Appropriate Polarization following Pharmacological Rescue of V2 Vasopressin Receptors Encoded by X-linked Nephrogenic Diabetes Insipidus Alleles Involves a Conformation of the Receptor That Also Attains Mature Glycosylation*

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To understand the mechanisms of G protein-coupled receptor delivery and steady state localization, we examined the trafficking itineraries of wild type (WT) and mutant V2 vasopressin receptors (V2Rs) in polarized Madin-Darby canine kidney II (MDCK II) cells and in COS M6 cells; the mutant V2Rs represent selected alleles responsible for X-linked nephrogenic diabetes insipidus. The WT V2R is localized on the plasma membrane and mediates arginine vasopressin (AVP)-stimulated cAMP accumulation, whereas the clinically relevant V2R mutants, L292P V2R, ΔV278 V2R, and R337X V2R, are retained intracellularly, are insensitive to extracellularly added AVP, and are not processed beyond initial immature glycosylation, manifest by their endoglycosidase H sensitivity. Reduced temperature and pharmacological, but not chemical, strategies rescue mutant V2Rs to the cell surface of COS M6 cells; surface rescue of L292P V2R and R337X V2R, but not of ΔV278 V2R, parallels acquisition of AVP-stimulated cAMP production. Pharmacological rescue of the L292P or R337X V2R by incubation with the membrane-permeant V2R antagonist, SR121463B, leads to a mature glycosylated form of the receptor that achieves localization on the basolateral surface of polarized MDCK II cells indistinguishable from that of the WT V2R. Surprisingly, however, the immature form of the mutant L292P V2R escapes to the apical, but not basolateral, surface of polarized MDCK II cells, even in the absence of SR121463B. These findings are consistent with the interpretation that the receptor conformation that allows appropriate processing through the N-linked glycosylation pathway is also essential for V2R targeting to the appropriate surface of polarized epithelial cells.

Extensive investigation has revealed several mechanisms that modulate G protein-coupled receptor (GPCR)* responsiveness following agonist occupancy, including receptor relocalization (reviewed in Refs. 1 and 2). However, less attention has focused on the molecular mechanisms accounting for how receptors achieve localization in the agonist-naïve state. A key determinant governing the specificity of GPCR signaling entails appropriate receptor localization on the cell surface, permitting access to requisite ligands and signal transduction machinery. Cell surface localization is governed by two predominant mechanisms: 1) receptor delivery to a particular site and 2) retention at that site. The functional importance of GPCR localization is emphasized by diseases that result from receptor mislocalization, such as retinitis pigmentosa, X-linked nephrogenic diabetes insipidus (NDI), and hypogonadotropic hypogonadism (3), which result from intracellular accumulation of mutant rhodopsin, the V2 vasopressin receptor (V2R), or the gonadotropin-releasing hormone receptor, respectively.

Our previous studies have exploited α2-AR subtypes as a model for characterization of GPCR trafficking and localization because of their different trafficking itineraries in polarized cells and in response to agonist (reviewed in Ref. 4). All three α2-AR subtypes (α2A-, α2B-, and α2C-AR) are located, at steady state, at the basolateral surface in polarized Madin-Darby canine kidney (MDCK) II cells, analogous to their localization in vivo (5, 6). However, they achieve this basolateral localization via different trafficking itineraries. Whereas the α2A-AR and α2C-AR are directly targeted to the basolateral surface, the α2B-AR is randomly distributed to both the apical and basolateral surface and then selectively retained at the lateral subdomain (6). Truncations of the α2A-AR and chimeras with the apically targeted A1 adenosine receptor reveal that α2A-AR targeting to the basolateral surface relies upon multiple, non-contiguous, membrane-embedded sequences within or near the lipid bilayer (7, 8), suggesting that a three-dimensional surface provides the basis for interaction with trafficking molecules. Consequently, it can be reasoned that no single linear sequence can be exploited to identify receptor targeting machinery. Thus, to elucidate the mechanisms involved in basolateral delivery of GPCRs, we explored the human V2R and naturally occurring point mutations in the V2R responsible for the pathogenesis of X-linked NDI. Many of the >160 human mutations described to date cause the receptor to be retained intracellularly (9), unresponsive to its physiological ligand arginine vasopressin (AVP). We examined the trafficking and localization

(as in ΔV278, deletion of Val278); Endo H, endoglycosidase H; HA, hemagglutinin; HERG, human ether-a-go-go-related gene; MDCK, Madin-Darby canine kidney; NDI, nephrogenic diabetes insipidus; NHS, sulf-o-N-hydroxyisuccinimide; PBS, phosphate-buffered saline; PNGase F, peptide-N-glycosidase F; X, Stop (as in R337X, Arg337Stop); V2R, vasopressin receptor; WT, wild type; DMEM, Dulbecco’s modified Eagle’s medium; TEMED, N,N,N’,N’-tetramethylethlenediamine.)

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of the wild type V2R and three intracellularly retained V2R mutants to assess whether these mutants could be spatially and functionally rescued and whether cell surface rescue was also paralleled by localization on the appropriate surface of polarized MDCK II cells.

EXPERIMENTAL PROCEDURES

Materials—Human wild type (WT) and mutant V2R cDNAs were graciously provided by Dr. Jurgen Wess or were constructed by overlap extension PCR mutagenesis. EASYTAG [³²P]-EXPRESSSS protein labeling mix (1175 Ci/mmol), [2,8-³H]adenine (30.4 Ci/mmol), 8-arginine density could not be determined for the mutant V2R cell lines because binding/mg of protein, estimated in saturation binding studies. Binding CO₂. Simian kidney fibroblast (COS M6) cells were maintained in -3,4,5-³H]vasopressin (68.5 Ci/mmol), [8-¹⁴C]adenosine phenylalanyl epitope-tagged WT and mutant V2Rs were developed using the CaPO₄ immobilized streptavidin-succinimide (NHS)-Biotin and Immunopure™ autoradiography enhancer, and Entensify™ autoradiography enhancer were purchased from PerkinElmer Life Sciences. DEAE-dextran was from Amersham Biosciences. Paraformaldehyde (16% solution, EM grade) was from Electron Microscopy Sciences (Washington, PA). Polyvinylidene difluoride nylon membranes were from Millipore Corp. (Bedford, MA). Dowex AG50 W-X4 resin, 40% acrylicamide, TEMED, and ammonium persulfate were from Bio-Rad. Adenosine 3',5'-cyclic monophosphate (sodium salt), alumina, aprotinin, [Arg⁸]vasopressin (acetate salt), bacitracin, bovine serum albumin, chloroquine (diphasochrome salt), fetal calf serum, 3-isobutyl-1-methylxanthine, leupeptin, phenylmethlysulfonyl fluoride, soya bean trypsin inhibitor, and Triton X-100 were from Sigma. The 12CA5 monoclonal antibody (100 µg/ml clone 3F10) against the HA epitope tag was obtained from Roche Applied Science, and the Alexafluor-488-conjugated goat anti-rat IgG (2 µg/ml) directed against the hemagglutinin (HA) epitope tagged engineered into the amino terminus of the various V2R structures was obtained from Babco, and the Cy3-conjugated donkey anti-mouse IgG (2 µg/ml) was from Jackson Immunochemicals (West Grove, PA). Rat anti-HA monoclonal antibody (100 µg/ml clone 3F10) against the HA epitope tag was obtained from Roche Applied Science, and the Alexafluor-488-conjugated goat anti-rat IgG (2 µg/ml) was from Molecular Probes, Inc. (Eugene, OR). Protein A-agarose beads were from Vector (Burlingame, CA). EZ-link™ sulfo-N-hydroxysuccinimide (NHS)-Biotin and Immunopure™ immobilized streptavidi- nin-conjugated agarose (for Figs. 1A and 1B) were from Pierce. The 12- and 24.5-mm polycarbonate cell filters (Transwell chambers; 0.4-µm pore size) were obtained from Costar (Cambridge, MA). Aqua-Poly/Mount was from PolySciences Inc. (Warrington, PA). Dulbecco’s modified Eagle’s medium (DMEM) and trypsin/EDTA were prepared by the cell culture core facility sponsored by the Diabetes Research and Training Center at Vanderbilt University Medical Center. All other chemicals were reagent grade.

Cell Lines—Permanent clonal MDCK II cell lines expressing HA epitope-tagged WT and mutant V2Rs were developed using the CaPO₄ method as described previously (7). Briefly, 10 µg of V2DrpD-N-HA, pCMV4N-V2R-L292P, pCMV4N-V2R-R337X, or pCMV4N-V2R-G418 (individual cDNAs encoding HA epitope-tagged human wild type (WT) or mutant V2R) were transfected into the 12- and 24.5-mm polycarbonate cell filters transduced with 2 µg of pRSVneo (cDNA encoding neomycin resistance) into MDCK II cells. Colonies were selected based on resistance to G418, a neomycin analog, and isolated as described previously (7). G418-resistant colonies were screened for WT V2R expression by asaying binding of the radioligand [³H]AVP. V2R mutant-expressing cell lines were screened via immunofluorescence against the HA epitope, using either the mouse 12CA5 monoclonal antibody or the rat anti-HA monoclonal antibody. Parental and stably expressing WT V2R MDCK II cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C, 5% CO₂. Simian kidney fibroblast (COS M6) cells were maintained in supplemented DMEM plus 20% fetal Calf serum. The cultured were membrane filters (Transwell chambers; 0.4-µm pore size) were obtained from Costar (Cambridge, MA). Aqua-Poly/Mount was from PolySciences Inc. (Warrington, PA). Dulbecco’s modified Eagle’s medium (DMEM) and trypsin/EDTA were prepared by the cell culture core facility sponsored by the Diabetes Research and Training Center at Vanderbilt University Medical Center. All other chemicals were reagent grade.

Surface Localization of V2R—Biotinylation studies also allow quantitation of the relative amount of surface versus internalized receptors (MDCK II; COS M6), or apical versus basolateral receptors (MDCK II), even without metabolic labeling. For these studies, one million of WT V2R, or 106 (150-mm dish), or 4.5 × 10⁵ (35-mm dish), 2.0 × 10⁶ (100-mm dish), or 4.5 × 10⁶ (150-mm dish). On the day of transfection, cells were rinsed in PBS prior to incubation with a mixture containing 0.67, 4, or 9 µg of plasmid DNA, respectively, with 500 µg/ml DEAE-dextran for 20 min at 37 °C, 5% CO₂. The DNA/DEAE-dextran mixture was aspirated and replaced with fresh DMEM supplemented with 20 µM HEPES and 100 µM chloroquine and maintained at 37 °C, 5% CO₂ for 2 h. At this time, the medium was aspirated, and the cells were rinsed with PBS and chased in DMEM, 3 mM HEPES, 5% CO₂, 0.28 of the density of WT-expressing MDCK II cells in the absence of SR121463B rescue and 0.31 of this density after pharmacological rescue. The R337X V2R is 0.55 of control V2R expression but increases to 1.18 of control V2R expression with SR121463B treatment. For comparison, the WT V2R increases its total cell content from 1.0 (as defined) under control conditions to 1.06 following overnight SR121463B treatment.

Transient Expression Studies—COS M6 cells were seeded the day prior to transfection at a density of 3.5 × 10⁵ (35-mm dish), 2.0 × 10⁶ (100-mm dish), or 4.5 × 10⁶ (150-mm dish). On the day of transfection, cells were rinsed in PBS prior to incubation with a mixture containing 0.67, 4, or 9 µg of plasmid DNA, respectively, with 500 µg/ml DEAE-dextran for 20 min at 37 °C, 5% CO₂. The DNA/DEAE-dextran mixture was aspirated and replaced with fresh DMEM supplemented with 20 µM HEPES and 100 µM chloroquine and maintained at 37 °C, 5% CO₂ for 2 h. At this time, the medium was aspirated, and the cells were rinsed with PBS and chased in DMEM, 3 mM HEPES, 5% CO₂, 0.28 of the density of WT-expressing MDCK II cells in the absence of SR121463B rescue and 0.31 of this density after pharmacological rescue. The R337X V2R is 0.55 of control V2R expression but increases to 1.18 of control V2R expression with SR121463B treatment. For comparison, the WT V2R increases its total cell content from 1.0 (as defined) under control conditions to 1.06 following overnight SR121463B treatment.

Sulfo-NHS-Biotin Surface Labeling Strategy—Assessment of steady-state localization of the WT V2R, R337X V2R, and L292P V2R in stably expressing MDCK II cell lines was accomplished via covariant labeling of the apical or basolateral surface of polarized MDCK II cells with sulfo-NHS-biotin exactly as described previously. Integrity of the cell monolayer was determined via [³H]methoxyinulin leak assays (7).

Delivery to Cell Surfaces of Polarized MDCK II Cells—Delivery of nascent V2R to the cell surface was examined by metabolic labeling and surface biotinylation strategies, essentially as described previously (6, 7). Stably transfected MDCK II cells expressing WT V2R were incubated in cysteine/methionine-free medium for 2 h prior to a 90-min pulse in Cy5/Met-free medium supplemented with 1 µM [³H]-EXPRESSSS protein labeling mix. At the end of the pulse phase, Transwells were biotinylated at either the apical or basolateral surface with two sequential rounds of sulfo-NHS-Biotin (1 mg/ml). Cells were extracted into ice-cold dodecyl-β-d-maltoside/cholesterol hemisuccinate buffer (4 % and 0.8 mg/ml, respectively, containing 20% glycerol, 25 mM glycyglycine, 20 mM Hepes, 100 mM NaCl, 5 mM EGTA, 100 µM phenylmethlysulfonyl fluoride, 1 µM soybean trypsin inhibitor, 1 µM leupeptin, pH 7.4) and centrifuged at 100,000 × g for 60 min, and the supernatant was sequentially incubated with mouse 12CA5 anti-HA antibody (1.50) and goat anti-mouse IgG (100 µg/ml). The washed resin was eluted with radiolabeled protein affinity assay buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) and the above protease inhibitors at 90 °C. The biotinylated protein eluate was subjected to SDS-PAGE on 7.5% polyacrylamide gels. The gels were incubated for 60 min in EN/HANCE prior to drying and exposure to Eastman Kodak Co. X-Omat film. Data shown are from autoradiograms.

Spatial and Functional Rescue of Mutant V2 Receptors

Immunolocalization of the Wild Type and Mutant V2R—Stably expressing WT V2R MDCK II cells or transiently transfected COS M6 cells were grown on 12-mm glass coverslips (COS M6 cells) or in 12-mm Transwell chambers (MDCK II cells) and maintained for 2 days (cov-
6 hours later the cell culture medium was aspirated and wells were day following transfection and seeded into 24-well plates. Twenty-four the apparent density (also /H11011 V2Rs in MDCK II cells (gt) for this observation other than the realization that the density of serum also did not successfully remove residual SR121463B from tended wash protocols with or without bovine serum albumin and/or were not sufficient in removing the V2R antagonist from MDCK II cells. Using the permeant antagonist. Rescue by lowering culture temperature is not served as controls.

Functional studies of AVP-stimulated cAMP production before and after rescue were restricted to COS M6 cells. Although we could achieve surface rescue with SR121463B in MDCK II cells, we were not able to observe AVP-stimulated cAMP accumulation in polarized (Transwell) or unpolarized (plated) MDCK II cultures following overnight treatment with SR121463B due to inadequate wash-out of this membranepenetrating antagonist. Rescue by lowering culture temperature is not efficient in MDCK II cells, in contrast to COS M6 cells. Using the concentration response for AVP-stimulated cAMP accumulation in WT cells as a monitor of residual SR121463B, we found that wash protocols that had successfully removed SR121463B in COS M6 cell experiments to allow subsequent detection of AVP-stimulated cAMP accumulation were not sufficient in removing the V2R antagonist from MDCK II cells. Residual antagonist masked AVP-stimulated cAMP accumulation, even in WT V2R-expressing cells, which are highly sensitive to AVP. Extended wash protocols with or without bovine serum albumin and/or serum also did not successfully remove residual SR121463B from MDCK II cells after overnight incubation. We do not have an explanation for this observation other than the realization that the density of V2Rs in MDCK II cells (~12 pmol/mg for WT V2R) represents the density of the receptor in all cells, since this is a clonal cell line, whereas the apparent density (also ~12 pmol/mg) for WT V2R in transiently expressing COS M6 cells represents a mean of all densities for cells overexpressing the receptor (i.e., ~12 pmol/mg) and cells expressing no receptor. Perhaps the high receptor expression per cell in COS M6 cells fosters receptor sensitivity to AVP even in the presence of some residual SR121463B. Forskolin does elevate cAMP in WT and mutant V2R-expressing MDCK II cells before and after SR121463B treatment, indicating that the adenyl cyclase system is operative in the cells and that forskolin is able to detect cAMP changes in MDCK II cells if they occur. AVP also readily stimulates cAMP production in WT V2R-expressing MDCK II cells when not pretreated with SR121463B as a “rescue” treatment.

On the day of the assay of cAMP accumulation, COS M6 cells were incubated with prewarmed cell culture medium containing 0.1% bovine serum albumin followed by a single wash with medium. Cells were then washed with fresh prewarmed PBS and incubated with PBS supplemented with 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, for 5 min. Following aspiration, cells were treated with selected concentrations of AVP for 15 min in a final assay volume of 200 μl. Reactions were terminated by adding 750 μl of an ice-cold solution containing 12% trichloroacetic acid, 2 mM cAMP, 2 mM ATP, and [γ-32P]cAMP (~1600 cpm) to permit an assessment of recovery of [γ-32P]cAMP in subsequent purification steps. Cells were then placed on ice for 10 min, after which the reactions were transferred to glass test tubes using disposable transfer pipettes. Each well was washed with 1.05 ml of H2O and combined with its respective harvested sample. After neutralization with 120 μl of 5% NaOH, cellular debris was pelleted by centrifugation (3000 rpm, 10 min at 4 °C). The supernatants were subjected to sequential Dowex and alumina column chromatography to isolate [γ-32P]cAMP. Data are presented as [γ-32P]cAMP/[γ-32P]ATP + [γ-32P]ADP. The value of [γ-32P]ATP + [γ-32P]ADP was estimated from the H cpm eluted from the Dowex column as pass-through (11).

RESULTS
Characterization of Surface Versus Internal Expression of WT V2R and Selected V2R Mutants in COS M6 Cells

To compare trafficking of the HA epitope-tagged WT V2R (referred to as WT V2R throughout) with clinically relevant V2R mutations, we examined the localization of these structures following transient expression in COS M6 cells. Immunostaining of intact cells (no Triton X-100 in the incubation with rat monoclonal anti-HA primary antibody) revealed that the WT V2R was readily detected on the cell surface. Immunostaining was performed on intact cells with rat anti-HA primary monoclonal antibody in the absence of 0.2% Triton X-100 detergent (A–D). Immunostaining to visualize receptors found both at the cell surface and within intracellular compartments was performed with rat anti-HA antibody in the presence of 0.2% Triton X-100 detergent to permeabilize cells (E–H). Labeling with the anti-HA primary monoclonal antibody was detected by an AlexaFluor-488-conjugated goat-anti-rat IgG secondary antibody.

with 120 μl of 5% NaOH, cellular debris was pelleted by centrifugation (3000 rpm, 10 min at 4 °C). The supernatants were subjected to sequential Dowex and alumina column chromatography to isolate [γ-32P]cAMP. Data are presented as [γ-32P]cAMP/[γ-32P]ATP + [γ-32P]ADP. The value of [γ-32P]ATP + [γ-32P]ADP was estimated from the H cpm eluted from the Dowex column as pass-through (11).
FIG. 2. Immunolocalization of the wild type V2R and V2R mutants in COS M6 cells following chemical, pharmacological, and temperature rescue strategies. COS M6 cells expressing WT V2R or the selected V2R mutants were grown on 12-mm glass coverslips and cultured for 2 days prior to fixation and immunostaining as described under “Experimental Procedures.” Twenty-four hours after transfection, cells were left untreated at 37 °C (A–D) or maintained in the presence of 10% glycerol for 12–16 h at 37 °C (E–H) or 10 μM SR121463B for 12–16 h at 37 °C (I–L). To assess the effectiveness of lower growth temperature to facilitate surface expression, cells were maintained for 12–16 h at 28 °C (M–P). Immunostaining was performed under conditions that permitted detection of V2R expression at the cell surface (i.e. where the cells were incubated with rat anti-HA monoclonal antibody in the absence of 0.2% Triton X-100 detergent). Labeling with the rat anti-HA primary monoclonal antibody was detected by an Alexafluor-488-conjugated goat anti-rat IgG secondary antibody.

FIG. 3. Assessment of the fraction of V2R reaching the biotinylated surface of COS M6 cells: Effect of pharmacologic rescue. COS M6 cells expressing WT V2R or the selected V2R mutants were left untreated (□) or incubated with 10 μM SR121463B (▪) overnight at 37 °C prior to incubation with sulfo-NHS-biotin to covalently label the cell surface. Cells were extracted into detergent, the V2R immunoprecipitated using the rat anti-HA antibody and protein A-agarose, and surface proteins subsequently isolated by streptavidin agarose, as described under “Experimental Procedures.” The fraction of receptors on the cell surface agarose was calculated by comparing the amount of V2R in the eluate of streptavidin-agarose with the total amount of V2R in the immunoprecipitate, as determined using Western blot analysis. Data represent the mean ± S.E. from three experiments performed under identical conditions.

Differential Sensitivity of V2R Mutants to Facilitated Rescue

Localization—To determine whether the intracellularly trapped V2R mutants could be rescued to the cell surface, where their functionality could be assessed, cells expressing mutant V2R were incubated with either chemical or pharmacological chaperones or grown at reduced (28 °C) temperature. Subjecting cells to 10% glycerol, a so-called “chemical chaperone” that can rescue some intracellularly trapped proteins (presumably by protein stabilization (13)), had no effect on the redistribution of mutant V2Rs nor on the WT V2R (Fig. 2, compare E–H with A–D). The lack of surface expression in glycerol-treated cells was not due to differences in protein expression level, since each of the V2R mutants was synthesized but was trapped intracellularly when examined in permeabilized cells (data not shown).

In contrast, treatment with the membrane-permeant V2R antagonist SR121463B dramatically increased detectable V2R expression at the surface for all mutant structures examined (Fig. 2, J–L). It had been previously demonstrated that application of SR121463B increased cell surface expression of other selected V2R mutants, presumably due to promotion or stabilization of properly folded receptors during their maturation in intracellular compartments (14, 15). Moreover, subjecting COS M6 cells to reduced temperature during culture enhanced cell surface localization of the V2R mutants to an extent comparable with pharmacological rescue (Fig. 2, N–P), suggesting that 28 °C is permissive for appropriate folding and trafficking events for these structures in COS M6 cells.

We utilized cell surface biotinylation strategies in COS M6 cells to provide quantitative information regarding the delivery of mutant V2R to the surface following rescue with temperature or SR121463B, using WT V2R as a comparator, as shown in Fig. 3. For these calculations, we defined the amount of WT V2R on the surface as 100%; in our studies, we observed that 38% of the total WT V2R expressed in COS M6 cells was on the surface in the absence of SR121463B. SR121463B treatment leads to rescue of the L292P V2R from 8.0 to 38.2% of WT V2R surface expression; the R337X V2R from 22.0 to 88.9% of WT V2R surface expression; and the ΔV278 V2R from 5.3 to 21.2% of WT V2R surface expression (Fig. 3). Interestingly, of the receptor expressed at the surface following exposure to SR121463B, virtually all of the WT V2R, R337X V2R, and ΔV278 V2R was in the mature glycosylated form, whereas none of the L292P V2R was detected in the mature glycosylated form (data not shown).

For V2R expressed in COS M6 cells and exposed to 28 °C culture, quantitatively less mutant V2R was expressed at the cell surface overall. Furthermore, in contrast to findings for rescue by SR121463B, only ~50% of the R337X and ΔV278 V2R that reaches the cell surface achieves the mature glycosylated
Spatial and Functional Rescue of Mutant V2 Receptors

Fig. 4. Functional assessment of wild type V2R and V2R mutants in COS M6 cells: Effect of temperature rescue. COS M6 cells expressing WT V2R or the selected V2R mutants were seeded in 24-well culture plates and labeled with [3H]adenine prior to assessment of cAMP accumulation as described under “Experimental Procedures.” Cells were left untreated at 37 °C (A) or maintained for 12–16 h at 28 °C (B) prior to assessment of basal (□) and AVP (1 μM, ■)-mediated cAMP accumulation to determine whether temperature-rescued V2R mutants were functionally responsive and capable of mediating cAMP production. Data represent the mean ± S.E. from 7–12 experiments performed in duplicate under identical conditions. *, p < 0.05 determined using one-way analysis of variance and the Student-Newman-Keuls multiple comparisons test.

Fig. 5. Functional assessment of wild type V2R and mutant L292P V2R in COS M6 cells: Effect of pharmacological rescue. COS M6 cells expressing WT V2R or the mutant L292P V2R were seeded in 24-well culture plates and labeled with [3H]adenine prior to assessment of cAMP accumulation as described under “Experimental Procedures.” Cells were left untreated at 37 °C or subjected to 10 μM SR121463B for 12–16 h at 37 °C. Cells were washed extensively prior to assessment of basal (□) or 1 μM AVP (■)-mediated cAMP accumulation to determine whether or not the membrane-permeant V2R antagonist-dependent rescue permitted functional V2R responsiveness to AVP. Data represent the mean ± S.E. from six experiments performed in duplicate under identical conditions. *, p < 0.05 determined using one-way analysis of variance and the Student-Newman-Keuls multiple comparisons test for AVP-mediated cAMP production in cells treated with the membrane-permeant V2R antagonist SR121463B versus control (no drug treatment) cells.

Function—To determine whether V2R mutants rescued to the surface were capable of binding AVP, coupling to G proteins, and activating effector systems, we examined AVP-mediated cAMP accumulation following rescue treatments. COS M6 cells expressing WT V2R or V2R mutant structures were maintained at 37 °C or 28 °C and subsequently assessed for basal or AVP-stimulated cAMP accumulation. Cells expressing WT or mutant V2Rs demonstrated no significant alterations in basal cAMP accumulation when maintained at 37 or 28 °C (Fig. 4, A and B, open bars). Whereas AVP treatment of intact cells expressing WT V2R resulted in receptor-stimulated cAMP accumulation, cells expressing each of the V2R mutants at 37 °C were markedly unresponsive to AVP-mediated cAMP production (Fig. 4A, black bars). However, when subjected to temperatures that facilitated proper plasma membrane receptor localization, the V2R mutants L292P V2R and R337X V2R were capable of binding AVP and mediating AVP-stimulated cAMP accumulation (Fig. 4B, black bars). Cells expressing the ΔV278 V2R were unresponsive to AVP following incubation at 28 °C (Fig. 4B), despite surface localization of this mutant receptor under these conditions (Fig. 2D). Thus, although the ΔV278 V2R can be spatially rescued to an extent apparently comparable with the L292P or R337X V2R structures, this X-linked NDI allele must also possess defective AVP binding, coupling, and/or signaling properties (16).

Despite temperature and pharmacological rescue of the L292P and R337X mutant V2R alleles, these mutant V2Rs were incapable of achieving AVP-mediated cAMP production quantitatively comparable with wild type V2R (Figs. 4B and 5). AVP dose response curves for mutant receptors rescued by incubation at 28 °C revealed that L292P V2R and R337X V2R display a rightward shift in their ability to elicit AVP-mediated cAMP production as well as a reduced maximal AVP stimulation (Fig. 6). These findings explain our inability to detect [3H]AVP binding to the L292P V2R and R337X V2R, even following surface rescue by culturing COS M6 cells at reduced (28 °C) temperature (data not shown). The reduced affinity of this receptor for agonist ligand, probably due either to alterations in the binding pocket or in coupling to G proteins, prevents detection of [3H]AVP-V2R complexes when assessed via radioligand binding assays. Due to the amplification of the receptor signal in the cAMP accumulation assay, we are nonetheless able to detect a functional receptor (as assessed by its ability to couple to adenylyl cyclase stimulation) once rescued to the cell surface (Figs. 4B and 5).

We were surprised that temperature and V2R antagonist treatment partially restored AVP-mediated signaling in cells expressing the R337X V2R, since previous studies had shown...
that the R337X V2R demonstrated poor, if any, functional recovery following SR121463B treatment (15). Nonetheless, whereas this mutation introduces a premature stop codon truncating the receptor upstream of the cysteine palmitoylation sites (17, 18), our findings that R337X V2R can activate adenyl cyclase when rescued to the surface suggest that V2R amino acid residues carboxyl to the palmitoylation sites are not absolutely required for V2R coupling to adenyl cyclase stimulation, consistent with previous findings employing V1R/V2R chimeras showing that V2R coupling to Gαs is principally determined by the third intracellular loop of the V2R (19).

Processing and Cell Surface Localization of WT and Mutant V2R in Polarized MDCK II Cells

To examine the properties of the human WT V2R and assess its steady state localization, we stably expressed the WT V2R in renal epithelial (MDCK II) cells, a model system that facilitates the examination of the trafficking of surface proteins to polarized surfaces (4–8). As shown in Fig. 7A, the mouse 12CA5 monoclonal antibody directed against the HA epitope of the V2R reveals that this receptor is expressed at the cell surface (right panels, XY scan); a Z scan of these cells reveals that this expression is enriched at the lateral subdomain (right panels, Z scan). The lack of immunofluorescence in parental (nontransfected) MDCK II cells provides confidence that the fluorescence detected is due to the HA epitope-tagged WT V2R expressed in this clonal MDCK II cell line (left panels). The laterally enriched pattern of V2R expression in polarized MDCK II cells is consistent with the basolateral localization of V2R in vivo.

To complement these morphologic studies, we employed metabolic labeling and surface biotinylation strategies to examine the delivery of the WT V2R to the basolateral surface. This strategy allows for the relative quantitation of the amount of newly synthesized receptor that is delivered to a particular surface (apical or basolateral) in polarized MDCK II cells and has been employed successfully for monitoring receptor delivery of each of the α2-AR subtypes as well as other GPCRs (4–8). As shown in Fig. 7B, the WT V2R appears to be directly delivered to the basolateral surface, since the majority of WT V2R is detected in streptavidin eluates from basolaterally biotinylated MDCK II cells, whereas little WT V2R is eluted from streptavidin-agarose when the apical membrane is biotinylated. These findings have been corroborated following varying metabolic labeling time periods (15–120 min; data not shown), demonstrating that the receptor is delivered principally to the basolateral surface and remains there at steady state. These data independently confirm our morphological findings (Fig. 7A) and those of previous investigators (20) that WT V2R is enriched on the basolateral surface of polarized MDCK II cells.

The V2R mutants that could achieve AVP signaling after surface rescue in COS M6 cells also were studied in permanent transformants of MDCK II cells. As shown in Fig. 8, when compared with the WT V2R, the mutant V2Rs are retained intracellularly following polarization in MDCK II cells grown in Transwell culture and are not detectable when the primary antibody is exposed to fixed MDCK II cells in the absence of 0.2% Triton X-100 (Fig. 8, A–C, respectively). In polarized MDCK II cells, like COS M6 cells, reduced temperature is not as efficacious as in COS M6 cells for surface rescue of mutant V2Rs, and rescue at this temperature for MDCK II cells is in fact difficult to demonstrate at all (data not shown). However, treatment with SR121463B indeed rescues at least a fraction of the mutant V2Rs to the surface (Fig. 8, compare E and F with H and I, respectively).

To more rigorously document the localization of these mutant V2Rs following pharmacological rescue, the basolateral versus the apical surfaces of V2R-expressing MDCK II cells were biotinylated, and the relative quantity of basolateral versus apical signal was evaluated for each V2R structure, with or without prior treatment with SR121463B (Fig. 9). As demonstrated in metabolic labeling studies (Fig. 7B), the WT V2R is selectively targeted to the basolateral surface independent of SR121463B treatment (Fig. 9A, lane 3 versus lane 4). Quantitatively, 79–82% of the WT V2R is expressed at this surface with or without SR121463B.

Unlike the WT V2R, 0.1% of the mutant R337X (Fig. 9B, lane 7) and none of the L292P (Fig. 9C, lane 11) V2Rs appear on the basolateral surface until rescue with SR121463B (Fig. 9, B.
Spatial and Functional Rescue of Mutant V2 Receptors

Fig. 8. L292R and R337X V2 vasopressin receptor are trapped intracellularly in polarized MDCK II cells but can be rescued to the cell surface with the V2R antagonist, SR121463B. MDCK II cells stably expressing the WT or mutant V2 vasopressin receptor were grown for 5 days on 12-mm Transwells at 37°C, fixed with 4% paraformaldehyde, and immunostained as described under “Experimental Procedures.” A–C, incubated with primary antibody in the absence of Triton X-100 and thus representing V2R available on the surface. D–I, incubated with primary antibody in the presence of 0.1% Triton X-100 and representing both surface and intracellular V2R. G–I, treated with a 10 μM concentration of the cell-permeant V2 vasopressin receptor antagonist SR121463B overnight. The rat anti-HA monoclonal antibody was detected by the Alexafluor-488-conjugated goat anti-rat IgG secondary antibody. Localization of the HA epitope tag was analyzed on a Zeiss LSM 410 laser confocal microscope, where the XY is presented in the lower panel of each image. Z scans shown in the upper panel display a laser-sectional side view of the V2R-expressing MDCK II cells. The yellow line across each XY scan represents the laser sectioning that led to the Z scan.

(lane 8) and C (lane 12)). Following SR121463B treatment, 25% of the total cellular R337X V2R achieves expression at the basolateral surface (Fig. 9B, lane 8); these rescued R337X V2R have achieved mature N-linked glycosylation indistinguishable from the WT receptor (cf. Fig. 10). For the L292P V2R, pharmacological rescue also results in basolateral expression of a mature glycosylated form of the receptor (Fig. 9C, lane 12), but this represents only 9% of the total cellular L292P V2R content. Surprisingly, in the absence of SR121463B, the L292P V2R appears at the apical surface (Fig. 9C, lane 9); SR121463B treatment enriches this apical expression by 5% (from 17% of the total cellular L292P V2R in the absence of SR121463B treatment to 22% of the total cellular L292P V2R content following antagonist treatment) (Fig. 9C, lane 10). This apically expressed L292P V2R represents the immature glycosylated form of the receptor, based on its relative migration (cf. Fig. 9C, lanes 9 and 10, and Fig. 10, lanes 4–6) and its sensitivity to Endo H (data not shown).

Maturation of the N-glycosylated V2R can be evaluated by sensitivity to Endo H and PNGase F. On SDS-PAGE, WT V2Rs migrate at ~45–55 kDa and ~38–40 kDa (Fig. 10A); V2R oligomers also can be detected and migrate at ~100 kDa (16). The 38–40-kDa lower molecular mass form is sensitive to endoglycosidase H, an enzyme that cleaves the mannose-rich immature glycosylated forms of cell surface glycoproteins (21), suggesting that this is a precursor of the WT V2R. In contrast, the higher mass WT V2R is sensitive to degradation by PNGase F, which cleaves all N-glycosylated moieties (21), but is insensitive to endoglycosidase H, suggesting that the 45–55-kDa form represents the mature, N-glycosylated form of the receptor. The PNGase F-sensitive form of the mature WT V2R is not fully cleaved to a mass consistent with nonglycosylated V2R (predicted mass based on the sum of the mass of component amino acids). This is because the V2R is also O-glycosylated, and these sites are resistant to the endoglycosidase H or PNGase F enzymes (22).

The mutant L292P V2R and R337X V2R migrate at ~38–40 and ~30 kDa, respectively, without temperature or pharmacological rescue. These forms correspond to the immature glycosylation forms of these two receptor structures, as evidenced by their sensitivity to both endoglycosidase H and PNGase F (Fig. 10, B and C).

DISCUSSION

Appropriate receptor localization constitutes an essential first step in allowing target cells to respond correctly to extracellular cues. Localization may also define, at least in part, selective interactions between receptors and effectors in surface microcompartments. Mislocalization of receptors due to inherited mutations plays a significant role in the etiology of multiple disease states, as reviewed by Edwards et al. (23). In the case of human alleles of the V2R (the model system evaluated in this study), more than 70% of the receptor-coding mutations that lead to X-linked NDI are due to intracellular receptor accumulation (24). V2Rs trapped inside cells are unresponsive to the physiological hormone, AVP, which does not readily cross the membrane surface. Retained mutant V2R cannot participate in coupling to adenyl cyclase and subsequent cAMP production and therefore cannot recruit preformed aquaporin 2 channels to the apical surface of renal epithelia (24).

The present studies evaluate three clinically relevant mutant V2Rs, L292P, A278V, and R337X, document that these alleles encode mutant receptors that are trapped intracellularly. Our findings are the first to show that these alleles are temperature-sensitive and can be rescued to the surface by incubation of mutant V2R-expressing COS M6 cells at reduced temperature (28°C). To our knowledge, these findings represent the first data identifying temperature-sensitive V2R mutants. Polytopic proteins that manifest temperature-sensitive protein folding defects have been described previously and include mutants of the lutropin/choriogonadotropin GPCR (25), the ΔF508 cystic fibrosis transmembrane regulator (26), and the N470D HERG potassium channel (13). These folding defects give rise to abnormal proteins that are nonfunctional and/or result in retention within cytosolic compartments. Reduction in growth temperature is interpreted to foster proper protein folding, manifest as increased cell surface expression, as is the case for the V2R mutants examined here.

The present studies also demonstrate that X-linked NDI V2R mutants can be rescued to the surface pharmacologically, by binding of a membrane-permeant V2R antagonist (SR121463B), by analogy with other pharmacologically rescued alleles of the V2R (15). We know, however, that rescue to the surface is not necessarily sufficient to achieve V2R function, as exemplified by the ΔV278 V2R. This mutant allele does not couple to cAMP production following surface rescue, suggesting that this molecule has deficient binding, deficient coupling to Gα12, or both (19). Even L292P V2R and R337X V2R, after rescue to the surface, do not manifest quantitatively similar cAMP stimulation responses as the WT V2R. Thus, the maximal stimulation of cAMP that these alleles can achieve is reduced compared with WT V2R, and the concentration-response curve for cAMP production in response to AVP is moved rightward, consistent with the interpretation that binding or coupling to Gα12 (or both) is still perturbed in the L292P and R337X V2R despite rescue at permissive temperatures.
As expected, the intracellularly trapped V2R mutants reflect immature glycosylated forms of the receptor and are sensitive to digestion by endoglycosidase H. Moreover, basolateral delivery of pharmacologically rescued L292P V2R and R337X V2R occurs principally for the mature glycosylated form of the receptor. Therefore, it appears that rescue (and presumably normal trafficking) to the appropriate surface of polarized renal epithelial cells involves a conformation of the receptor that is capable of migration through the Golgi network to achieve mature N-linked glycosylation as well as delivery to basolaterally targeted vesicles. However, this interpretation is not meant to imply that receptor glycosylation is an absolute requirement for basolateral delivery, since mutations of the WT V2R that eliminate N-linked glycosylation of the receptor do not alter basolateral targeting (27). These findings are similar to those for other GPCRs, such as the α2A-adrenergic receptor; mutations that eliminate N-linked glycosylation of the receptor do not perturb direct basolateral delivery of the receptor (7). We further substantiated the lack of a requirement for N-linked glycosylation of the V2R for direct basolateral delivery by demonstrating that overnight incubation with 5 μg/ml tunicamycin resulted in the elimination of N-linked glycosylation of nascent V2R, but these V2Rs (~40 kDa) remained correctly targeted to the basolateral surface of polarized MDCK II cells (data not shown).

In light of our finding that appropriate delivery to the basolateral surface involves a conformation of the V2R that is also capable of mature glycosylation and migration through the Golgi to basolaterally targeted vesicles in the trans-Golgi network, we were surprised to observe that a fraction of the immature glycosylated form of the L292P V2R is delivered to the cell surface in the absence of temperature-sensitive or pharmacological rescue but that this delivery is to the "wrong" (i.e. apical) surface of polarized MDCK II cells (Fig. 9C). In fact, occasionally we could detect this apical L292P V2R on the apical surface in confocal images (Fig. 8, compare A and I). We do not know by what vesicular pathway the immature glycosylated form of the L292P V2R reaches the apical surface; however, this apically delivered L292P V2R remains sensitive to degradation by Endo H, confirming that it represents an immature glycosylated form of the receptor. Notably, the mature, glycosylated form of the L292P V2R is found only on the basolateral surface after pharmacological rescue. These data are consistent with binding of SR121463B to intracellularly accumulated L292P V2R, resulting in stabilization of a fraction of the V2R in a conformation capable of attaining both mature glycosylation and correct localization to the appropriate (i.e. basolateral) surface. We note that it is only for the L292P V2R that we see significant escape to the apical surface. This may be because this mutation is predicted to occur within the bilayer region of the V2R and may lead to substantial misfolding of the receptor, of which only a small fraction is successfully refolded in the presence of SR121463B. In contrast, R337X V2R represents a structure with only a truncation in the C-terminal tail, and misfolding may not be as extreme as for the L292P V2R. Indeed, we always observe quantitatively more rescue of the mature form of R337X than L292P V2R to the basolateral surface when comparing total cell extracts in the absence versus the presence of SR121463B, especially following the V2R antagonist treatment.

Several mutants, such as ΔF508 cystic fibrosis transmembrane regulator in cystic fibrosis (26) and the N470D HERG potassium channel in congenital long QT syndrome (13), have been demonstrated to be defective in protein trafficking but are capable of wild type-like function when rescued to the cell surface. We interpret the rescue of cell surface expression and AVP-mediated receptor function of the L292P and R337X V2Rs to indicate that the proposed use of intermittent therapy with membrane-permeant V2R antagonists for treatment of X-linked NDI has the potential to be effective in patients with...
these particular alleles, because the mature form of the receptor is selectively delivered appropriately to the basolateral surface. Furthermore, therapeutic intervention would not be confounded by the unexpected findings of apical delivery of immature products of mutant V2R alleles, because AVP is only delivered physiologically to the basolateral surface of renal epithelial cells. Identification here of temperature-sensitive and pharmacologically rescued mutant V2R alleles provides tools for further dissection and elucidation of molecular players involved in appropriate V2R (and GPCR in general) maturation and delivery to the cell surface of polarized cells.

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