transiently transfected with the indicated fusion proteins expressed at a constant receptor-EYFP/receptor-RLuc ratio. WT V3R and MUT V3R BRET ratios are significantly different from V3R/GABABR1 BRET ratios. ***, p < 0.0001 (LSD test). B, dose-dependent effect of SSR149415 on BRET ratio of WT V3R (squares) and MUT V3R (circles). Data are represented as % of BRET ratio in the absence of SSR149415. C, saturation curves with (white squares) and without (black squares) 10^{-5} M SSR149415 obtained in HEK-293 transiently transfected with 50 ng of WT-V3R-RLuc and increasing amounts of WT-V3R-EYFP from 30 to 750 ng of the plasmid. Saturation curves were obtained by plotting the BRET ratio as a function of the EYFP/RLuc ratio. As a specificity control, GABABR1-RLuc (50 ng) was also monitored with increasing quantities of WT-V3R-EYFP (30 to 750 ng) (black triangle). Values represent data from three different experiments performed in duplicate (± S.D.).

FIGURE 6. SSR149415 enhances V3R expression and prevents V3R proteasomal degradation in HEK cells. A, effect of 10^{-5} M SSR149415 on WT V3R and MUT V3R total expression measured by FACS in HEK-293 cells transiently expressing the Myc-EGFP-tagged-WT V3R (WT) and the mutant Myc-EGFP-tagged-MUT V3R (MUT). B, dose-dependent effect of SSR149415 on WT V3R (squares) and MUT V3R (circles) expression measured by FACS. Values are the means (± S.D.) of three different experiments. ***, p < 0.0001 (LSD test). C, effect of 10^{-5} M SSR149415 on ubiquitination of the Myc-tagged-WT V3R (WT) and the mutant Myc-tagged-MUT V3R (MUT) transiently transfected in HEK-293 cells. The incorporation of ubiquitin into immunoprecipitated Myc-tagged-V3R and total amount of receptor was assayed by probing the same membrane with anti-ubiquitin (left panel) and anti-c-Myc antibodies (right panel), respectively. The immature V3R (lower arrowhead) and the mature V3R (upper arrowhead) forms are shown. Nontransfected HEK-293 cells (HEK) were immunoprecipitated with anti-c-Myc antibody and used as a control. Representative experiment of three separate experiments.
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We have previously reported that mutations in the hydrophobic sequence FN(X)3LL(X)3L in the proximal C terminus of the V3 receptor result in this receptor being retained in the ER (13). However, it was not clear whether this motif is a targeting signal for ER export or is required for transport-competent folding of the receptor. In the present study, we analyzed the interactions of the ER-retained V3R mutant with the quality control machinery using a specific non-peptide antagonist of the V3R. This antagonist behaves as a pharmacological chaperone. These small molecules permeate the cell membranes and have been shown to bind several unstable misfolded receptors in the ER and to restore their maturation, cell-surface expression, and biological functions.

Evidence from the present study suggests that the mutant V3 receptor has a folding defect; it accumulates in a precursor form, interacts for longer durations with calnexin, and is more ubiquitinated compared with the wild-type receptor. During their biosynthesis in the ER, proteins interact with several molecular chaperones that ensure the correct folding of the molecule before being delivered to the export machinery. For example, calnexin, the ER lectin, interacts with monoglucosylated oligosaccharide structures, N-linked to the nascent glycoproteins. A number of glycoproteins, including several members of the GPCR family, such as the V2 (20), luteinizing hormone, and follicle-stimulating

FIGURE 7. SSR149415 reduces association of V3R with calnexin. A, HEK-293 cells expressing the Myc-tagged-WT V3R (WT) and the Myc-tagged-MUT V3R (MUT) were treated or not with 10−5 M SSR149415. The associated calnexin in immunoprecipitated Myc-tagged-V3R and the total amount of receptor were assessed by probing the same membrane with anti-Calnexin (upper panel) and anti-c-Myc antibodies (lower panel), respectively. The immature V3R (lower arrowhead) and the mature V3R (upper arrowhead) forms are shown. Calnexin is present in nontransfected HEK-293 cells (HEK) but not in nontransfected HEK-293 cells immunoprecipitated with anti-c-Myc antibody (IP HEK). B, graphical representation of the association of the immature forms of the WT V3R and the MUT V3R with calnexin quantified by densitometric analysis of the SDS-PAGE from A. The calnexin/immature V3R ratio was calculated, with 100% being the untreated calnexin/immature WT V3R ratio. Values are the means (± S.E.M.) of three different experiments. *** p < 0.0001 (LSD test).

FIGURE 8. SSR149415 modifies the kinetics of association of V3R with calnexin. A, metabolic labeling was carried out by incubation with 100 Ci/ml [35S]methionine/cysteine for 15 min followed by the indicated times of chase. Quantification was by densitometric analysis of the SDS-PAGE from A, with 100% being the signal determined at the time 0 for each receptor. The data were fitted to a one-phase exponential decay equation. Values are the means (± S.E.M.) of three different experiments.
The mutant V3 receptor is highly ubiquitinated, suggesting it undergoes ubiquitination, and then its degradation by the 26 S proteasome (16–18). the retro-translocation of the misfolded protein in the cytosol, its ubiquitination, and the ER-associated degradation pathway. This degradation is initiated by prolonged association with calnexin (20).

Therefore, we suggest that the mutated sequence of the V3 receptor may disrupt the association of the receptor dissociation with calnexin and decreases its subsequent degradation efficiency in the ER, which is a limiting step in their processing. It is possible that in some cases, interacting proteins such as the previously mentioned escort proteins could regulate this limiting step.

In conclusion, the non-peptide antagonist SSR149415 is a pharmacological chaperone for the vasopressin V3 receptor and is able to rescue the maturation, cell-surface expression, and signaling properties of a misfolded GPCR. The mechanism by which the pharmacological chaperone restores the expression of the V3 receptor on the cell surface is certainly linked to the stabilization of protein conformation. A three-dimensional molecular model of the interaction between the human V3R and SSR149415 has recently been described based on the structure of crystallized bovine rhodopsin (12). From this model, it has been proposed that the Thr-203 (TM5) and/or Met-324 (TM7) residues are crucial for the selectivity of the V3R/SSR149415 interaction with hydrogen bonding and hydrophobic interactions occurring with the antagonist. Therefore, SSR149415 may restore the correct rearrangement of the receptor transmembrane domains that are disrupted by the C-terminal mutations. SSR149415 accelerates the kinetics of the receptor dissociation with calnexin and decreases its subsequent degradation by the proteasome. The half-life of precursor is also reduced, and the mature protein appears. Therefore, a simple “general” stabilization can reroute the misfolded receptor from an ER-associated degradative pathway to a secretory pathway, which restores the receptor functions. Therefore, we suggest that the mutated sequence of the V3 receptor may only destabilize the general conformation of the receptor.

An alternative, but unlikely hypothesis is that the pharmacological chaperone modifies the conformation of the receptor and reveals a motif that is present in the wild-type receptor but is hidden in the mutant receptor. This motif would interact with a specific chaperone, either an escort protein or a protein of the export machinery. For example, RTP1 and RTP2 have been suggested to promote the correct folding of odorant receptors (38). Cyclophilin-related ninaA and RanBP2 have been shown to act as specific molecular chaperones for opsin (39, 40). Another possibility is that the pharmacological chaperone bypasses some of the quality control steps or avoids the necessity for the receptor to be associated to an escort protein.

Whatever mechanism the pharmacological chaperone uses to restore the expression of the receptor on the cell surface, our study shows for the first time that the mechanism does not involve modifying the oligomerization of the receptor. We unambiguously show using the BRET technique that, like the wild-type V3 receptor, the mutant receptor forms homodimers and that SSR149415 does not modify the BRET signal at concentrations that induce maturation and expression of the mutant on the cell surface. Therefore, the V3 receptor can form constitutive homodimers during its biosynthesis in the ER. This has recently been demonstrated for many GPCRs, such as the β2-adrenergic (41), the gonadotropin releasing hormone (42), the dopamine D2 (43), or the oxytocin and the vasopressin V1a and V2 (28) receptors. Recently, it has been demonstrated that homodimerization of the β2-adrenergic receptor in the ER is a prerequisite for cell-surface targeting (29). Although an oligomerization defect, which would be suppressed by the pharmacological chaperone, could explain the retention of the MUT V3R in the ER, our results clearly showed that this is not the case.

We also found that SSR149415 was able to increase the BRET signal of the wild-type V3 receptor in a dose-dependent manner. Further analysis of the BRET signal showed that the maximal response increased with no change in the BRET$_{\text{max}}$ values, which reflects the affinity between the monomers. This suggests that SSR149415 causes a conformational change of the preexisting receptor oligomers by modifying the distance and/or orientation between the two partners. This further suggests a general conformational role of the pharmacological chaperone.

Finally, we also found that SSR149415 was able to increase the maturation of the wild-type V3 receptor and its expression on the cell surface. Quantitative analysis showed that this required a pharmacological chaperone dose 100 times lower than for the mutant receptor. This shows that the WT V3R is partially but significantly retained in the ER. A low export efficiency, which is increased by pharmacological chaperones, has been reported for the wild-type mouse V2 receptor (34), the wild-type γ opioid receptor (44, 45), and the wild-type gonadotropin-releasing hormone receptor (25). This is in contrast with other studies in which the pharmacological chaperones SR121463A, naloxone, or 11-cis-7-ring did not affect the maturation of the wild-type human V2 receptor (27), the wild-type μ opioid receptor (36), or the wild-type rhodopsin (26), respectively. Therefore, some wild-type GPCRs may have a constitutive defect in their maturation efficiency in the ER, which is a limiting step in their processing. It is possible that in some cases, interacting proteins such as the previously mentioned escort proteins could regulate this limiting step.

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