Charged Residues of the Conserved DRY Triplet of the Vasopressin V1a Receptor Provide Molecular Determinants for Cell Surface Delivery and Internalization

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ABSTRACT

The highly conserved “Asp-Arg-Tyr” triplet in the distal region of the third transmembrane region of most G-protein-coupled receptors is implicated in their activation process and mediation of G-protein signaling. The aim of this study was to determine whether specific features at this locus are important for the vasopressin V1a receptor (V1aR) by performing site-directed mutagenesis. In transfected HEK 293T cells, mutation of Asp (D148A) resulted in a misfolded receptor that was nonfunctional, localized intracellularly, and not constitutively active. Nonconservative (D148R) substitution was not expressed, whereas asparagine (D148N) partially restored cell surface expression, although no specific ligand-binding or inositol phosphate signaling was detected. In contrast, conservative (D148E) substitution was expressed moderately higher, bound ligands, and signaled similarly to a hemagglutinin epitope-tagged wild-type receptor. However, D148E showed a greater tendency to be internalized once it was delivered to the membrane. Individual replacements of the conserved argine and tyrosine (R149A, Y150A) led to decreased signal transduction without affecting surface expression, agonist affinity, or internalization or increasing basal signaling activity. Incorporation of aspartate (R149D) or reversal of charges (D148R/R149D) were nonfunctional, localized intracellularly, and indicated the absence of an ionic interaction between Asp-148 and Arg-149. It is noteworthy that an important role of arginine was identified for regulating agonist-mediated internalization when a histidine (R149H) was present. This mutant was expressed on the cell surface but was rapidly internalized after agonist treatment. This study highlights the importance of specific charged residues within this motif that provide important determinants for cell surface delivery, internalization and for normal V1aR function.

G-protein-coupled receptors (GPCRs) form a large and functionally diverse superfamily that represents ∼1 to 2% of encoded genes in the human genome. Despite being activated by a variety of stimuli, from photons to glycoproteins (Kristiansen, 2004), these receptors exhibit primary sequence homology and a conserved tertiary structure comprising a bundle of seven transmembrane (TM) domains. Although much is known about some of the structural features involved in the binding of ligands, the actual mechanism for ligand activation is less well defined. Agonist occupancy of GPCRs is believed to result in conformation changes that lead to activation of specific G-proteins (Karnik et al., 2003; Wong, 2003). Studies with mutant GPCRs suggest that intracellular loops (particularly the second and third) and the cytoplasmic C terminus provide important epitopes for a number of signaling and regulatory proteins, including G-proteins, arrestins, and G-protein-receptor kinases (Wong, 2003; Tan et al., 2004). One highly conserved triplet of amino acids (Asp-Arg-Tyr) is located at the interface of TM-III and second intracellular loop (IC2) in class I “rhodopsin-like” GPCRs (Fig. 1). This “DRY” motif has been described to provide a pivotal role in signal transduction of GPCRs. The aspartate (or glutamate in rhodopsin) has been reported to be important.
tant for stabilizing intramolecular interactions, notably with the neighboring arginine, thereby constraining GPCRs in the inactive (R) conformation. Mutation of this Asp/Glu disrupts this constraint and has resulted in the ability of some GPCRs to adopt an active conformation (R*) state (Scheer et al., 1996, 1997). This conformational change is hypothesized to reposition the arginine from a polar pocket and is considered to be important for interaction with G-proteins (Ballesteros et al., 1998; Scheer et al., 2000).

Mutagenesis studies in a number of receptors have demonstrated the importance of this arginine, including the well documented α₁b-adrenergic receptor (AR) (Scheer et al., 2000), which showed increased agonist-binding affinities but impaired receptor signaling by decreasing its ability to couple to G-proteins. In other receptors, mutation has resulted in impaired receptor signaling with decreases in agonist binding (Jones et al., 1995; Chung et al., 2002; Capra et al., 2004).

Naturally occurring mutations have been identified that result in receptor dysfunction and are responsible for certain diseases [e.g., nephrogenic diabetes insipidus (NDI) (Morello et al., 1996, 1997). This conformational change is hypothesized to reposition the arginine from a polar pocket and is considered to be important for interaction with G-proteins (Ballesteros et al., 1998; Scheer et al., 2000).

The position of the engineered HA-epitope tag is indicated. Branched structures indicate the positions of some receptors [e.g., histamine H₂ receptor (H₂R) (Alewijnse et al., 2000), α₁b-AR (Scheer et al., 1997), and β₂-AR (Rasmussen et al., 1999)] but not for others, where only effects on receptor expression were reported [e.g., m1 muscarinic receptors (mACHr); Lu et al., 1997]. The tyrosine residue is the least conserved and studied among this triad sequence, with cysteiny1, histidyl, and serine residues occurring in some GPCRs, such as oxytocin (OT), V₂ vasopressin (AVP), and gonadotropin-releasing hormone (GnRH) receptors, respectively. Together, these observations have given rise to the possibility that the DRY motif may not have the same function in all GPCRs.

The neurohypophysial hormones AVP and OT are structurally-related nonapeptides that mediate a plethora of physiological functions, including vasopressor and antiuretic actions, by binding to specific receptors (Gimpl and Fahrendholz, 2001; Thibonnier et al., 2001a). At present, four AVP/OT receptor subtypes (V₁αR, V₁bR, V₂R, and OTR) have been cloned from different species and constitute a subfamily of the larger GPCR superfamily that possesses discrete but related pharmacological profiles. The V₁αR is widely expressed and mediates nearly all of the actions of AVP, except for antiureasis (renal V₂R) and corticotropin secretion (pituitary V₁bR). AVP mediates vascular smooth muscle (V₁aRs) contraction and regulates cardiovascular function (Thibonnier et al., 2001a). In contrast, OT results in contraction of uterine myometrium (OTRs) during labor and mammary myoepithelium to elicit lactation (Gimpl and Fahrendholz, 2001). With the exception of the V₁bR (which couples to adenylyl cyclase), these receptors couple to Gα₉/₁₁, thereby generating inositol 1,4,5-trisphosphate and diacylglycerol as second messengers. So far, identification of domain(s) involved in G-protein coupling and in receptor activation has been limited. The importance of the IC2 region in Gα₉/₁₁ coupling (Liu and Wess, 1996) and C terminus for the V₁aR has been proposed (Hawtin et al., 2001; Thibonnier et al., 2001b). However, the functional significance of the highly conserved DRY motif for the V₁aR has not yet been determined. The aims of the present study were to examine the functional consequences of inserting conservative and nonconservative mutations in this motif for the V₁aR.

**Materials and Methods**

**Materials.** The cyclic antagonist 1-β-mercaptoprol-β,β-cyclopentamethylene proponic acid, 2-O-methyltyrosine AVP (CA) was purchased from Bachem (St. Helens, UK). AVP and the linear antagonist phenyl acetyl-D-Tyr(Me)2Arg6Tyr(NH2)9AVP were from Sigma (Poole, UK). SR 49059 was obtained from Sanofi Recherche (Toulouse, France). Cell culture media and supplements were purchased from Invitrogen (Uxbridge, UK). Modifying enzymes including BspTI and BshTI were obtained from MBI Fermentas (Sunderland, UK). All other reagents were of analytic grade and obtained from various commercial suppliers.

**Mutant Receptor Constructs.** Mutation of the V₁aR was made using a PCR approach as described previously (Hawtin et al., 2002). The wild-type rat V₁aR was modified to contain a unique BshTI restriction site (underlined) using the primer 5'-ACA-GCC-GAC-CGG-TAC-ATC-GCC-GTGCG-CAC-GGC-GAT-GTA-CCG-GC-3' containing the appropriate base change without affecting the coding sequence (bold). The PCR product was subcloned (HindIII and KpnI) into the mammalian expression vector pcDNA3 containing a previously engineered hemagglutinin (HA)-epitope tag incorporated after the initiation methionine in the amino terminus of the wild-type V₁aR sequence (Hawtin and Wheatley, 1997). This construct was further modified by incorporating a single base change (bold) for a unique BspTI restriction site (underlined) using the primer 5'-GCC-GAC-CGG-TAC-ATC-GCC-GTGCG-CAC-GGC-GAT-GTA-CCG-GC-3'. A BshTI/KpnI product was subcloned into the pcDNA3 construct above to give pcDNA3-[BshTI/BspTI]V₁aR.

GTT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CCG-GT

using [BshTI/BspTI]V1αR-pcDNA3 as template. Each primer con-

ferred previously (Hawtin et al., 2002). In brief, 16 h after trans-

non-specific binding was determined with 3% (w/v) BSA in TBS for 45

duced by liquid scintillation spectroscopy. EC50 values were deter-

Cell Surface Expression of Mutant Receptors. Cell surface expres-

incubation with secondary goat anti-mouse conjugated alkaline phosphatase (Sigma) diluted to

indicated) and incubated at 25°C for 90 min to establish equilibrium. Each well

Cells were seeded at a density of 7.5 × 104 cells/well in poly-d-lysine–coated 24-well

preparataion of HEK 293T cells was prepared 36 h after transfection as

Materials and Methods.

Hawtin and BspTI restriction sites. All receptor constructs were

Radioligand Binding Assays. A washed cell membrane prepa-

Radioligand binding assays were performed in MultiScreen HTS 96-well opaque plates

K

trapping was defined by liquid scintillation spectroscopy. EC50 values were deter-

myo-2-3H]inositol (20.0 Ci/mmol; MP Biomedicals, Irvine, CA) and diluted in 10 ml of water. After the

Expression of mutant V1αR constructs was determined by using an indirect ELISA-based method. In brief, HEK 293T cells were seeded at a density of 7.5 × 104 cells/well in poly-d-lysine–coated 24-well plates and transfected as described above. After 36 h, cells were fixed with 3.7% (v/v) formaldehyde in TBS (20 mM Tris and 150 mM NaCl, pH 7.5) for 15 min at 25°C. Cells were washed three times with TBS. Nonspecific binding was blocked with 3% (w/v) BSA in TBS for 45 min. The anti-HA primary antibody (HA-7; Sigma) was diluted to 1:30,000 in 3% (w/v) BSA/TBS before the addition to each well for 60 min at room temperature with occasional shaking, followed by three gentle washes with TBS. Cells were briefly reblocked with 3% (w/v) BSA in TBS for 15 min, before incubation with secondary goat anti-mouse conjugated alkaline phosphatase (Sigma) diluted to 1:20,000 in 3% (w/v) BSA/TBS for 60 min with occasional shaking. Cells were washed three times with TBS, before the addition of 250 µl of the colorimetric alkaline phosphate substrate p-nitrophenol phosphate (5 mM) dissolved in diethanolamine buffer (1 M dietha-

Eagle's medium (Invitrogen) containing 1% (v/v) fetal calf serum and

expression was based on the following equation:

AVP-induced Inositol Phosphate Production. HEK 293T cells were seeded at a density of 7.5 × 104 cells/well in poly-d-lysine–

EC50 was defined as 100 × [(ODstimulated – ODmock)/(ODbasal – ODmock)]. All experiments were performed in triplicate for each condition and values were obtained from at least three separate experiments.

Immunohistochemistry. HEK 293T cells were seeded in 24-well plates and transfected with receptor mutant cDNA using Transfast as described above. After 36 h, media from cells was replaced with fresh, prewarmed growth media. To promote V1αR internalization, cells were exposed to AVP receptor mutant cDNA using Transfast as described above. After

ELISA. Cells were fixed and washed with PBS before blocking with 10% (v/v) goat serum in PBS for 30 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

24 h using Transfast (Promega Corp., Southampton, UK) as de-

the elution of inositol and glycerophosphoinositol (10 ml of 25 mM NH4COOH containing 0.1 M HCOOH), a mixed inositol fraction containing mono-, bis-, and trisphosphates was eluted with 5 ml of 850 mM NH4COOH containing 0.1 M HCOOH, mixed with Ultima-

Radioactive binding was measured using a TopCount NXT scintillation counter (PerkinElmer) and radioactivity-quant-

Non-specific binding was determined in parallel incubations using 10 nM radioligand and a 100-fold excess of unlabeled radioligand.

Cells were seeded at a density of 7.5 × 104 cells/well in poly-d-lysine–coated 24-well

EC50 values were determined by nonlinear regression after fitting of logistic sigmoidal curves to the experimental data.

The data was analyzed by nonlinear regression to fit theoretical Langmuir binding isotherms to the experimental data using Prism 4 (GraphPad Software Inc., San Diego, CA). Individual IC50 values obtained for competing ligands were corrected for radioligand occupancy as described previously (Cheng and Prusoff, 1973) using the radioligand affinity (K) experimentally determined for each construct.

Ligand binding parameters were determined by Scatchard plots or

Incubations were terminated by addition of ice-cold 0.1 M HCOOH for 30 min. Samples were loaded onto Bio-Rad AG1-X8 columns (formate form; Hercules, CA) and diluted in 10 ml of water. After the

Immunohistochemistry. HEK 293T cells were seeded in 24-well plates containing poly-d-lysine-coated glass cover slips (12 mm) and transfected using Transfast as described above. Cells were fixed and washed with TBS as described previously for the ELISA. Cells were blocked with 3% (w/v) BSA/TBS containing glycerine [1% (w/v)] for 45 min, followed by incubation with anti-HA primary antibody (diluted to 1:3000 in 3% (w/v) BSA/glycine/TBS for 60 min). Cells were washed three times with TBS before blocking 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, cover slips were mounted on glass slides before confocal microscopy.

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 in humidified 5% (v/v) CO2 in air at 37°C. Cells were seeded at a density of approximately 5 × 104 cells/100 mm dish and transfected after 48 h using a calcium phosphate precipitation protocol with 10 µg of DNA/dish.

Radioligand Binding Assays. A washed cell membrane prepa-

Radioactive binding was measured using a TopCount NXT scintillation counter (PerkinElmer) and radioactivity-quantified by liquid scintillation spectroscopy. EC50 values were determined by nonlinear regression after fitting of logistic sigmoidal curves to the experimental data.

V1αR-pcDNA3 as template. Each primer con-

Radioligand binding assays were performed in MultiScreen HTS 96-well opaque plates

Cell Culture and Transfection. Human embryonic kidney (HEK) 293T cells were routinely cultured in 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 twice with PBS, then incubated in inositol-free media containing 10 mM LiCl for 30 min, after which AVP was added at the concentrations indicated for a further 30 min.
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 HeNe laser at 543 nm and a long pass filter at 560 nm. 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 LSM software (Zeiss, Jena, Germany).

### Results

**Pharmacological Characterization of Alanyl-Substituted DRY Mutant V1aR Constructs.** The aim of this study was to establish whether the highly conserved DRY motif is important for ligand binding, signaling, and agonist-mediated internalization of receptors. Based on the crystal structure of rhodopsin (Palczewski et al., 2000), the DRY motif is embedded within TM3 at the interface of the IC2 region (Fig. 1). Indeed, the Arg149 within this motif [i.e., Arg(3.50)] is one of the most conserved residues in GPCRs. All members of the neurohypophysial peptide hormone receptor family cloned so far have an aspartyl (3.49) and arginyl (3.51) at these loci (Fig. 1). In contrast, the residue at position 3.51 is less conserved within this family. A histidyl residue is found only in V2Rs, with cysteines present for all species of OTRs. In the case of V1aRs and V1bRs, a tyrosine residue is absolutely conserved (Fig. 1).

To identify the contribution to ligand binding provided by individual residues within this DRY motif, residues Asp148, Arg149, and Tyr150 of the V1aR were individually mutated to alanine to give [D148A]V1aR, [R149A]V1aR, and [Y150A]V1aR, respectively. Each mutant receptor construct was expressed in HEK 293T cells and characterized pharmacologically. Based on the crystal structure of rhodopsin (Palczewski et al., 2000), the DRY motif is embedded within TM3 at the interface of the IC2 region (Fig. 1). Indeed, the Arg149 within this motif [i.e., Arg(3.50)] is one of the most conserved residues in GPCRs. All members of the neurohypophysial peptide hormone receptor family cloned so far have an aspartyl (3.49) and arginyl (3.51) at these loci (Fig. 1). In contrast, the residue at position 3.51 is less conserved within this family. A histidyl residue is found only in V2Rs, with cysteines present for all species of OTRs. In the case of V1aRs and V1bRs, a tyrosine residue is absolutely conserved (Fig. 1).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist Antagonists</th>
<th>Cell Surface Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA</td>
<td>LA</td>
</tr>
<tr>
<td>Wt</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>D148A</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>R149A</td>
<td>2.6 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Y150A</td>
<td>1.3 ± 0.3</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

CA, cyclic peptide antagonist; LA, linear peptide antagonist; SR 49059, nonpeptide antagonist; N.D., not detected.

**Fig. 2.** Comparison of functional coupling of alanyl-substituted DRY mutant receptors. A, AVP-induced accumulation of mono-, bis-, and trisphosphates in HEK 293T cells transiently transfected with wild-type V1aR (●); [R149A]V1aR (■); or [Y150A]V1aR (▲). Values are stimulation induced by AVP at the stated concentrations expressed as percentage maximum. B, EC50 and Emax (fold maximum stimulation over basal) values of Wt and mutant receptors. Data shown are mean ± S.E.M. of three individual experiments [unless otherwise stated (n)] each performed in triplicate. a, not a true Emax. -fold stimulation of [D148A]V1aR was determined with 10 μM AVP. ND, none detected. **, p < 0.01 compared with wild-type V1aR using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism 4).
slightly higher than the Wt receptor in each case, reflecting the slight decrease in affinity of AVP at these constructs (Table 1). In the case of [D148A]V1αR, this mutant failed to signal even when challenged with high concentrations (10 μM) of AVP (Fig. 2B). Furthermore, the $E_{\text{max}}$ for all mutant constructs was at least 50% lower than Wt (Fig. 2B). It is also noteworthy that the basal level of Inos accumulation was not significantly different [using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism4)] between each of the mutants and Wt, an indication that none of the mutants displayed an enhanced level of constitutive activity. Therefore, the disruption of ligand binding and intracellular signaling of [D148A]V1αR may be due to the failure of this mutant to be trafficked efficiently to the plasma membrane.

**Mutation of Asp**$^{148}$ **Resulted in Impaired Cell Surface Receptor Expression.** The mutant receptors [D148A]V1αR, [R149A]V1αR, and [Y150A]V1αR each contained the HA-epitope tag incorporated at the N terminus (Fig. 1). An ELISA-based assay was developed to quantify expression of mutant receptors at the cell-surface compared with HA-tagged Wt expression. This technique offers considerable advantages compared with other techniques (e.g., whole cell binding) in that it does not rely on the binding interaction of a tracer ligand that may or may not be altered with a specific mutation. Furthermore, this technique allows more accurate quantification of receptors at the cell surface to be assessed by direct comparison with other receptors in parallel experiments compared with microscopy. To validate that the ELISA was measuring cell surface expression, we compared the cell surface localization of HA-tagged Wt V1αR and a mock-transfected vector using immunofluorescence confocal microscopy (Fig. 3). The transfected control vector gave no background signal (Fig. 3, A and B). In contrast, the Wt V1αR was clearly shown to be expressed at high levels on

**Fig. 3.** Cell surface localization of wild-type and Ala$^{148}$ receptors. HEK 293T cells were transiently transfected with either pcDNA3 vector alone (A and B), HA-tagged Wt V1αR (C and D), or [D148A]V1αR (E and F). Cells were fixed in 3% (v/v) paraformaldehyde and processed for immunocytochemistry as described under Materials and Methods. Phase images (A, C, and E) or excitation (543 nm) with the HeNe laser and a long pass filter for emission at 560 nm (B, D, and F) are shown. Images shown are representative from three separate experiments.
the cell surface (Fig. 3, C and D) and confirmed that the ELISA technique provides a high signal-to-noise ratio for quantification of cell surface receptors.

The mutant constructs [R149A]V1aR and [Y150A]V1aR were all expressed on the cell surface at levels similar to that of Wt as determined by ELISA (Table 1). In contrast, [D148A]V1aR was not expressed on the cell surface shown by ELISA (Table 1) or with confocal microscopy (Fig. 3, E and F). Permeabilization of the cell membrane with 0.1% (v/v) Triton X-100 before quantification of receptor revealed that the mutant [D148A]V1aR was actually expressed at low levels but was retained inside the cell (data not shown; Fig. 3D). Furthermore, we also tested whether increasing the cDNA during transfection (0.125–2.0 μg/well) was able to increase cell-surface expression or signaling of the [D148A]V1aR mutant. However, cell surface expression or inositol phosphate signaling was not increased above levels shown in Tables 1 and 2 (data not shown). These results show that Asp^{148} is critical for trafficking and/or delivery of the V1aR to the cell surface.

**Agnost-Mediated Internalization of Wild-Type and V1aR Mutants Ala^{149} and Ala^{150}.** Most GPCRs are internalized in response to prolonged agonist stimulation. For V1aR, these are internalized via a β-arrestin-dependent pathway (Bowen-Pidgeon et al., 2001) and are both agonist- and time-dependent (Fig. 4A). Indeed, as little as 1 nM AVP is able to promote internalization of ~30% of receptors over 60 min (data not shown). To evaluate whether Arg^{149} and/or Tyr^{150} provide important epitope(s) for AVP-mediated V1aR internalization, we compared the mutations engineered at these sites to the internalization kinetics of Wt receptors using the ELISA-based assay. Using HEK 293T cells transiently expressing Wt V1aR, [R149A]V1aR, and [Y150A]V1aR at equivalent abundance, it was found that AVP (1 μM) promoted internalization of all receptors (Fig. 4A). After exposure to AVP for 60 min, the percentage of cell surface receptors internalized (~60%) was the same in each case (Fig. 4B). Furthermore, the rate of internalization (time for 50% of receptors that are sensitive to internalization) was not significantly different compared with Wt V1aR using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism 4) (Fig. 4B).

**Specific Requirements at Position-148 for Cell Surface Delivery and Functional Recovery.** To evaluate the properties of the Asp^{148} residue that underlie its importance for V1aR cell surface expression and function, the constructs [D148N]V1aR, [D148E]V1aR, and [D148R]V1aR were engineered. These mutant receptors probed the importance of the charge of Asp^{148}, by 1) removing the charge but still maintaining the overall side chain length ([D148N]V1aR), 2) pre-

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TABLE 2
Pharmacological profile of substituted Asp^{148} and Arg^{149} mutant V1aRs

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist binding (K_i)</th>
<th>Antagonists (K_i)</th>
<th>Cell Surface Expression (% Wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA LA SR 49059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1 0.2 ± 0.0 0.7 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>D148N</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>D148E</td>
<td>4.5 ± 1.1 (4)</td>
<td>1.4 ± 0.3 0.3 ± 0.1 1.7 ± 0.5</td>
<td>43 ± 4 (9)</td>
</tr>
<tr>
<td>D148R</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>R149D</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>R149H</td>
<td>3.5 ± 0.3</td>
<td>1.0 ± 0.2 0.4 ± 0.0 2.0 ± 0.2</td>
<td>63 ± 5 (8)</td>
</tr>
<tr>
<td>D148R/R149D</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

CA, cyclic peptide antagonist; LA, linear peptide antagonist; SR 49059, nonpeptide antagonist; N.D., not detected.
serving the negative charge ([D148E]V₁₄₉R) while extending
the side chain length with an additional methylene, or 3) substitution with a positively charged residue [D148R]V₁₄₉R. The [D148R]V₁₄₉R was not detected on the cell surface (Table 2), whereas both [D148N]V₁₄₉R and [D148E]V₁₄₉R mutants showed a significant increase in cell surface expression compared with [D148A]V₁₄₉R (Fig. 5A). However, their cell surface expression was reduced to ~20% ([D148N]V₁₄₉R) and ~40% ([D148E]V₁₄₉R) of normal Wt levels and this was identical at 24 h (data not shown) and 36 h after transfection (Fig. 5A; Table 2). It was important to ascertain whether recovery of cell surface expression with these mutants was a necessary prerequisite for restoration of ligand binding and signaling that was absent for [D148A]V₁₄₉R (Table 1). Although the [D148N]V₁₄₉R mutant showed an increased surface expression compared with [D148A]V₁₄₉R, it was unable to bind [³H]AVP tracer ligand (Table 2) or signal in response to AVP (10 μM) challenge (Fig. 6C). Likewise, [D148E]V₁₄₉R was unable to bind tracer (Table 2) or signal (Fig. 6C). In contrast, the [D148E]V₁₄₉R mutant was able to bind both agonist and antagonist ligands and exhibited a pharmacological profile

Fig. 5. Cell surface expression of Asp¹⁴₈ and Arg¹⁴₉ substituted mutant receptors. HEK 293T cells were transiently transfected with wild-type V₁₄₉R, [D148A]V₁₄₉R, [D148N]V₁₄₉R, and [D148E]V₁₄₉R (B) or wild-type V₁₄₉R, [R149A]V₁₄₉R, [R149D]V₁₄₉R, and [R149H]V₁₄₉R (B). Total number of receptors remaining at the cell surface was quantified by ELISA as described under Materials and Methods. Data shown is mean ± S.E.M. of (n) individual experiments, each performed in triplicate. **, p < 0.01 compared with Wt V₁₄₉R using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism 4).

Fig. 6. Comparison of functional coupling of Asp¹⁴₈- and Arg¹⁴₉-substituted mutant receptors. AVP-induced accumulation of mono-, bis-, and trisphosphates in HEK 293T cells transiently transfected with wild-type V₁₄₉R (●) and [D148E]V₁₄₉R (▲) (A) or wild-type V₁₄₉R (●) and [R149H]V₁₄₉R (□) (B). Values are stimulation induced by AVP at the stated concentrations expressed as percentage maximum, CEC₅₀ and E₅₀ (fold maximum stimulation over basal) values of Wt and mutant receptors. Data shown are mean ± S.E.M. of three individual experiments (unless otherwise stated (n)) each performed in triplicate. ND, none detected. **, p < 0.01 compared with Wt V₁₄₉R using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism 4): a, not a true E₅₀-fold stimulation of [D148N]V₁₄₉R, [D148R]V₁₄₉R, [R149H]V₁₄₉R, and [D148R/R149D]V₁₄₉R were determined with 10 μM AVP.
very similar to that of Wt (Table 2), although the $K_i$ for AVP was slightly raised (~5-fold). The ability of [D148E]$V_{1a}$R to generate an intracellular signal was also assessed (Fig. 6A). From the resulting dose-response curve, the EC$_{50}$ value for [D148E]$V_{1a}$R was almost identical to that of Wt receptor, despite having a reduced $E_{max}$ (Fig. 6C). The basal level of InsP$_3$ signaling was not significantly increased [ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism4)] in either mutant relative to Wt (data not shown). Together, these results show the importance of a negative charge at position-148 for cell surface delivery and subsequent restoration of ligand binding and $V_{1a}$R signaling capacity.

Conservative Mutation of Glu$^{148}$ Displayed Enhanced Receptor Internalization. The recovery of cell surface expression with [D148E]$V_{1a}$R allowed us to probe whether this conservative substitution at position-148 was important for AVP-mediated $V_{1a}$R internalization. As described previously, the internalization kinetics of [D148E]$V_{1a}$R was compared with Wt $V_{1a}$Rs using the ELISA-based assay. After exposure to AVP for 60 min, the percentage of cell surface receptors present at the cell surface that was able to be internalized was significantly increased (~80%) for [D148E]$V_{1a}$R compared with ~60% for Wt receptor (Fig. 7A). However, the rate at which this mutant receptor was internalized ($t_{1/2}$ ~ 7 min) was very similar to that of Wt (Fig. 7C).

Charge Specific Requirements at Position-149 for Surface Expression and Normal $V_{1a}$R Function. The observation that a negatively charged residue was important at position 148 raised the possibility that charged residues within this TM-III-IC2 interface might have a wider importance in cell surface delivery and also raised the possibility of a mutual interaction between Asp$^{148}$ and Arg$^{149}$. To evaluate this, we engineered the constructs [R149D]$V_{1a}$R, [R149H]$V_{1a}$R, and [D148R/R149D]$V_{1a}$R. These mutant receptors probed the importance of Arg$^{149}$ by 1) reversing the charge with substitution of an aspartyl (i.e., [R149D]$V_{1a}$R), 2) preserving the positive charge with a histidyl (i.e., [R149H]$V_{1a}$R); or 3) switching the charged residues at positions 148 and 149, respectively (i.e., [D148R/R149D]$V_{1a}$R). The mutants [R149D]$V_{1a}$R and [D148R/R149D]$V_{1a}$R were not expressed on the cell surface determined by ELISA (Fig. 5B; Table 2) or confocal microscopy (data not shown). Therefore, these mutants were unable to bind tracer ligand (Table 2) or signal when challenged with AVP (10 $\mu$M; Fig. 6C). In contrast, the [R149H]$V_{1a}$R mutant was expressed on the cell surface. However, this was consistently reduced to ~60% of Wt surface expression levels (Fig. 5B; Table 2). This [R149H]$V_{1a}$R mutant was able to bind both agonist and antagonist ligands and exhibited a pharmacological profile comparable with wild-type (Table 2), although the $K_i$ for AVP was slightly higher (~4-fold), which was also observed for [R149A]$V_{1a}$R (Table 1). The ability of [R149H]$V_{1a}$R to increase second messenger generation was assessed (Fig. 6B). The dose-response curves for AVP-induced accumulation of InsPs for [R149H]$V_{1a}$R and [R149A]$V_{1a}$R (Fig. 2A) was equally right-shifted compared with Wt, with EC$_{50}$ values increasing by approximately ~3-fold (Fig. 6C), and reflecting the slight decrease in affinity of AVP for both of these constructs (Tables 1 and 2). The basal level of InsPs signaling for all mutants was not increased relative to Wt $V_{1a}$R using ANOVA with a post hoc Dunnett’s test analysis (GraphPad Prism 4).

Rapid Internalization Kinetics of a Conservative His$^{149}$ Mutation. An inherited mutation in the related $V_2$R ([R137H]$V_2$R) is found in some patients suffering with the
Discussion

In this report, site-directed mutagenesis of the V1aR was used to study the role of the conserved DRY motif located at the cytosolic end of TM-III (Fig. 1). Mutation of the conserved aspartyl (3.49) residue ([D148A]V1aR) resulted in a receptor that was not expressed on the cell surface and consequently unable to bind tracer ligand or increase AVP-mediated InsP signaling. Despite the lack of surface expression, this mutant was localized within intracellular compartments after permeabilization. However, these levels were still below detectable limits to establish specific binding in membrane samples. A similar situation has been reported for other members of AVP/OT receptor family. For example, replacement of Asp142 in H2R (Alewijnse et al., 2000) and m1 mAChR (Lu et al., 1997) have reported structural instabilities. The role of Asp148 mutations may be related to their structural instability within the receptor architecture. The mutants may display differential retention within the ER and/or insertion into the plasma membrane. This may render the receptor more susceptible to degradation and/or internalization once trafficked to the membrane. Only [D148E]V1aR was expressed at sufficient levels to investigate changes in internalization. Once delivered to the surface, a greater proportion of [D148E]V1aR was internalized compared with Wt without affecting the rate. It is interesting that a similar situation was observed for D136E/N mutations in GnRH receptors (Arora et al., 1997). This enhanced level of internalization or degradation may contribute to reduced expression for other Asp(3.49) mutant GPCRs. Indeed, mutant GPCRs that have been described as constitutively active [e.g., H2R (Alewijnse et al., 2000) and β2-AR (Rasmussen et al., 1999)] have reported structural instabilities. The role of Asp(3.49) for V1aRs does not belong to the subgroup of GPCRs that when mutated result in constitutive activity. Instead, they have properties similar to those described for m1 mAChR (Lu et al., 1997), α2a-AR (Chung et al., 2002), and GnRH receptors (Arora et al., 1997).

Another reason for the lack of surface expression with Asp(3.49) mutations may be related to their structural instability within the receptor architecture. The mutants may display differential retention within the ER and/or insertion into the plasma membrane. This may render the receptor more susceptible to degradation and/or internalization once trafficked to the membrane. Only [D148E]V1aR was expressed at sufficient levels to investigate changes in internalization. Once delivered to the surface, a greater proportion of [D148E]V1aR was internalized compared with Wt without affecting the rate. It is interesting that a similar situation was observed for D136E/N mutations in GnRH receptors (Arora et al., 1997). This enhanced level of internalization or degradation may contribute to reduced expression for other Asp(3.49) mutant GPCRs. Indeed, mutant GPCRs that have been described as constitutively active [e.g., H2R (Alewijnse et al., 2000) and β2-AR (Rasmussen et al., 1999)] have reported structural instabilities. The role of Asp(3.49) for V1aRs does not belong to the subgroup of GPCRs that when mutated result in constitutive activity. Instead, they have properties similar to those described for m1 mAChR (Lu et al., 1997), α2a-AR (Chung et al., 2002), and GnRH receptors (Arora et al., 1997).
do not fully support this general hypothesis in that the alanyl substitution was able to bind agonist, signal, and internalize as normal. Although this mutant did show a reduced maximal signaling ability. This reduction-of-function phenotype may be caused by the uncoupling of V1aRs from the G-protein but was not a result of 1) reduced surface expression, 2) reduced agonist potency, or 3) elevated basal signaling activity. Evidence of a possible ionic interaction between Asp149/H9251 and Arg149/H9252 is unlikely because the double mutant (D148R/R149D) V1aR failed to reach the surface. On the other hand, the conservative histidyl substitution was an effective replacement for Arg149, which reversed these two charges, failed to be expressed on the surface and was unable to bind ligands or signal.

Charged residues located near the boundaries of TM regions are often important for the topology of membrane-spanning proteins (Rutz et al., 1999). For GPCRs, Asp(3.49) and Arg(3.50) residues are located at the interface of TM-III/IC2. It is probable that mutations of either residue disturb the charge balance at this position and destabilize the α-helical structure of TM-III and/or with phospholipids. This could exert deleterious effects on cell surface expression and impair interactions with G-protein(s), access for phosphorylation by kinases, and/or recruitment of regulatory proteins mediating internalization. In this regard, GluAsp residues are always conserved and, where studied, glutamyl is often an effective substitution for Asp(3.49) (Auger et al., 2002). This is consistent with the V1aR, where Glu148 was able to bind ligands and signal but not with other substitutions. Conservative replacements of Arg(3.50) are well tolerated among some GPCRs [e.g., Lys129 in thromboxane A2 receptors (Capra et al., 2004)]. In contrast, opposing charges can be detrimental, demonstrated by the lack of surface expression when an aspartyl residue was present. Furthermore, the position of these residues within the motif was crucial in that [D148R/R149D]V1aR failed to reach the surface. On the other hand, the conservative histidyl substitution was an effective replacement for Arg149 albeit with a slightly reduced expression and signaling ability. A mutation identified in V1aRs (R137H) of some patients with NDI was reported to be constitutively internalized and colocalized with β-arrestin (Barak et al., 2001). It is noteworthy that the corresponding mutant reported here (R149H/V1aR) showed a significantly increased rate and amount of receptor internalized without any elevated signaling activity. One possibility is that this mutant may display an enhanced affinity for β-arrestin and/or for other regulatory proteins involved in this process. It is interesting that a mutant (R123G) in the DRY motif of the N-formyl peptide receptor disrupted normal β-arrestin binding but was still able to internalize (Bennett et al., 2000). Therefore, the role of the Arg(3.50) in the V1aR (and other GPCRs) may extend to regulating other important aspects of receptor function.

Mutation of tyrosyl (3.51) had little effect on binding ligands, signaling, and receptor internalization. A reduced maximal signaling ability was observed that was similar to other Arg149 substitutions. The functional role of Tyr(3.51) has been studied in detail for m1 mACHRs, which reported reduced expression and a strong preference for aromatic residues (Lu et al., 1997). In general, Tyr(3.51) has not been extensively studied; only minor effects on receptor function have been reported.

In summary, this study has demonstrated the importance of specific residues within the highly conserved DRY motif in the V1aR for ligand-binding, signaling, cell surface delivery, and agonist-mediated internalization. An aspartyl (3.49) was critical for surface delivery and function. A glutamyl partially restored expression, but receptor stability at the surface was reduced, with a greater tendency of receptors to be internalized. In contrast to most GPCRs, Arg(3.50) (and Tyr(3.51)) was not essential for expression, agonist-binding, or coupling to Gα11, although maximal signaling responses were impaired. A histidyl at position (3.50) did reveal a role for agonist-mediated internalization of V1aRs. Although the DRY motif among GPCRs is highly conserved, its role in the general mechanism of GPCR activation and signaling are likely to be receptor and subtype specific.

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