Pharmacological Chaperone Activity of SR49059 to Functionally Recover Misfolded Mutations of the Vasopressin V1a Receptor*

Received for publication, October 26, 2005, and in revised form, March 14, 2006. Published, JBC Papers in Press, March 24, 2006, DOI 10.1074/jbc.M511610200

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Pharmacological chaperones represent a new class of ligand with the potential to facilitate the delivery of misfolded, but still active, G-protein-coupled receptors to the cell surface. Using transfected HEK 293T cells, treatment with a nonpeptide antagonist, SR49059, dramatically increased (~60-fold) the surface expression of a misfolded, nonfunctional and intracellularly localized vasopressin V1a receptor (V1aR) mutant (D148A). This rescue of surface expression (111 ± 7%) was almost identical to wild type assessed by confocal microscopy and quantitative enzyme-linked immunosorbent assay-based techniques. Recovery was not specific to D148A, since other surface-impaired mutations, D148N and D148E, and wild type were also increased following SR49059 exposure. However, surface delivery was specific to SR49059, since V1aR-selective peptide ligands or unrelated ligands were unable to mimic this action, suggesting that SR49059 acts intracellularly. SR49059-mediated surface rescue was time-, mutant-, and concentration-dependent but not directly related to its binding affinity. Maximal recovery was achieved following 12 h of treatment and did not involve de novo receptor synthesis or a consequence of preventing endogenous constitutive activity and/or internalization. Once at the surface, all mutants displayed enhanced signaling ability, and D148A was able to undergo agonist-mediated internalization. SR49059 was not effectively removed from the receptor, since signaling (EC50) of both wild type and D148A was reduced ~40-fold. This is the first report of a pharmacological chaperone ligand to act on misfolded mutant V1aRs. This work provides an excellent model to understand the mechanistic action of an important new class of drug that may have potential in the treatment of diseases caused by inherited mutations.

An important role of the endoplasmic reticulum (ER)² is to provide quality control mechanisms to ensure correctly folded proteins enter the secretory pathways (for reviews, see Refs. 1 and 2). This complex sorting system provides an environment for folding, initial post-translational modifications and oligomeric assembly of proteins prior to their translocation to other regions of the cell. This regulated system also prevents misfolded proteins and/or accumulation of defective proteins that potentially can interfere with other cellular processes and promote their degradation via ubiquitination/proteasome pathways (reviewed in Refs. 3 and 4). Protein misfolding can be caused by genetic mutation, fluctuations in temperature, cellular stress, and activation of signaling pathways that result in major diseases (4). Accumulation and aggregation of misfolded proteins are responsible for neurodegenerative pathologies, such as Alzheimer (β-amyloid) and Parkinson disease (α-synuclein). Inherited mutations within cell surface receptors, such as ΔF508 in the cystic fibrosis transmembrane conductance regulator gene (cystic fibrosis), enzymes (e.g. β-glucosidase) (lysosomal storage diseases), and G-protein coupled receptors (GPCRs) vasopressin V2 receptor (V2R) (nephrogenic diabetes insipidus), retinol pigments (rhodopsin), melatonin 4-receptor (familial obesity (5)), and gonadotropin-releasing hormone receptor (impaired gonadal function), all result from impaired intracellular trafficking and protein mislocalization (6, 7).

Strategies to reverse or rescue the effects of misfolded proteins represent major avenues for experimental and therapeutic intervention. Among these strategies is manipulation of the pathways involving endogenous ER molecular chaperone proteins (e.g. calnexin) (1, 2, 8), which regulate the binding, folding, stabilizing protein conformations, assembly, and/or signals for degradation of newly synthesized or misfolded proteins. Chemical agents, such as Me3SO, glycerol, and trimethylamine-N-oxide, can exert nonspecific chaperone effects by stabilizing some misfolded proteins and increasing their trafficking (2, 6). More recently, small ligands that represent a new class of compound termed “pharmacological chaperones” provide a more specific approach to target individual misfolded proteins (6, 7). These nonpeptidic cell-permeable compounds have been suggested to stabilize a conformation within the protein architecture that can bypass ER quality control and reverse the effects of the defective protein. In the case of GPCRs, pharmacological ligands (agonists and antagonists) provide an alternative strategy to functionally rescue disease-causing mutations that are intracellularly retained. These include receptors V1aR (9–11), gonadotropin-releasing hormone (12, 13), μ-opioid (14), melanin-concentrating hormone receptor 1 (15), and rhodopsin (16). The action of chaperone ligands is not only restricted to mutant GPCRs, since maturation of wild-type (WT) 6-opioid receptors can also be increased upon exposure (17). In general, very little is known about their mechanism(s) of action and if monohydride (cis-isomer); SSRI49145, (2S,4R)-1-[5-chloro-1-[2,4-dimethoxyphenyl]sulfonyl]-3-[2-methoxyphenyl]-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydrop-hydroxymethylpyridin-2-carboxamide (isomer (−)).
the pharmacology of receptor is normal following administration of this type of ligand.

The structurally related nonapeptides hormones vasopressin (AVP) and oxytocin (OT) mediate a plethora of physiological functions, including vasopressor and antidiuretic actions, by activation of specific receptors (18, 19). Four AVP/OT receptor subtypes (V1aR, V1bR, V2R, and OTR) have been cloned from different species and constitute a subfamily of the larger GPCR superfamily. The V1aR is widely expressed and mediates nearly all of the actions of AVP with the exception of antidiuresis (renal V2R) and adrenocorticotropic hormone secretion (pituitary V1bR). AVP mediates vascular smooth muscle (V2R) contraction and regulates cardiovascular function (19). In contrast, OT results in contraction of uterine myometrium (OTRs) during labor and mammary myoepithelium to elicit lactation (18). With the exception of the V1aR (which couples to adenylyl cyclase), these receptors couple to Gαq/11, thereby generating inositol 1,4,5-trisphosphate and diacylglycerol as second messengers.

Ligands developed toward specific receptors within the AVP/OT receptor family have been reported to act as pharmacological chaperones (9–11). The aim of this study was to determine (i) if these and other related ligands can act as a pharmacological chaperones and rescue the surface expression of previously identified misfolded mutant V1aR (20), and if so (ii) the conditions required to do this and (iii) if their pharmacology is normal once delivered at the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials—**The cyclic antagonist 1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(O-methyl)tyrosine AVP (CA) was purchased from Bachem (St. Helens, UK). AVP and linear antagonist phenylactetyl-tyr(Arg2)avp (LA) were from Sigma (Poole, UK). (23S)-[25S]-inosine 3′,5′-monophosphate substrate (SNAP) was from PerkinElmer Life Sciences (Boston, MA). All other reagents were of analytic grade and obtained from various commercial suppliers.

**Mutant Receptor Constructs—**All mutations of the V1aR were made previously by PCR and have been described in detail (20). All mutations (including the wt rat V1aR) in the mammalian expression vector pcDNA3 contained a previously engineered N-terminal hemagglutinin (HA) epitope tag (21). The wt rat A1R and mutant constructs (A1RΔ22 and A1RΔ34) in pcDNA3 (22) were a kind gift from Dr. Edin Ibrisimovic and Dr. Christian Nanoff (University of Vienna, Austria).

**Cell Culture and Transfection—**Human embryonic kidney (HEK) 293T cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, glutamine (2 mM), and sodium pyruvate (1 mM) in humidified 5% (v/v) CO2 in air at 37°C. Cells were seeded at a density of ~5 × 10⁴ cells/100-mm dish and transfected after 36 h using a calcium phosphate precipitation protocol using 10 μg of DNA/dish.

**Radioligand Binding Assays—**A washed cell membrane preparation of HEK 293T cells, transfected with the appropriate receptor construct, was prepared 36 h post-transfection as previously described (23), and the protein concentration was determined using the BCA protein assay kit (Sigma) with bovine serum albumin (BSA) as a standard. Competition radioligand binding assays were performed in MultiScreen™ HTS 96-well opaque plates (Millipore, Watford, UK) containing 1.0-μmol glass fiber (GF/B) filters and using the natural agonist [3H]AVP (73 Ci/mmol; PerkinElmer Life Sciences) as tracer ligand. Radioligand binding assays were performed as described previously (20). Essentially, each well contained radioligand (1.0–1.6 nm), cell membranes (120–190 μg), and competing ligand and was incubated at 25°C for 90 min. Nonspecific binding was determined using 10 μM unlabeled AVP. Bound radioligand was separated from free ligand by filtration, and radioactivity was measured using a Topcount NXT scintillation counter (PerkinElmer Life Sciences) following the addition of Microscint™ (PerkinElmer Life Sciences). Binding data were analyzed by nonlinear regression to fit theoretical Langmuir binding isotherms to the experimental data using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Individual IC₅₀ values obtained for competing ligands were corrected for radioligand occupancy as described (24), using the radioligand affinity (Kᵣ) experimentally determined for each construct.

**AVP-induced Inositol Phosphate Production—**HEK 293T cells were seeded at a density of 7.5 × 10⁴ cells/well in poly-D-lysine-coated 24-well plates and transfected after 24 h using Transfast™ (Promega Corp., Southampton, UK). The assay for AVP-induced accumulation of inositol phosphates (InsPs) has been described in detail recently (20). Briefly, 16 h post-transfection, medium was replaced with inositol-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 1% (v/v) fetal calf serum and 1 μCi/ml myo-[2-3H]inositol (20.0 Ci/mmol; MP Biomedicals, Irvine, CA) for 24 h. Cells were washed five times with PBS and then incubated in inositol-free Dulbecco’s modified Eagle’s medium containing 10 mM LiCl for 30 min, after which AVP was added at the concentrations indicated for a further 30 min. Incubations were terminated by the addition of ice-cold 0.1 M HCOOH for 30 min. Samples were loaded onto AG1-X8 columns (formate form; Bio-Rad). A mixed inositol fraction containing mono-, bis-, and trisphosphates (InsPᵣ-inositol 1,4,5-trisphosphate) was eluted with 5 ml of 850 mM NH₄COOH containing 0.1 M HCOOH, mixed with UltimaFlo AF scintillation mixture (PerkinElmer Life Sciences), and radioactivity was quantified by liquid scintillation spectroscopy. EC₅₀ values were determined by nonlinear regression after fitting of logistic sigmoidal curves to the experimental data using GraphPad Prism.

**Cell Surface Expression of Mutant Receptors—**Cell surface expression of mutant V1aR constructs was determined by performing an indirect ELISA-based method as described (20). Briefly, HEK 293T cells were seeded at a density of 7.5 × 10⁴ cells/well in poly-D-lysine-coated 24-well plates and transfected after 24 h using Transfast™. After 36 h, cells were fixed with 3% (v/v) formaldehyde in Tris-buffered saline (TBS) for 15 min at 25°C. Cells were washed (3 × 5 min in TBS) prior to the addition of 3% (v/v) BSA in TBS for 45 min to block nonspecific binding. The anti-HA primary antibody (HA-7; Sigma) diluted to 1:30,000 in 3% (w/v) BSA/TBS was incubated on cells for 60 min at room temperature. Cells were washed and reblocked (15 min) prior to incubation with secondary goat anti-mouse conjugated alkaline phosphatase (Sigma) diluted to 1:20,000 in 3% (v/v) BSA/TBS for 60 min. Cells were gently washed prior to the addition of colorimetric alkaline phosphatase substrate p-nitrophenol phosphate. Plates were incubated at
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37 °C (45 min) prior to removal of samples for colorimetric reading at 405 nm using an MRX plate reader (Dynatech Technologies, Chantilly, VA). For each experiment, mock conditions corresponding to the transfection of empty vector were included. The percentage of mutant receptor expressed at the cell surface is defined as 100 × (ODmutant − ODmock)/(ODstimulated − ODmock) × ODWT/ODmock. All experiments were performed in triplicate for each condition, and values were obtained from at least three separate experiments.

Agonist-mediated Internalization of Mutant Receptors—HEK 293T cells were seeded in 24-well plates and transfected as described above. After 36 h, medium from cells was replaced with fresh growth medium prior to the addition of AVP to induce V1aR internalization at various time intervals and incubated at 37 °C with 5% (v/v) CO2 in air. Quantification of receptors remaining at the cell surface was determined using ELISA as described above. The percentage of mutant receptor internalized is defined as 100 × (ODbasal − ODmock) − (ODstimulated − ODmock)/(ODbasal − ODmock). All experiments were performed in triplicate for each condition, and values are from at least three separate experiments.

Immunohistocytochemistry—HEK 293T cells were seeded in 24-well plates containing poly-d-lysine-coated glass coverslips (12 mm) and transfected using Transfast™ as described above. Cells were fixed and washed with TBS as described for ELISA. Cells were blocked with 3% (w/v) BSA/TBS containing glycine (1% (w/v)) for 45 min, followed by incubation with anti-HA primary antibody (diluted to 1:3,000 in 3% (w/v) BSA/glycine/TBS for 60 min. Cells were washed with TBS (3 × 5 min) prior to rebloking with 10% (v/v) goat serum in PBS for 15 min at room temperature. Cells were labeled with secondary antibody goat anti-mouse rhodamine red X (Molecular Probes, Leiden, The Netherlands) (diluted to 1:500 in 10% (v/v) goat serum in PBS) for 60 min at room temperature in the dark. After a further three washes, coverslips were mounted on glass slides prior to confocal microscopy.

Confocal Microscopy—Confocal microscopy was performed using a Zeiss LSM 510 laser-scanning microscope with a Zeiss Plan-Apo 63 × 1.4 numerical aperture oil immersion objective. The HA-tagged receptors were visualized by exciting the rhodamine red X secondary antibody with a 543-nm HeNe laser and a 560-nm long pass filter. For each slide, images were captured at random sites from three separate experiments. The gains and offsets were kept constant for each image that was generated using the Zeiss LSM software (Jena, Germany).

RESULTS

Recovery of Cell Surface Expression of a Misfolded Vasopressin V1a Receptor Using a Nonpeptide Antagonist—We have previously reported that a mutant [D148A]V1aR within the highly conserved DRY motif resulted in a misfolded receptor that was nonfunctional and localized intracellularly (Fig. 1) (20). In light of recent reports of ligands acting as pharmacological chaperones (9–17), an initial aim of this study was to investigate if a V1aR-specific ligand could act as a pharmacological chaperone by facilitating the trafficking/delivery of the [D148A]V1aR to the cell surface. The nonpeptide ligand SR49059 was originally developed as a high affinity antagonist specific for V1aRs (25). As was previously reported, the [D148A]V1aR is poorly expressed on the cell surface of transfected HEK 293T cells (Fig. 2B) (20). Following a post-transfection treatment with SR49059 (10 μM; 20 h), a dramatic increase in localization of [D148A]V1aR was observed at the cell surface (Fig. 2D). This restoration of surface receptor localization was comparable with that observed for the WT V1aR when performed in parallel experiments (Fig. 2B). In contrast, treatment with SR49059 had little effect on localization of WT receptor compared without treatment (Fig. 2B) or cells expressing a control vector lacking receptor (data not shown).

Immunofluorescence confocal microscopy is not a reliable technique for quantification of receptors at the cell surface. To overcome this, an ELISA-based assay with a high signal/noise ratio was developed to quantify surface expression of receptors incorporating an HA epitope...
SR49059 Is Not Specific to Ala148, since Other Mutations Can Also Be Rescued—The side chain at position 148 of the V1aR was reported to influence the level of surface expression (20). Substitution of Asp148 with either an asparaginyl (i.e. [D148N]V1aR) or glutamyl (i.e. [D148E]V1aR) led to significantly reduced surface expression of ~20 and ~40% of normal WT levels, respectively (Table 1) (20). This impaired surface expression with both [D148N]V1aR and [D148E]V1aR was apparent over a range of receptor cDNA concentrations transfected (Fig. 4A) and never exceeded the maximum saturable levels of ~20 and ~40% of WT, respectively. It was important to determine (i) if these mutations with impaired surface expression can also be rescued with SR49059 and (ii) if any recovery was influenced by the amount of receptor cDNA transfected. Following treatment with SR49059, cell surface expression of each mutation (at each cDNA concentration) was increased to levels almost identical to WT V1aR expression (Fig. 4B, Table 1). These results establish that SR49059 is able to rescue the surface expression of other mutations with impaired surface expression levels.

Ligand-specific Delivery of Misfolded Mutations to the Cell Surface—The complete recovery of cell surface expression of Asp148-substituted mutant V1aRs with SR49059 allowed investigation of whether specific properties of this ligand are essential for the recovery of surface expression and testing if other specific high affinity ligands can act as pharmacological chaperones. Following transfection, WT V1aR and mutant [D148A]V1aR were treated with the natural agonist AVP or two structurally different nanomolar affinity peptide antagonist ligands: (i) cyclic peptide antagonist (CA (d(CH2)5Tyr(Me)2AVP) (26)) containing a 20-membered ring formed by a disulfide bond between Cys2 and Cys6 \( (K_i = 0.5 \pm 0.1 \text{ nM}) \) (V1aR) (20) and (ii) linear peptide antagonist (LA; phenyl acetyl-D-Tyr(Me)2Arg6Tyr(NH2)9-AVP (27)) \( (K_i = 0.2 \pm 0.0 \text{ nM}) \) (V1aR) (20). Pretreatment with both peptide antagonists had a relatively small (~10–20%) but still noticeable increase in cell surface expression of the WT receptor above control (Table 1). Similarly, both antagonists increased surface expression (~10% of total WT level) of the [D148A]V1aR mutant compared with conditions without ligand (Fig. 3B, Table 1). These results clearly show that the two antagonists exert minor increases in receptor expression but do not mimic the pharmacological chaperone activity of SR49059. Prolonged exposure to AVP resulted in a ~70% loss of cell surface WT receptor, consistent with the notion of V1aRs being desensitized and/or downregulated (data not shown). In contrast, AVP had no effect on the cell surface expression of the [D148A]V1aR mutant (data not shown), an indication that the peptide agonist AVP does not possess any pharmacological chaperone activity. Furthermore, pretreatment with the peptide antagonist LA prior to the addition of SR49059 had no effect on the level of recovery with [D148A]V1aR compared with when SR49059 was used alone (Fig. 3B). Treatment of mutations [D148N]V1aR and [D148E]V1aR with either of the peptide ligands LA or CA had only relatively minor 10–25% minor increases in receptor surface expression (Table 1), confirming that the pharmacological chaperone activity of SR49059 is very specific.

The nonpeptide ligand SR121463 (28) was recently shown to act as a pharmacological chaperone on V1aR mutations with impaired surface expression (9, 10). This compound and a V1aR-selective ligand SSR149415 (29) were tested for their ability to rescue surface expression of each of the three Asp148-substituted mutations. Both SR121463 and SSR149415 ligands had little effect on increasing surface expression of the mutations and were unable to mimic the recovery obtained with SR49059 (Fig. 3B, Table 1). It is noteworthy that these two compounds have lower binding affinities for the WT V1aR following transient expression in HEK 293T cells \( (K_i = 900 \pm 200 \text{ nM}) \) (n = 3) and \( K_i = \ldots \) sequence engineered at their amino terminus (Fig. 1) (20). Incorporation of this 9-amino acid sequence was previously shown not to affect ligand binding or signaling compared with an untagged WT V1aR (21) and was used in all subsequent experiments. Using the ELISA-based assay, SR49059 was able to significantly increase (56-fold) the surface expression of [D148A]V1aR compared with cells without treatment (Fig. 3A, Table 1). This dramatic increase in surface expression using SR49059 was not only specific to the [D148A]V1aR mutant, since WT receptor expression was also slightly increased (~20%) compared with the level without treatment using the ELISA assay (Fig. 3A, Table 1). Together, these results show that the nonpeptide antagonist SR49059 is capable of acting as a pharmacological chaperone ligand by rescuing the surface expression of a misfolded mutant receptor.
Table 1

Ligand-specific recovery of cell surface expression of WT and mutant V\textsubscript{1a}R

| Receptor       | Control (mean ± S.E.) | SR49059 (mean ± S.E.) | CA (mean ± S.E.) | LA (mean ± S.E.) | LA/SR49059 (mean ± S.E.) | SR121463 (mean ± S.E.) | SSR149415 (mean ± S.E.) |
|----------------|-----------------------|------------------------|-----------------|----------------|--------------------------|------------------------|------------------------|
| WT             | 100 (8)               | 122 ± 4 (6)*           | 106 ± 6         | 114 ± 3        | 136 ± 11*                | 90 ± 7 (4)             | 113 ± 11 (4)           |
| D148A          | 2 ± 2 (7)*            | 109 ± 7 (6)*           | 12 ± 2 (4)      | 13 ± 2         | 109 ± 5*                 | 2 ± 3 (4)              | 4 ± 4 (4)              |
| D148N          | 19 ± 2 (5)*           | 94 ± 8 (4)*            | 30 ± 2          | 30 ± 1         | 94 ± 5*                  | 11 ± 2                 | 28 ± 3                 |
| D148E          | 43 ± 4 (9)*           | 95 ± 6 (4)*            | 71 ± 8*         | 64 ± 3*        | 100 ± 3*                 | 28 ± 4                 | 56 ± 2                 |

* p < 0.05 compared with each V\textsubscript{1a}R without drug treatment using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism4).

Values taken from Ref. 20.

† p < 0.01 compared with each V\textsubscript{1a}R without drug treatment using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism4).

FIGURE 4

SR49059-mediated cell surface recovery of Asp\textsuperscript{148}-substituted mutant receptors. HEK 293T cells were transiently transfected with WT V\textsubscript{1a}R ( ), D148A V\textsubscript{1a}R ( ), D148N V\textsubscript{1a}R ( ), and D148E V\textsubscript{1a}R ( ) using different amounts of cDNA (0.125–1.0 µg/well). Post-transfection (16 h), cells were treated with either vehicle (open symbols) or SR49059 (10 µM) (closed symbols) and incubated for a further 20 h at 37 °C. Quantification of receptor at the cell surface was determined by an ELISA-based assay as described under “Experimental Procedures.” Data are the mean ± S.E. of three separate experiments (unless stated otherwise), each performed in triplicate. Values shown represent surface expression as a percentage of WT receptor in the absence of drug treatment. Cell surface expression of 100% corresponds to ~0.8 pmol/mg membrane protein.
mutant and increased with the rank order of their initial expression level (i.e. [D148A]V1aR < [D148N]V1aR < [D148E]V1aR) (Fig. 6C).

Rescue of Cell Surface Expression with SR49059 Is Independent of Protein Synthesis and by Preventing Receptor Internalization—The increased surface expression of the Asp148-substituted mutants following SR49059 treatment may involve up-regulation of newly synthesized receptor. To investigate this, HEK 293T cells expressing each mutant receptor were pretreated with the cycloheximide inhibitor for 30 min prior to the addition and incubation with SR49059 for 10 h (Fig. 7A). The surface expression of each mutation in the presence of cycloheximide (6 ± 5% (n = 3), 25 ± 5% (n = 3), and 45 ± 6% (n = 3)) for [D148A]V1aR, [D148N]V1aR, and [D148E]V1aR, respectively) was similar to the level without inhibitor (Table 1). In contrast, an increase in surface expression for all mutations was observed in the presence of both cycloheximide and SR49059 (63 ± 2% (n = 3), 57 ± 12% (n = 3), and 62 ± 10% (n = 3) for [D148A]V1aR, [D148N]V1aR, and [D148E]V1aR, respectively) (Fig. 7A). These results suggest that the pharmacological chaperone activity of SR49059 acts independently of stimulating newly synthesized receptor. In an attempt to determine if SR49059 directly facilitates the trafficking of the mutants to the cell surface, the inhibitors brefeldin A (which blocks newly synthesized transport vesicles fusing with the Golgi (31)) and monensin (which blocks trafficking from Golgi to membrane (32)) were used to inhibit translocation of receptors to the cell surface in the presence and absence of SR49059 (Fig. 7, B and C). Prolonged treatment (and reapplication after 5 h; data not shown) with these inhibitors prevented cell surface recovery of each mutant in the presence of SR49059 (Fig. 7, B and C). However, these inhibitors had dramatic effects on reducing normal WT expression levels compared with control levels after 16 h post-transfection (data not shown), indicating that they may be cytotoxic under these conditions.

An additional possibility is that chaperone ligands act by preventing endogenous receptor internalization/recycling, which ultimately leads to the accumulation of receptor at the surface. To investigate this possibility, HEK 293T cells expressing WT V1aR or [D148A]V1aR mutant were treated with an internalization inhibitor concanavalin A (which inhibits ~40% AVP-mediated V1aR internalization; data not shown) for 30 min or 20 h following transfection (36 and 16 h, respectively). Treatment with concanavalin A did not increase the surface expression of WT (99 ± 2%; n = 3) or [D148A]V1aR (2 ± 2%; n = 3) after 30 min. However, prolonged concanavalin A treatment actually reduced WT expression (49 ± 9%; n = 3) and had no effect on [D148A]V1aR expression compared with control. Since inhibition of receptor internalization did not mimic the action of SR49059 on the [D148A]V1aR mutant (Table 1), this suggests that this is not a major site for SR49059 to mediate its chaperone activity.

Functional Rescue and Signaling of WT and Misfolded Receptors following SR49059-mediated Cell Surface Delivery—Having established that SR49059 was able to restore cell surface expression of all three mutations, it was important to determine if the mutant receptors were now able to signal in response to agonist AVP stimulation. Each mutant receptor was expressed in HEK 293T cells and treated with SR49059 (10 μM; 20 h) prior to measuring AVP-induced accumulation of InsPs (Fig. 8A). The signaling ability (E_{max}) of all mutations was significantly enhanced (~2–3-fold) following SR49059 treatment. This was particularly important for both [D148A]V1aR and [D148N]V1aR mutants, since these were previously reported to be nonfunctional (20). Signaling was also slightly elevated with WT V1aRs compared with receptor without SR49059 treatment (Fig. 8), presumably as a result of the slightly higher cell surface expression (Fig. 3). It is also noteworthy that the basal level of InsPs accumulation was not significantly different between each of the mutants and WT (ANOVA with a post hoc Dunnett’s test analysis.

![Figure 5](image_url)
Furthermore, SR49059 did not possess any endogenous agonist or inverse agonist signaling activity on both [D148A]V1aR and WT V1aRs (data not shown).

The enhanced signaling ability of [D148A]V1aR following SR49059 treatment was assessed over a range of transfected cDNA concentrations (data not shown). Increasing the amount of cDNA of WT V1aR increased the fold stimulation of InsPs as expected as a result of increasing the surface expression of receptor until saturation (Fig. 4A).

Following SR49059 treatment, WT V1aR signaling was also increased in a manner very similar without SR49059 treatment. A slightly higher signaling capacity was observed when 0.5 μg/well cDNA was used, which was consistent with an increased level of surface expression (Fig. 3). In contrast, the signaling ability of [D148A]V1aR following SR49059

### GraphPad Prism4

(GraphPad Prism4) following SR49059 treatment (data not shown). Furthermore, SR49059 did not possess any endogenous agonist or inverse agonist signaling activity on both [D148A]V1aR and WT V1aRs (data not shown).

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### Table

| Receptor | Concentration (nM) | Time $t_{1/2}$ (h) |
|----------|-------------------|-------------------|
| D148A    | 1100 ± 100        | 3.7 ± 0.3         |
| D148N    | 420 ± 70          | 2.9 ± 0.2         |
| D148E    | 190 ± 80          | 2.4 ± 0.1         |

### Figure 6

Concentration- and time-dependent recovery of cell surface expression of mutant receptors. HEK 293T cells were transiently transfected with [D148A]V1aR (●), [D148N]V1aR (▲), and [D148E]V1aR (▼) using 0.5 μg of cDNA/well. A. post-transfection (16 h), cells were treated with SR49059 (at the concentrations indicated) and incubated for a further 20 h at 37 °C. B. post-transfection (16–36 h), cells were treated and incubated with SR49059 (10 μM) for various time periods (indicated on the x axis) at 37 °C. Quantification of receptor at the cell surface was determined by ELISA as described under “Experimental Procedures.” Data are the mean ± S.E. of three separate experiments, each performed in triplicate.

### Figure 7

The effect of SR49059 mediated recovery of surface expression in the presence of specific inhibitors. HEK 293T cells transiently transfected with wild-type V1aR, [D148A]V1aR, [D148N]V1aR, and [D148E]V1aR. Post-transfection (16 h), cells were treated with cycloheximide (20 μg/ml) (A), brefeldin A (5 μg/ml) (B), or monensin (10 μM) (C) for 30 min prior to the addition of SR49059 (10 μM; 10-h exposure) or vehicle control at 37 °C. Receptor expressed at the cell surface was quantified by ELISA as described under “Experimental Procedures.” Data are the mean ± S.E. of three separate experiments, each performed in triplicate. Values shown are surface expression as a percentage of WT V1aR without drug treatment. *, p < 0.05; **, p < 0.01 compared with each V1aR without drug treatment using Student’s paired t test analysis (GraphPad Prism4).
treatment was increased compared with the ability without treatment with all concentrations of cDNA (data not shown).

The dose-response curves for AVP-induced accumulation of InsPs for WT V1aR and [D148A]V1aR following SR49059 treatment were also assessed. As shown in Fig. 9A, the curves for WT V1aR and [D148A]V1aR following SR49059 treatment were equally right-shifted compared with WT without SR49059, with EC50 values increasing by ~40-fold (Fig. 9B). These reduced potencies were observed despite extensive wash steps (five washes) and when an additional acid wash step (5 mM acetic acid, 150 mM NaCl, pH 5) was included in the procedure (data not shown). To determine if SR49059 was acting as a competing antagonist, transfected cells expressing WT V1aR were stimulated with AVP following incubation with increasing concentrations of SR49059 (Fig. 10A). A progressive decrease in maximum response was observed, indicating that only a hemiequilibrium was achieved with this slowly dissociating antagonist. Although a Schild analysis could not be performed, values obtained from the first shift with the lowest concentration of antagonist (10 nM) was calculated as Ks = 1.6 ± 0.4 (n = 3) and similar to Ks = 0.7 ± 0.1 nM determined by radioligand binding (20). It is noteworthy that no specific increase in tracer-ligand binding could be detected following SR49059 treatment with the D148A mutation (data not shown). The response curve for WT following overnight SR49059 treatment (performed in parallel) displayed an increase in maximum signaling (Fig. 10B) as a result of increased surface expression (Fig. 3A).

Together, these results show that following SR49059 treatment, signaling of mutant V1aRs can be significantly increased. However, the efficiency of the agonist AVP to mediate this effect is compromised with a reduced potency, which is partly due to the incomplete removal of SR49059 from the receptor.

Internalization of WT and Misfolded Receptor following SR49059 Treatment—It was important to address if rescued mutations were able to be regulated as normal following SR49059 treatment. The [D148A]V1aR mutant provides an excellent model to address this, since this mutant is poorly expressed on the cell surface without chaperone treatment (Fig. 3). V1aRs are internalized via receptor arrestin-dependent pathways and are both agonist- and time-dependent (20).

Internalization of mutant V1aRs was assessed by ELISA, and the percentage of V1aRs remaining on the cell surface was determined (Fig. 11). The percentage of V1aRs remaining on the cell surface was determined (Fig. 11). The percentage of V1aRs remaining on the cell surface was determined (Fig. 11). The percentage of V1aRs remaining on the cell surface was determined (Fig. 11).

DISCUSSION

In this report, SR49059 a nonpeptide antagonist originally developed as a high affinity V1aR-selective ligand (25) was identified to act as a pharmacological chaperone on V1aRs. This new class of ligands has the potential to rescue misfolded receptors trapped within ER/secretory...
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The site of SR49059 to rescue mutant receptors is not merely due to the occupancy and accumulation of receptors at the cell surface by preventing endogenous receptor internalization. Evidence to support this was that neither peptide ligands nor incubation with an inhibitor of internalization was able to mimic the actions of SR49059. Since peptide ligands are generally not cell-permeable, this suggested that SR49059 mediates its activity intracellularly. This was also evident when SR49059 increased the surface expression (with all mutants) even at high peptide concentrations (10 μM) and with a ~10-fold higher binding affinity than SR49059 (WT V₁₃R (20)). Peptides alone did slightly elevate expression, which was most likely by preventing receptor turnover. Cell permeability of SR49059 and its relatively small size are likely to be important factors for its chaperone activity.

One possibility for the mechanism of chaperone ligands is to stabilize a conformation within the receptor architecture. Consequently, this may bypass cellular quality control mechanisms and/or facilitate a correct folding process, which diverts misfolded receptors from degradation pathways (3). Our results support this general hypothesis in that SR49059 is able to interact specifically with the receptor. In some studies, recovery of surface expression/signaling with chaperone treatment was suggested to correlate well with the binding affinity (Kᵢ) of the ligand to the receptor (10, 17). In this study, the Kᵢ of SR49059 for V₁₃Rs is nanomolar, whereas its chaperone activity was at least 100-fold lower. By directly comparing the recovery of a series of mutations, it was clearly seen that the amount of SR49059 to mediate this increase was very dependent on the initial expression of each mutant. The higher the initial surface expression level of the mutant (i.e., ~40% for D148E), the less SR49059 and time that was required to mediate a complete recovery. In contrast, D148A (~0% surface) required significantly higher amounts (~6-fold) of SR49059 and a longer time to mediate an identical recovery. These results show that a correlation between binding affinity and recovery are not directly linked. Furthermore, the fact that surface recovery of all mutants achieved normal WT expression (which is not always the case in some studies) and only at high concentrations is likely to influence both the amount of ligand required and its ability to rescue specific mutations. This phenomenon was also observed for mutant V₂Rs, where recovery of both surface expression and signaling with SR121463 was ~100-fold lower than its binding affinity on V₂Rs (9).

SR49059 may interact differently with each mutant within the ER/early secretory pathways (10, 32). Post-translational maturation through the Golgi may also contribute additional rate-limiting steps for their recovery. In this regard, SR121463 and naltrexone were both shown to promote increased glycosylation of a mutant V₁₃R (9) and δ-opioid receptor (17), respectively. Consistent with these reports, surface recovery was independent on newly synthesized receptor, since SR49059 was able to increase expression of each mutant in the presence of cycloheximide. However, the ER-Golgi disruption agents brefeldin A and monensin were not useful tools to determine which part of the secretory pathway SR49059 mediates its effects, since these inhibitors had dramatic effects on reducing normal WT surface expression.

The mutants in this study are not constitutively active, since agonist-independent signaling was not enhanced compared with WT (20). Furthermore, SR49059 did not act as an agonist or inverse agonist (by decreasing basal signaling of WT or mutant V₁₃Rs). Since a constitutively active mutant has not yet been reported for the V₁₃R, it is not possible to test if SR49059 has any inverse agonist properties. For some GPCRs, inverse agonists can increase expression of both WT (e.g., H₂R

![Image](374x26 to 402x38)

**FIGURE 10.** AVP-mediated signaling in the presence of SR49059. A, HEK 293T cells transiently transfected with WT V₁₃R were treated (post-transfection; 36 h) with vehicle control (□), or increasing concentrations of SR49059 (10 nM (△), 100 nM (▲), 1000 nM (○), and 10,000 nM (□)) for 30 min at 37 °C. B, post-transfection (16 h), cells were treated with vehicle control (open symbols) or SR49059 (10 μM (closed symbols) and incubated for a further 20 h at 37 °C. Accumulation of InsPs was measured following stimulation with AVP for 30 min at 37 °C, as described under “Experimental Procedures.” Values are stimulation induced by AVP at the stated concentrations expressed as a percentage of the maximum (included in each plate). Data shown are the mean ± S.E. (triplicate determinations) from a single experiment and are representative of three separate experiments.

pathways (33) and facilitate their trafficking to the surface (6, 7). For some GPCRs, ligands have rescued intracellularly retained mutant GPCRs (12–16), including the V₂R (9–11). Since these ligands are not always receptor-specific, mutations within subfamilies may also be rescued. An initial aim of this study was to investigate if such ligands (including V₁₃R/V₁₃R/V₂R-selective ligands) can rescue misfolded mutants of the V₁₃R. To address this, it is important to separate the recovery of surface expression from other aspects of receptor function, such as ligand-binding, signaling, and/or regulation, which may be influenced directly by mutation.

Recently, mutations of Asp₁₄₈ within the highly conserved DRY motif resulted in misfolded receptors that were poorly expressed on the cell surface (20). Upon exposure to SR49059, a dramatic recovery of surface expression was observed for a D148A mutant that was previously intracellular. This rescue was very specific, since other ligands (peptide and nonpeptide antagonists or peptide agonist) did not mimic the effects of SR49059. Furthermore, SR49059 increased the surface expression of a series of mutations with impaired expression to levels almost identical to WT after 10–12 h. Interestingly, SR49059 also increased WT receptor expression (~20%) and when transfected at different levels. Together these provide evidence that SR49059 acts as a chaperone, as described for other GPCRs (9–16). Interestingly, SR121463 (a known chaperone ligand on V₂R mutations (9)) and the V₁₃R-selective ligand SSR149415 had no effects on the V₁₃R mutants used in this study.

V₁₃R from a single experiment and are representative of three separate experiments.
with cimetidine) (38) and constitutively active mutants by up-regulation and/or translocation of intracellular localized receptors (39). In this study, deletion of either 22 or 34 residues from the C terminus region of the A2R, which both had impaired surface expression (22) could not be rescued with SR49059, confirming that SR49059 was receptor-specific. However, A2R reverse agonists (30) were able to stimulate surface expression of A2RΔ22 but not A2RΔ34. The recovery of only A2RΔ22 suggests that not all mutants can be rescued and that some regions (in this case a segment within the C terminus) are involved in the correct folding process and/or ER export and/or provide key protein contacts (40, 41). Understanding which mutations can be rescued and within which region of the receptor is likely to provide future challenges. Chaperone ligands may decrease the exposure time of endogenous chaperones (e.g. calnexin (42–44)) and/or partners within ER compartments (44). Alternatively, chaperone ligands may promote receptor dimerization and/or recruitment of G-protein(s), which may be necessary prior to normal expression. Interestingly, co-expression of both WT and an ER-retained gonadotropin-releasing hormone mutant led to a dominant-negative effect that reduced WT surface expression but was reversed with a chaperone ligand (45, 46).

To date, chaperone ligands have been reported to rescue or increase receptor-mediated signaling. However, the ability of these rescued mutants to behave as “normal” is not well documented. Following SR49059 treatment, all mutants displayed an increased signaling capacity ($E_{\text{max}}$) as a direct consequence of their increased surface expression. However, the efficiency of D148A to signal was impaired, resulting in a 40-fold reduction of potency. This decreased signaling was also present with WT, suggesting that this was not merely a reflection of a reduced binding affinity with the mutant. This reduced potency was also observed despite extensive washes indicating that SR49059 does not dissociate efficiently from the receptor and/or that SR49059 altered a specific receptor conformation necessary for efficient G-protein coupling. Preincubation of increasing concentrations of SR49059 prior to AVP stimulation on WT clearly established that SR49059 did not act as a competitive antagonist at high concentrations in this system. It will be interesting to see if other chaperone ligands share this property and contribute to their mode of action. Indeed, it was recently reported that SR121463 (28) would offer greater promise, being more selective for V2Rs without the chaperone effects on V1aRs. The efficiency of D148A to signal was impaired, resulting in a reduced level of internalized receptor was present for both WT and D148A with SR49059 treatment. This provides further evidence that SR49059 interacts longer with receptor.

Despite SR49059 having a reduced binding affinity ($K_i \sim 200 \text{ nM}$) on human V$_2$R (25), it is currently being pursued as an alternative treatment for nephrogenic diabetes insipidus (47). This disease is characterized by the inability to concentrate urine in the kidney as a direct result of inheriting mutation(s) (>150 identified) within the V$_2$R (48). The chaperone activity of SR49059 on V$_2$Rs reported here may restrict its use clinically. In contrast, SR121463 (28) would offer greater promise, being more selective for V$_2$Rs without the chaperone effects on V$_2$Rs. For other diseases, chaperone ligands may provide treatments for retina pigmentosa (16), impaired gonadal function (12, 13), pain (10, 17), nephrogenic diabetes insipidus (9, 10), and feeding disorders (15). Understanding which mutations can be rescued and within which region is likely to provide future challenges. Chaperone ligands may decrease the exposure time of endogenous chaperones (e.g. calnexin (42–44)) and/or partners within ER compartments (44). Alternatively, chaperone ligands may promote receptor dimerization and/or recruitment of G-protein(s), which may be necessary prior to normal expression. Interestingly, co-expression of both WT and an ER-retained gonadotropin-releasing hormone mutant led to a dominant-negative effect that reduced WT surface expression but was reversed with a chaperone ligand (45, 46).

In summary, this is the first report of a nonpeptide antagonist that acts as a chaperone ligand to rescue surface expression of mutant V$_2$Rs. SR49059 chaperone activity was very specific and dependent on time, concentration, and initial expression level of each receptor. This recovery resulted in receptors that were now able to signal and internalize once delivered to the cell surface. The development of chaperone-based compounds may provide alternative strategies for diseases that result from inappropriate surface expression.

Acknowledgements—I am grateful to Dr. Claudine Serradeil-Le Gal (Sanofi Recherche, France) for providing samples of SR49059, SR121463, and SSR149415 and to Dr. Edin Ibrisimovic and Dr. Christian Nanoff (University of Vienna, Austria) for providing the adenosine A1 receptor constructs. I thank Dr. Jillian Baker for providing samples of DPPX, DPCPX, XAC, and CGS159453. I am grateful to Prof. Steve Hill for critical reading and comments on the manuscript and Tim Self (Institute of Cell Signalling, University of Nottingham, UK) for excellent technical assistance with the confocal microscopy.

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