

# Charged Residues of the Conserved DRY Triplet of the Vasopressin V<sub>1a</sub> 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 V<sub>1a</sub> receptor (V<sub>1a</sub>R) 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 arginine 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 V<sub>1a</sub>R 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 ac-

tivation 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 impor-

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**ABBREVIATIONS:** GPCR, G-protein-coupled receptor; TM, transmembrane helix; IC2, second intracellular loop; AR, adrenergic receptor; NDI, nephrogenic diabetes insipidus; mAChR, muscarinic acetylcholine receptor; OT, oxytocin; OTR, oxytocin receptor; AVP, [arginine<sup>8</sup>]vasopressin; GnRH, gonadotropin releasing hormone; H<sub>2</sub>R, histamine H<sub>2</sub> receptor; CA, 1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-(O-methyl)tyrosine AVP; SR 49059, (2S)-[(2R,3S)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide; HA, hemagglutinin; PCR, polymerase chain reaction; HEK, human embryonic kidney; BSA, bovine serum albumin; InsP, inositol phosphates; TBS, Tris-buffered saline; BSA, bovine serum albumin; Wt, wild-type; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; V<sub>1a</sub>R, vasopressin V<sub>1a</sub> receptor; V<sub>1b</sub>R, vasopressin V<sub>1b</sub> receptor; V<sub>2</sub>R, vasopressin V<sub>2</sub> receptor.

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  $\alpha_{1b}$ -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 and Bichet, 2001) and hypogonadotropic hypogonadism (Costa et al., 2001)]. The significance of the aspartate has been studied and has resulted in constitutive activity for some receptors [e.g., histamine H<sub>2</sub> receptor (H<sub>2</sub>R) (Alewijns et al., 2000),  $\alpha_{1b}$ -AR (Scheer et al., 1997), and  $\beta_2$ -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 cysteinyl, histidyl, and serine residues occurring in some GPCRs, such as oxytocin (OT), V<sub>2</sub> 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.

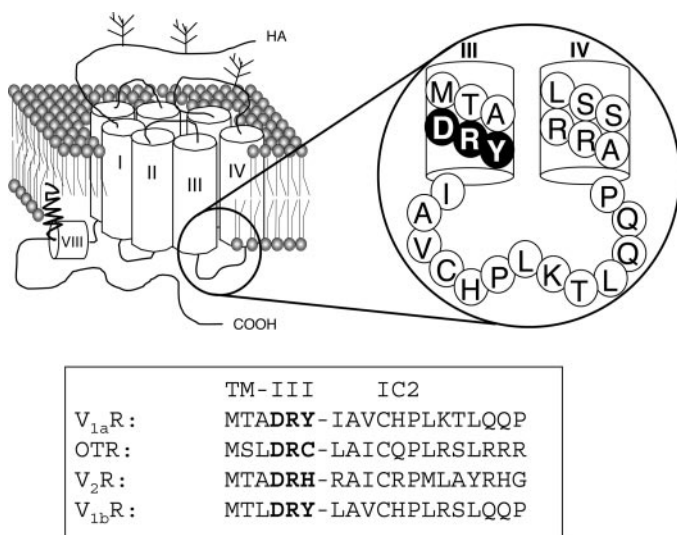
The neurohypophysial hormones AVP and OT are structurally-related nonapeptides that mediate a plethora of physiological functions, including vasopressor and antidiuretic actions, by binding to specific receptors (Gimpl and Fahrenholz, 2001; Thibonnier et al., 2001a). At present, four AVP/OT receptor subtypes (V<sub>1a</sub>R, V<sub>1b</sub>R, V<sub>2</sub>R, 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 V<sub>1a</sub>R is widely expressed and mediates nearly all of the actions of AVP, except for antidiuresis (renal V<sub>2</sub>R) and corticotropin secretion (pituitary V<sub>1b</sub>R). AVP mediates vascular smooth muscle (V<sub>1a</sub>Rs) 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 Fahrenholz, 2001). With the exception of the V<sub>2</sub>R (which couples to adenylyl cyclase), these receptors couple to G $\alpha_{q/11}$ , 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 G $\alpha_{q/11}$  coupling (Liu and Wess, 1996) and C terminus for the V<sub>1a</sub>R has been proposed (Hawtin et al., 2001; Thibonnier et al., 2001b). However, the functional significance of the highly conserved DRY motif for the V<sub>1a</sub>R 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 V<sub>1a</sub>R.

## Materials and Methods

**Materials.** The cyclic antagonist 1-( $\beta$ -mercapto- $\beta$ , $\beta$ -cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine AVP (CA) was purchased from Bachem (St. Helens, UK). AVP and the linear antagonist phenyl acetyl-D-Tyr(Me)<sup>2</sup>Arg<sup>6</sup>Tyr(NH<sub>2</sub>)<sup>9</sup>AVP 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 V<sub>1a</sub>R was made using a PCR approach as described previously (Hawtin et al., 2002). The wild-type rat V<sub>1a</sub>R was modified to contain a unique BshTI restriction site (underlined) using the primer 5'-G-ACA-GCC-GAC-CGG-TAC-ATC-GCC-GTG-TGC-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 V<sub>1a</sub>R 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-GTG-TGC-CAC-CCG-CTT-AAG-ACC-3'. A BshTI/KpnI product was subcloned into the pcDNA3 construct above to give pcDNA3-[BshTI/BspTI]V<sub>1a</sub>R.

The mutant constructs [D148A]V<sub>1a</sub>R, [R149A]V<sub>1a</sub>R, [Y150A]V<sub>1a</sub>R, [D148E]V<sub>1a</sub>R, [D148N]V<sub>1a</sub>R, [D148R]V<sub>1a</sub>R, [R149D]V<sub>1a</sub>R, [R149H]V<sub>1a</sub>R, and [D148R/R149D]V<sub>1a</sub>R were made using the following antisense oligonucleotides: 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CCG-GGC-GGC-TGT-CAT-C-3'; 5'-G-



**Fig. 1.** Schematic diagram of the V<sub>1a</sub>R. TMs are shown as cylinders straddling the lipid bilayer. The position of the engineered HA-epitope tag is indicated. Branched structures indicate the positions of N-glycosylation sites. The couplet comprising Cys<sup>371</sup>/Cys<sup>372</sup> is palmitoylated (Hawtin et al., 2001) and is shown forming an additional membrane anchor point that results in a fourth intracellular loop containing an  $\alpha$ -helical segment parallel to the membrane. The sequence and position in the receptor of the DRY motif and IC2 is indicated by the enlarged insert. Sequence alignment (obtained from SwisProt PDB and GenEMBL) of the distal region of TM-III, the conserved DRY motif (bold) and IC2 for human V<sub>1a</sub>R, OTR, V<sub>1b</sub>R, and V<sub>2</sub>Rs are shown.

GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CGC-GTC-GGC-TGT-C-3'; 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GGC-CCG-GTC-GG-3'; 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CCG-TTC-GGC-TGT-CAT-C-3'; 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CCG-GTT-GGC-TGT-CAT-C-3'; 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-CCG-GCG-GGC-TGT-CAT-C-3'; 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-ATG-GTC-GGC-TGT-C-3' and 5'-G-GGT-CTT-AAG-CGG-GTG-GCA-CAC-GGC-GAT-GTA-GTC-GCG-GGC-TGT-C-3' respectively and using [BshTI/BspTI]V<sub>1a</sub>R-pcDNA3 as template. Each primer contained the unique BspTI restriction site (underlined) and appropriate base changes (bold) to introduce specific mutation(s) at the desired location within the HA-tagged V<sub>1a</sub>R coding sequence. Each PCR product was subcloned into pcDNA3-[BshTI/BspTI]V<sub>1a</sub>R using HindIII and BspTI restriction sites. All receptor constructs were confirmed by automated fluorescent sequencing (University of Nottingham, UK).

**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) CO<sub>2</sub> in air at 37°C. Cells were seeded at a density of approximately  $5 \times 10^5$  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 preparation of HEK 293T cells was prepared 36 h after transfection as described previously (Hawtin et al., 2002), and the protein concentration was determined using the bicinchoninic acid protein assay kit (Sigma) with BSA as standard. Competition radioligand binding assays were performed in MultiScreen HTS 96-well opaque plates (Millipore, Watford, UK) containing 1.0-µm glass fiber (GF/B) filters and using the natural agonist [<sup>3</sup>H]-Phe<sup>3</sup>-3,4,5-AVP, (73 Ci/mmol; PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands) as tracer ligand. Radioligand binding assays were performed in binding buffer [20 mM HEPES, 10 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, and 1 mM EGTA, pH 7.4] and supplemented with 0.05% (w/v) BSA. Each well (final volume, 250 µl) contained radioligand (0.4–2.4 nM), cell membranes (100–330 µg), and competing ligand (at the concentrations indicated) and incubated at 25°C for 90 min to establish equilibrium. Nonspecific binding was determined in parallel incubations using 10 µM unlabeled AVP as appropriate. Bound radioligand was separated from free ligand by filtration using a vacuum manifold system (Millipore). Filters were washed twice with ice-cold binding buffer without BSA and sealed with opaque backing tape. After the addition of MicroScint-20 (PerkinElmer Life and Analytical Sciences), radioactivity was measured using a TopCount NXT scintillation counter (PerkinElmer Life and Analytical Sciences). Binding data were analyzed by nonlinear regression to fit theoretical Langmuir binding isotherms to the experimental data using Prism 4 (GraphPad Software Inc., San Diego, CA). Individual IC<sub>50</sub> values obtained for competing ligands were corrected for radioligand occupancy as described previously (Cheng and Prusoff, 1973) using the radioligand affinity (K<sub>i</sub>) experimentally determined for each construct.

**AVP-Induced Inositol Phosphate Production.** HEK 293T cells were seeded at a density of  $7.5 \times 10^4$  cells/well in poly-D-lysine-coated 24-well plates and transfected with cDNA (0.5 µg/well) after 24 h using Transfast (Promega Corp., Southampton, UK) as described in the manufacturer's protocol. The assay for AVP-induced accumulation of inositol phosphates (InsPs) was based on that described previously (Hawtin et al., 2002). In brief, 16 h after 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 [<sup>3</sup>H]-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.

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 elution of inositol and glycerophosphoinositol (10 ml of 25 mM NH<sub>4</sub>COOH containing 0.1 M HCOOH), a mixed inositol fraction containing mono-, bis-, and triphosphates was eluted with 5 ml of 850 mM NH<sub>4</sub>COOH containing 0.1 M HCOOH, mixed with Ultima-Flo AF scintillation cocktail (PerkinElmer), and radioactivity-quantified by liquid scintillation spectroscopy. EC<sub>50</sub> values were determined by nonlinear regression after fitting of logistic sigmoidal curves to the experimental data.

**Cell Surface Expression of Mutant Receptors.** Cell surface expression of mutant V<sub>1a</sub>R constructs was determined by using an indirect ELISA-based method. In brief, HEK 293T cells were seeded at a density of  $7.5 \times 10^4$  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 diethanolamine, 280 mM NaCl, and 0.5 mM MgCl<sub>2</sub>, pH 9.4) to each well. Plates were incubated at 37°C until an adequate color change had occurred (45 min), at which time a 100-µl sample was taken, mixed with an equal volume of 0.4 M NaOH before colorimetric reading at 405 nm using an MRX plate reader (Dynatech Technologies, Chantilly, VA). For each experiment, mock conditions corresponding to the transfection of vector without receptor were included. The percentage of mutant receptor expressed at the cell surface is defined as  $100 \times [(OD_{\text{mutant}} - OD_{\text{mock}})/(OD_{\text{wt}} - OD_{\text{mock}})]$ . 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 with receptor mutant cDNA using Transfast as described above. After 36 h, media from cells was replaced with fresh, prewarmed growth media. To promote V<sub>1a</sub>R internalization, cells were exposed to AVP for different time intervals over a maximum period of 60 min at 37°C with 5% (v/v) CO<sub>2</sub> in air. Cells were fixed and quantification of receptors remaining at the cell surface was determined using the ELISA-based assay as described above. The percentage of mutant receptor internalized is defined as  $100 \times [(OD_{\text{basal}} - OD_{\text{mock}}) - (OD_{\text{stimulated}} - OD_{\text{mock}})]/(OD_{\text{basal}} - OD_{\text{mock}})$ . All experiments were performed in triplicate for each condition and values from at least three separate experiments.

**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 glycine [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.



**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).

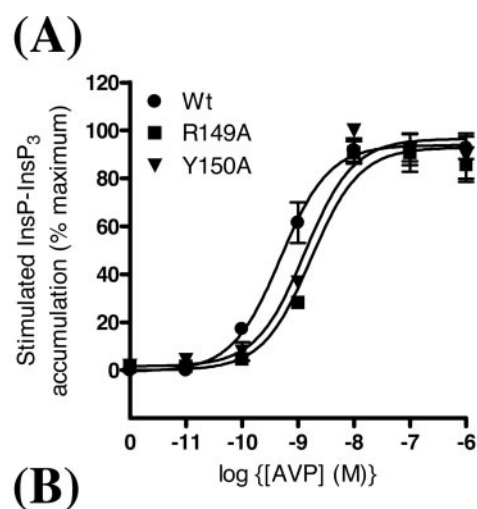
## Results

**Pharmacological Characterization of Alanyl-Substituted DRY Mutant V<sub>1a</sub>R 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 Arg<sup>149</sup> within this motif [i.e., Arg(3.50), using nomenclature proposed by Ballesteros and Weinstein (1995)] 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 (3.50) 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 V<sub>2</sub>Rs, with cysteines present for all species of OTRs. In the case of V<sub>1a</sub>Rs and V<sub>1b</sub>Rs, a tyrosine residue is absolutely conserved (Fig. 1).

To identify the contribution to ligand binding provided by individual residues within this DRY motif, residues Asp<sup>148</sup>, Arg<sup>149</sup>, and Tyr<sup>150</sup> of the V<sub>1a</sub>R were individually mutated to alanine to give [D148A]V<sub>1a</sub>R, [R149A]V<sub>1a</sub>R, and [Y150A]V<sub>1a</sub>R, respectively. Each mutant receptor construct was expressed in HEK 293T cells and their pharmacological characteristics were compared with those of a HA-tagged wild-type (Wt) V<sub>1a</sub>R. Incorporation of the HA-epitope sequence at the amino terminus was previously shown to not affect ligand binding or signaling compared with an untagged Wt V<sub>1a</sub>R (Hawtin and Wheatley, 1997) and was used in all subsequent experiments. Pharmacological characterization was aided by the fact that four different classes of ligand are available for probing changes in the ligand binding profile of V<sub>1a</sub>R constructs. In each case, competition radioligand binding curves were determined using a recently developed 96-well filtration assay with the natural agonist AVP and three

different structural classes of antagonist: 1) cyclic peptide antagonist [d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)<sup>2</sup>AVP (Kruszynski et al., 1980)] containing a 20-membered ring formed by a disulfide bond between Cys<sup>1</sup> and Cys<sup>6</sup>; 2) linear peptide antagonist [[PhAcD-Tyr(Me)<sup>2</sup>Arg<sup>6</sup>Tyr(NH<sub>2</sub>)<sup>9</sup>]AVP (Schmidt et al., 1991)]; and 3) nonpeptide antagonist (SR 49059; Serradeil-Le Gal et al., 1993). The K<sub>i</sub> values are presented in Table 1, corrected for radioligand occupancy. The mutant constructs [R149A]V<sub>1a</sub>R and [Y150A]V<sub>1a</sub>R, exhibited a pharmacological profile very similar to Wt, although the K<sub>i</sub> for AVP and the three different classes of antagonist was slightly raised (2- to 6-fold) in each case (Table 1). In marked contrast, [D148A]V<sub>1a</sub>R was unable to bind tracer ligand. With the exception of [D148A]V<sub>1a</sub>R, Wt and remaining mutant constructs were all expressed at approximately the same level of 1 to 2 pmol/mg of protein.

The capability of each of the mutant receptor constructs to generate an intracellular signal in response to the natural agonist AVP was also investigated. In each case, AVP-induced accumulation of InsPs was assayed (Fig. 2A). From the resulting dose-response curves, the EC<sub>50</sub> and E<sub>max</sub> were determined for each construct, and these are presented in Fig. 2B. The EC<sub>50</sub> value for [R149A]V<sub>1a</sub>R and [Y150A]V<sub>1a</sub>R was



**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 V<sub>1a</sub>R (●); [R149A]V<sub>1a</sub>R (■); or [Y150A]V<sub>1a</sub>R (▼). Values are stimulation induced by AVP at the stated concentrations expressed as percentage maximum. B, EC<sub>50</sub> and E<sub>max</sub> (-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 E<sub>max</sub>; -fold stimulation of [D148A]V<sub>1a</sub>R was determined with 10 μM AVP. ND, none detected. \*\*, *p* < 0.01 compared with wild-type V<sub>1a</sub>R using ANOVA with a post hoc Dunnett's test analysis (GraphPad Prism 4).

TABLE 1

Pharmacological profile of alanyl-substituted DRY mutant V<sub>1a</sub>Rs. Mutant V<sub>1a</sub>Rs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K<sub>i</sub>) were calculated from IC<sub>50</sub> values and corrected for radioligand occupancy as described under Materials and Methods. Cell surface expression of each mutant was quantified in parallel by ELISA and expressed as percentage of HA-tagged Wt V<sub>1a</sub>R. Data shown are mean ± S.E.M. of three individual experiments [unless otherwise stated (n)] with each performed in triplicate. Cell surface expression of 100% corresponds to ~0.8 pmol/mg of membrane protein.

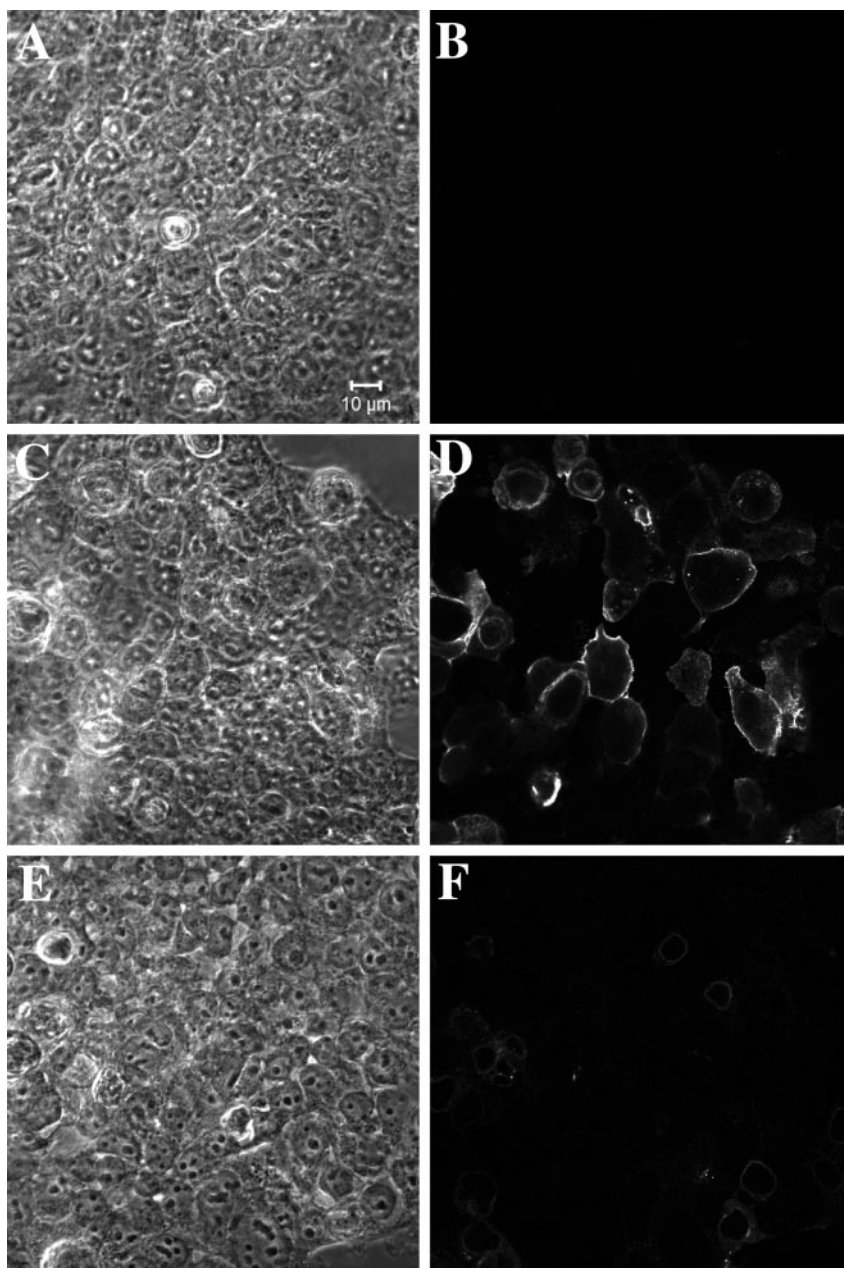
Receptor	Binding affinities (K <sub>i</sub> )				Cell Surface Expression
	Agonist AVP	Antagonists			
		CA	LA	SR 49059	
		nM			% Wt
Wt	1.0 ± 0.1	0.5 ± 0.1	0.2 ± 0.01	0.7 ± 0.1	100
D148A	N.D.	N.D.	N.D.	N.D.	2 ± 2 (7)
R149A	2.6 ± 0.4	1.4 ± 0.3	1.2 ± 0.1	1.9 ± 0.2	101 ± 5 (5)
Y150A	1.3 ± 0.3	1.0 ± 0.2	0.4 ± 0.1	1.3 ± 0.2	91 ± 4 (6)

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

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] $V_{1a}$ R, this mutant failed to signal even when challenged with high concentrations (10  $\mu$ M) of AVP (Fig. 2B). Furthermore, the  $E_{max}$  for all mutant constructs was at least 50% lower than Wt (Fig. 2B). It is also noteworthy that the basal level of InsPs 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] $V_{1a}$ R may be due to the failure of this mutant to be trafficked efficiently to the plasma membrane.

**Mutation of Asp<sup>148</sup> Resulted in Impaired Cell Surface Receptor Expression.** The mutant receptors [D148A] $V_{1a}$ R, [R149A] $V_{1a}$ R, and [Y150A] $V_{1a}$ R each con-

tained 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  $V_{1a}$ R 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  $V_{1a}$ R was clearly shown to be expressed at high levels on



**Fig. 3.** Cell surface localization of wild-type and Ala<sup>148</sup> receptors. HEK 293T cells were transiently transfected with either pcDNA3 vector alone (A and B), HA-tagged Wt  $V_{1a}$ R (C and D), or [D148A] $V_{1a}$ R (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.

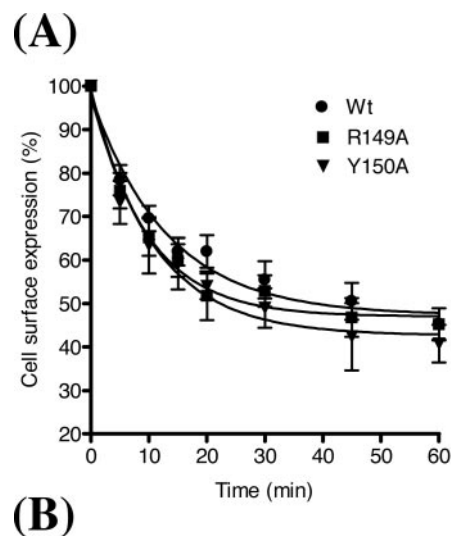
the cell surface (Fig. 3, C and D) and confirmed that the ELISA technique provides a high signal-to-noise ratio for quantification of cell surface receptors.

The mutant constructs [R149A]V<sub>1a</sub>R and [Y150A]V<sub>1a</sub>R were all expressed on the cell surface at levels similar to that of Wt as determined by ELISA (Table 1). In contrast, [D148A]V<sub>1a</sub>R 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]V<sub>1a</sub>R 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]V<sub>1a</sub>R 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<sup>148</sup> is critical for trafficking and/or delivery of the V<sub>1a</sub>R to the cell surface.

**Agonist-Mediated Internalization of Wild-Type and V<sub>1a</sub>R Mutants Ala<sup>149</sup> and Ala<sup>150</sup>.** Most GPCRs are internalized in response to prolonged agonist stimulation. For V<sub>1a</sub>Rs, these are internalized via a  $\beta$ -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<sup>149</sup> and/or Tyr<sup>150</sup> provide important epitope(s) for AVP-mediated V<sub>1a</sub>R 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 V<sub>1a</sub>R, [R149A]V<sub>1a</sub>R, and [Y150A]V<sub>1a</sub>R 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 V<sub>1a</sub>R 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<sup>148</sup> residue that underlie its importance

for V<sub>1a</sub>R cell surface expression and function, the constructs [D148N]V<sub>1a</sub>R, [D148E]V<sub>1a</sub>R, and [D148R]V<sub>1a</sub>R were engineered. These mutant receptors probed the importance of the charge of Asp<sup>148</sup>, by 1) removing the charge but still maintaining the overall side chain length ([D148N]V<sub>1a</sub>R), 2) pre-



**Fig. 4.** Internalization of wild-type and alanyl-substituted DRY mutant receptors. A, AVP-induced internalization of HEK 293T cells transiently transfected with wild-type V<sub>1a</sub>R (●), [R149A]V<sub>1a</sub>R (■), or [Y150A]V<sub>1a</sub>R (▼). Cells were stimulated with 1 µM AVP at 37°C to promote internalization and incubated (with AVP) for appropriate time intervals indicated (over a maximum 60-min period). Total number of receptors remaining at the cell surface was measured by ELISA as described under *Materials and Methods*. B, maximum (percentage) of each receptor internalized after AVP treatment (1 µM) for 60 min is shown. The time (t<sub>1/2</sub> min) for 50% of each receptor that can be internalized is indicated. Data shown are the mean ± S.E.M. of three separate experiments [unless otherwise stated (n)] each performed in triplicate.

TABLE 2

Pharmacological profile of substituted Asp<sup>148</sup> and Arg<sup>149</sup> mutant V<sub>1a</sub>Rs

Mutant V<sub>1a</sub>Rs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K<sub>d</sub>) were calculated from IC<sub>50</sub> values and corrected for radioligand occupancy as described under *Materials and Methods*. Cell surface expression of each mutant was quantified in parallel by ELISA and expressed as percentage of HA-tagged Wt V<sub>1a</sub>R. Data shown are mean ± S.E.M. of three individual experiments [unless otherwise stated (n)] with each performed in triplicate. Cell surface expression of 100% corresponds to ~0.8 pmol/mg of membrane protein.

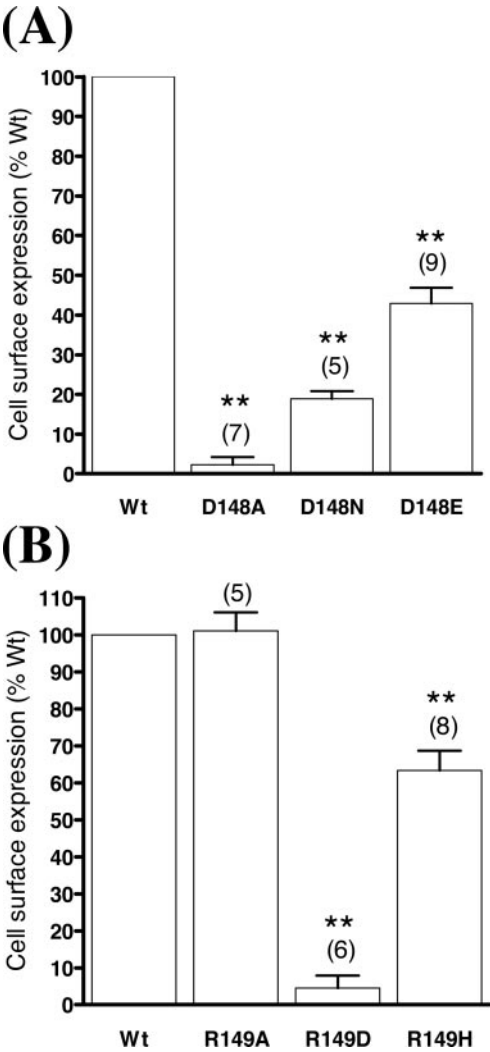
Receptor	Binding affinities ( $K_i$ )				Cell Surface Expression
	Agonist AVP	Antagonists			
		CA	LA	SR 49059	
		<i>nM</i>			<i>% Wt</i>
Wt	1.0 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	100
D148N	N.D.	N.D.	N.D.	N.D.	19 ± 2 (5)
D148E	4.5 ± 1.1 (4)	1.4 ± 0.3	0.3 ± 0.1	1.7 ± 0.5	43 ± 4 (9)
D148R	N.D.	N.D.	N.D.	N.D.	3 ± 0 (4)
R149D	N.D.	N.D.	N.D.	N.D.	5 ± 4 (6)
R149H	3.5 ± 0.3	1.0 ± 0.2	0.4 ± 0.0	2.0 ± 0.2	63 ± 5 (8)
D148R/R149D	N.D.	N.D.	N.D.	N.D.	2 ± 1 (4)

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

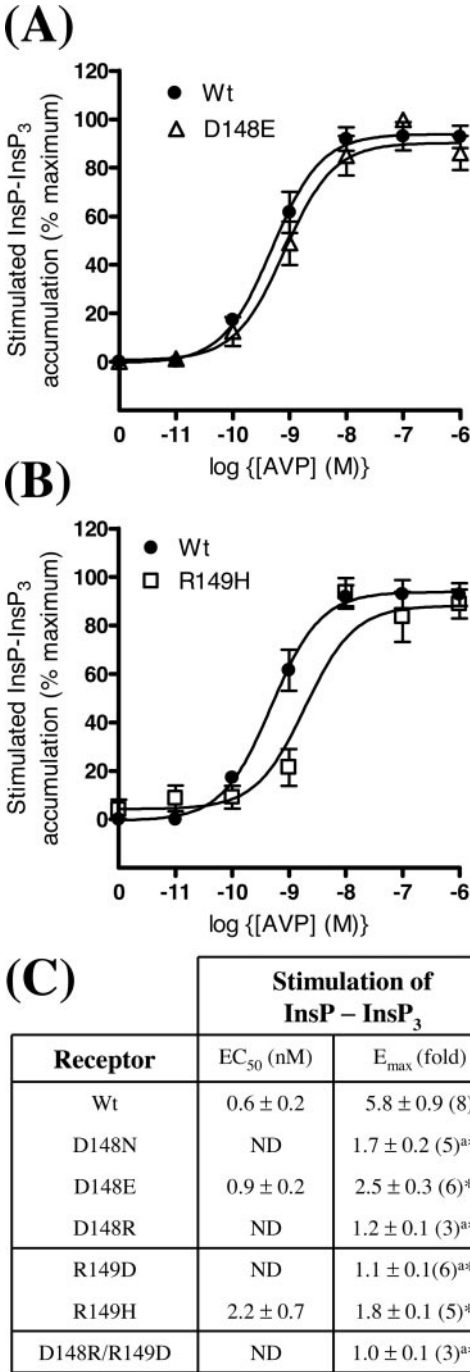


serving the negative charge ([D148E]V<sub>1a</sub>R) while extending the side chain length with an additional methylene, or 3) substitution with a positively charged residue [D148R]V<sub>1a</sub>R. The [D148R]V<sub>1a</sub>R was not detected on the cell surface (Table 2), whereas both [D148N]V<sub>1a</sub>R and [D148E]V<sub>1a</sub>R mutants showed a significant increase in cell surface expression compared with [D148A]V<sub>1a</sub>R (Fig. 5A). However, their cell surface expression was reduced to ~20% ([D148N]V<sub>1a</sub>R) and ~40% ([D148E]V<sub>1a</sub>R) 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]V<sub>1a</sub>R (Table 1). Although the [D148N]V<sub>1a</sub>R mutant showed an increased surface expression compared with [D148A]V<sub>1a</sub>R, it was unable to bind [<sup>3</sup>H]AVP tracer ligand (Table 2) or signal in response to AVP (10 μM) challenge (Fig. 6C). Likewise, [D148R]V<sub>1a</sub>R was

unable to bind tracer (Table 2) or signal (Fig. 6C). In contrast, the [D148E]V<sub>1a</sub>R mutant was able to bind both agonist and antagonist ligands and exhibited a pharmacological profile



**Fig. 5.** Cell surface expression of Asp<sup>148</sup> and Arg<sup>149</sup> substituted mutant receptors. HEK 293T cells were transiently transfected with wild-type V<sub>1a</sub>R, [D148A]V<sub>1a</sub>R, [D148N]V<sub>1a</sub>R, and [D148E]V<sub>1a</sub>R (A) or wild-type V<sub>1a</sub>R, [R149A]V<sub>1a</sub>R, [R149D]V<sub>1a</sub>R, and [R149H]V<sub>1a</sub>R (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 V<sub>1a</sub>R using ANOVA with a post hoc Dunnett's test analysis (GraphPad Prism 4).



**Fig. 6.** Comparison of functional coupling of Asp<sup>148</sup>- and Arg<sup>149</sup>-substituted mutant receptors. AVP-induced accumulation of mono-, bis-, and trisphosphates in HEK 293T cells transiently transfected with wild-type V<sub>1a</sub>R (●) and [D148E]V<sub>1a</sub>R (△) (A) or wild-type V<sub>1a</sub>R (●) and [R149H]V<sub>1a</sub>R (□) (B). Values are stimulation induced by AVP at the stated concentrations expressed as percentage maximum. C, EC<sub>50</sub> and E<sub>max</sub> (-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 V<sub>1a</sub>R using ANOVA with a post hoc Dunnett's test analysis (GraphPad Prism4). a, not a true E<sub>max</sub>, -fold stimulation of [D148N]V<sub>1a</sub>R, [D148R]V<sub>1a</sub>R, [R149D]V<sub>1a</sub>R, and [D148R/R149D]V<sub>1a</sub>R were determined with 10 μM AVP.

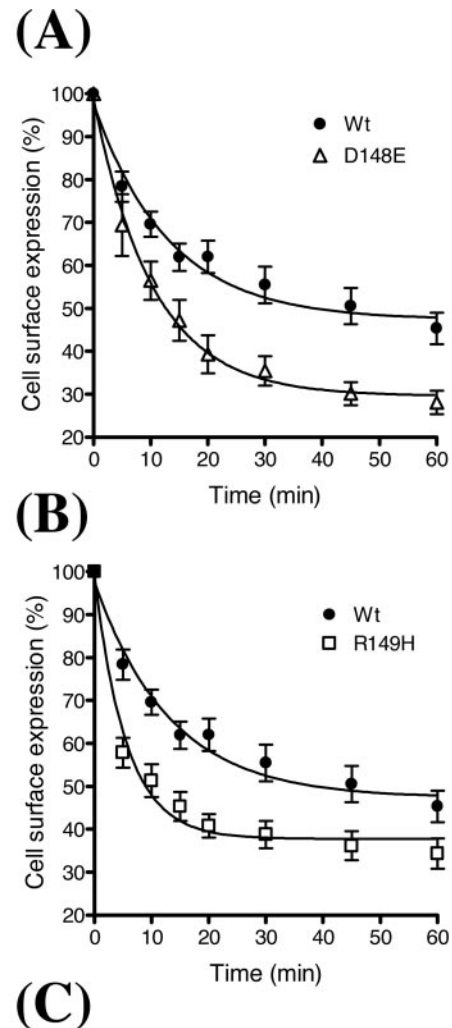
very similar to that of Wt (Table 2), although the  $K_i$  for AVP was slightly raised ( $\sim 5$ -fold). The ability of [D148E]V<sub>1a</sub>R to generate an intracellular signal was also assessed (Fig. 6A). From the resulting dose-response curve, the EC<sub>50</sub> value for [D148E]V<sub>1a</sub>R was almost identical to that of Wt receptor, despite having a reduced  $E_{\max}$  (Fig. 6C). The basal level of InsP 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<sub>1a</sub>R signaling capacity.

**Conservative Mutation of Glu<sup>148</sup> Displayed Enhanced Receptor Internalization.** The recovery of cell surface expression with [D148E]V<sub>1a</sub>R allowed us to probe whether this conservative substitution at position-148 was important for AVP-mediated V<sub>1a</sub>R internalization. As described previously, the internalization kinetics of [D148E]V<sub>1a</sub>R was compared with Wt V<sub>1a</sub>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 ( $\sim 80\%$ ) for [D148E]V<sub>1a</sub>R compared with  $\sim 60\%$  for Wt receptor (Fig. 7A). However, the rate at which this mutant receptor was internalized ( $t_{1/2} \sim 7$  min) was very similar to that of Wt (Fig. 7C).

**Charge Specific Requirements at Position-149 for Surface Expression and Normal V<sub>1a</sub>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<sup>148</sup> and Arg<sup>149</sup>. To evaluate this, we engineered the constructs [R149D]V<sub>1a</sub>R, [R149H]V<sub>1a</sub>R, and [D148R/R149D]V<sub>1a</sub>R. These mutant receptors probed the importance of Arg<sup>149</sup> by 1) reversing the charge with substitution of an aspartyl (i.e., [R149D]V<sub>1a</sub>R), 2) preserving the positive charge with a histidyl (i.e., [R149H]V<sub>1a</sub>R); or 3) switching the charged residues at positions 148 and 149, respectively (i.e., [D148R/R149D]V<sub>1a</sub>R). The mutants [R149D]V<sub>1a</sub>R and [D148R/R149D]V<sub>1a</sub>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<sub>1a</sub>R mutant was expressed on the cell surface. However, this was consistently reduced to  $\sim 60\%$  of Wt surface expression levels (Fig. 5B; Table 2). This [R149H]V<sub>1a</sub>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 ( $\sim 4$ -fold), which was also observed for [R149A]V<sub>1a</sub>R (Table 1). The ability of [R149H]V<sub>1a</sub>R to increase second messenger generation was assessed (Fig. 6B). The dose-response curves for AVP-induced accumulation of InsPs for [R149H]V<sub>1a</sub>R and [R149A]V<sub>1a</sub>R (Fig. 2A) was equally right-shifted compared with Wt, with EC<sub>50</sub> values increasing by approximately  $\sim 3$ -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<sub>1a</sub>R using

ANOVA with a post hoc Dunnett's test analysis (GraphPad Prism4) (data not shown).

**Rapid Internalization Kinetics of a Conservative His<sup>149</sup> Mutation.** An inherited mutation in the related V<sub>2</sub>R ([R137H]V<sub>2</sub>R) is found in some patients suffering with the



**Fig. 7.** Internalization of wild-type and Glu<sup>148</sup>- and His<sup>149</sup>-substituted mutant receptors. AVP-induced internalization of HEK 293T cells transiently transfected with wild-type V<sub>1a</sub>R (●) and [D148E]V<sub>1a</sub>R (Δ) (A) or wild-type V<sub>1a</sub>R (●) and [R149H]V<sub>1a</sub>R (□) (B). Cells were stimulated with 1  $\mu$ M AVP at 37°C to promote internalization and incubated (with AVP) for appropriate time intervals indicated (over a maximum 60-min period). Total number of receptors remaining at the cell surface was measured by ELISA as described under *Materials and Methods*. C, maximum (percentage) of each receptor internalized after continuous AVP stimulation (1  $\mu$ M) at 37°C for 60 min. The time ( $t_{1/2}$ ) min for 50% of each receptor that can be internalized is indicated. Data shown are the mean  $\pm$  S.E.M. of three separate experiments [unless otherwise stated ( $n$ )] each performed in triplicate. \*,  $p < 0.05$  and \*\*,  $p < 0.01$  compared with Wt V<sub>1a</sub>R using ANOVA with a post hoc Dunnett's test analysis (GraphPad Prism 4).



disease NDI, a condition that results in their inability to retain water in the kidney (Morello and Bichet, 2001). Pharmacological assessment of [R137H]V<sub>2</sub>R in recombinant cells showed that this mutant failed to respond to AVP by having a reduced cell surface expression and intracellular localization of receptors (Barak et al., 2001). This subsequently resulted in the phenomenon of some mutations being described as constitutively internalized (Wilbanks et al., 2002). The analogous substitution in the V<sub>1a</sub>R (i.e., [R149H]V<sub>1a</sub>R) showed a ~40% reduced cell surface expression but was expressed at sufficient levels to perform internalization experiments. Agonist-mediated internalization of [R149H]V<sub>1a</sub>R was compared in parallel to Wt V<sub>1a</sub>Rs. After exposure to AVP stimulation for 60 min, the percentage of cell surface receptors that was internalized was increased for [R149H]V<sub>1a</sub>R (~70%) relative to Wt (Fig. 7B). It is noteworthy that the rate at which this mutant receptor was internalized ( $t_{1/2}$  ~ 4 min) was significantly increased compared with Wt (Fig. 7C).

## Discussion

In this report, site-directed mutagenesis of the V<sub>1a</sub>R 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]V<sub>1a</sub>R) 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 Asp<sup>136</sup> [Asp(3.49)] with an alanyl in the OTR also displayed reduced expression (Fanelli et al., 1999). This mutant was unable to bind [<sup>3</sup>H]OT tracer or increase OT-mediated InsP production. This phenomenon of reduced expression (usually <10% of Wt) has been observed in other Asp(3.49) mutant receptors such as H<sub>2</sub>R (Alewijns et al., 2000), m1 mAChR (Lu et al., 1997), V<sub>2</sub>R (Morin et al., 1998), and GnRH receptors (Arora et al., 1997). The reasons for this impaired expression at this locus is not yet clear; however, reduction in receptor stability, mis-folding, and receptor desensitization and internalization have been proposed (Wilbanks et al., 2002).

One possibility for the lack of [D148A]V<sub>1a</sub>R surface expression could be explained by the receptor being down-regulated as a consequence of constitutive activity. This mutant displayed no increased basal signaling activity (indication of constitutive activity). In some studies involving mutation of Asp(3.49), any subsequent low expression has often been normalized or the amount of cDNA transfected increased to allow direct comparison of equivalent Wt expression. Increasing the cDNA of [D148A]V<sub>1a</sub>R did not further increase surface expression or signaling. It is important to note that this approach must be taken with caution, because a proportion of the receptor may be localized intracellularly. GPCRs are considered to undergo a basal level of endogenous synthesis/re-cycling (Parnot et al., 2002; Milligan, 2003). Accumulation or increased levels of intracellular receptors may alter this equilibrium and induce a degree of constitutive activity as an artifact of adjusting expression levels using values deter-

mined by binding. Indeed, Wt receptors can adopt an enhanced constitutive active profile after overexpression. Moreover, translocation of intracellular localized H<sub>2</sub>Rs to the cell surface can be dramatically increased with inverse agonists (Smit et al., 1996).

To probe the importance of Asp<sup>148</sup> that is crucial for surface delivery, mutants that removed ([D148N]V<sub>1a</sub>R), preserved ([D148E]V<sub>1a</sub>R) or reversed this charge ([D148R]V<sub>1a</sub>R) were engineered. The [D148N]V<sub>1a</sub>R and [D148R]V<sub>1a</sub>R mutants were expressed at too low levels to detect significant specific-binding and signaling. In contrast, the glutamyl substitution was able to bind ligands and signal, albeit with a reduced maximal coupling ability. This implies that the negative charge at Asp(3.49) is important, at least in part, for increasing cell-surface delivery and subsequent restoration of normal V<sub>1a</sub>R function. A similar scenario was observed for D130E/N mutations in eotaxin CCR3 receptors (Auger et al., 2002). The importance of side chain substitutions at Asp(3.49) has been extensively studied in m1 mAChRs that severely reduce expression levels (Lu et al., 1997). In contrast, replacement of Asp<sup>142</sup> in  $\alpha_{1b}$ -ARs with all possible 19 encoding amino acids revealed the importance of side-chains for influencing the degree of constitutive activity (Scheer et al., 1997). If a mutant is constitutively active, then an increase in agonist-binding affinity, elevated basal and potency for second-messenger generation is often observed as a result of mimicking the active conformation (R\*) of a receptor. For [D148E]V<sub>1a</sub>R, agonist-binding affinity was in fact the opposite, with a slight ~4-fold reduction, suggesting that the glutamyl is exerting a subtle conformational change only required for agonist binding. This occurred without increasing basal signaling activity.

Another reason for the loss of surface expression with Asp<sup>148</sup> 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]V<sub>1a</sub>R was expressed at sufficient levels to investigate changes in internalization. Once delivered to the surface, a greater proportion of [D148E]V<sub>1a</sub>R 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., H<sub>2</sub>R (Alewijns et al., 2000) and  $\beta_2$ -AR (Rasmussen et al., 1999)] have reported structural instabilities. The role of Asp(3.49) for V<sub>1a</sub>Rs 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),  $\alpha_{2a}$ -AR (Chung et al., 2002), and GnRH receptors (Arora et al., 1997).

The region of the DRY motif has been modeled by Scheer et al., (2000) and suggests that the arginine is embedded within the receptor in the inactive state. Upon ligand-mediated receptor activation, the conformation change results in the movement of the arginine side chain to the cytoplasmic surface. This suggests that exposure of the arginine is a crucial event in G-protein binding and/or activation. These results

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 V<sub>1a</sub>Rs 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 Asp<sup>148</sup>-Arg<sup>149</sup> seems unlikely because the double mutant (D148R/R149D), 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  $\alpha$ -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, Glu/Asp 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 V<sub>1a</sub>R, where Glu<sup>148</sup> 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., Lys<sup>129</sup> in thromboxane A<sub>2</sub> 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]V<sub>1a</sub>R failed to reach the surface. On the other hand, the conservative histidyl substitution was an effective replacement for Arg<sup>149</sup> albeit with a slightly reduced expression and signaling ability. A mutation identified in V<sub>2</sub>Rs (R137H) of some patients with NDI was reported to be constitutively internalized and colocalized with  $\beta$ -arrestin (Barak et al., 2001). It is noteworthy that the corresponding mutant reported here ([R149H]V<sub>1a</sub>R) 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  $\beta$ -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  $\beta$ -arrestin binding but was still able to internalize (Bennett et al., 2000). Therefore, the role of the Arg(3.50) in the V<sub>1a</sub>R (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 Arg<sup>149</sup> 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 V<sub>1a</sub>R 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 G $\alpha_{q/11}$ , although maximal signaling responses were impaired. A histidyl at position (3.50) did reveal a role for agonist-mediated internalization of V<sub>1a</sub>Rs. 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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