CHARGED EXTRACELLULAR RESIDUES, CONSERVED THROUGHOUT A G-PROTEIN-COUPLED RECEPTOR FAMILY, ARE REQUIRED FOR LIGAND BINDING, RECEPTOR ACTIVATION AND CELL-SURFACE EXPRESSION.

Stuart R. Hawtin¶†, John Simms¶†, Matthew Conner¶, Zoe Lawson¶, Rosemary A. Parslow¶, Julie Trim§, Andrew Sheppard§ and Mark Wheatley¶

From the ¶School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK and §Ferring Research Limited, Southampton Science Park, 1 Venture Road, Southampton, SO16 7NP, UK. †Joint first authors.

Running title: Conserved Exofacial Charged Residues.

Address correspondence to: Dr. Mark Wheatley, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. Tel. 0044-121-4143981; FAX: 0044-121-414-5925; e-mail: m.wheatley@bham.ac.uk

For G-protein-coupled receptors (GPCRs) in general, the roles of extracellular residues are not well-defined compared to residues in transmembrane helices (TMs). Nevertheless, extracellular residues are important for various functions in both peptide-GPCRs and amine-GPCRs. In this study, the \( \text{V}_{1a} \) vasopressin receptor was used to systematically investigate the role of extracellular charged residues which are highly conserved throughout a sub-family of peptide-GPCRs, using a combination of mutagenesis and molecular modelling. Of the thirteen conserved charged residues identified in the extracellular loops (ECLs), Arg\(^{116} \) (ECL1), Arg\(^{125} \) (top of TMIII) and Asp\(^{204} \) (ECL2) are important for agonist binding and/or receptor activation. Molecular modelling revealed that Arg\(^{125} \) (and Lys\(^{125} \)) stabilizes TMIII by interacting with lipid head-groups. Charge reversal (Asp\(^{125} \)) caused re-ordering of the lipids, altered helical packing and increased solvent penetration of the TM bundle. Interestingly, a negative charge is excluded at this locus in peptide-GPCRs whereas a positive charge is excluded in amine-GPCRs. This contrasting conserved charge may reflect differences in GPCR binding modes between peptides and amines, with amines needing to access a binding site crevice within the receptor TM bundle whereas the binding site of peptide-GPCRs comprises more extracellular domains. A conserved negative charge at residue-204 (ECL2), juxtaposed to the highly conserved disulfide bond, was essential for agonist binding and signaling. Asp\(^{204} \) (and Glu\(^{204} \)) establishes TMIII contacts required for maintaining the \( \beta \)-hairpin fold of ECL2, which if broken (Ala\(^{204} \) or Arg\(^{204} \)) resulted in ECL2 unfolding and receptor dysfunction. This study provides mechanistic insight into the roles of conserved extracellular residues.

G-protein-coupled receptors (GPCRs)\(^1 \) exhibit a common tertiary structure comprising seven transmembrane helices (TMs) linked by extracellular loops (ECLs) and intracellular loops. The atomic detail of this general GPCR fold has been elucidated for bovine rhodopsin (bRho) using X-ray crystallography (1). This confirmed that the chromophore 11-cis-retinal is covalently linked to Lys\(^{296} \) (7,33) in transmembrane helix VII (TMVII) via a protonated Schiff-base and projects into a binding pocket formed within the TM bundle where it interacts with amino acid side-chains and water molecules (1, 2). Likewise, the binding pocket for small biogenic amine neurotransmitters such as acetylcholine and norepinephrine is buried deep within the TM bundle (3). Nevertheless, it is known from the bRho X-ray structure that the extracellular domains possess defined structure and are orientated to interact with each other and with the TM helices. Indeed ECL2 of bRho forms a \( \beta \)-hairpin which plunges down into the helical bundle to form a plug over the chromophore. Furthermore, the orientation of ECL2 in the majority of GPCRs is restrained by a conserved disulfide bond between ECL2 and the top of TMIII (1, 2).
The neurohypophysial peptide hormones vasopressin (AVP) and oxytocin (OT) generate a wide range of physiological effects, including vasopressor, antidiuretic and uterotonic actions (4, 5). The effects of AVP/OT are mediated by a family of receptors (V1aR, V1bR, V2R and OTR) which together with the vasotocin receptor (VTR), mesotocin receptor and isotocin receptor from lower vertebrates, constitute a sub-family of the rhodopsin/β-adrenergic receptor class of GPCRs (Family A). The V1aR, V1bR and OTR couple to phospholipase C thereby generating inositol 1,4,5 trisphosphate and diacylglycerol as second messengers, whereas the V2R stimulates adenyl cyclase. The V1aR is widely distributed and mediates nearly all of the actions of AVP with the exceptions of antidiuresis (V2R) and ACTH secretion (V1bR). Activation of the OTR stimulates contraction of the uterine myometrium during labor and causes lactation. In addition to the characteristic architecture of GPCRs, members of the neurohypophysial peptide hormone receptor family share certain sequence motifs and exhibit related pharmacologies (5-7). The hormone binding site of these receptors includes residues in the TM bundle (8) and ECL1 (9-11). It has also be reported that the N-terminus of the V1aR and OTR are required for agonist binding (12, 13). In particular, two charged residues (Arg46 and Glu54) in the V1aR N-terminus are required for high affinity agonist binding but not antagonist binding (14, 15). Likewise, Arg54 in the N-terminus of the OTR is required for agonist binding (16).

For GPCRs in general, the roles of extracellular residues are not well understood compared to residues in the TM domain. Nevertheless, extracellular residues are important for binding both amine (17) and peptide (18) ligands and have been implicated in ligand receptor-subtype specificity (19), binding allosteric modulators (20) switching ligand agonist/antagonist properties (21) and human immunodeficiency virus co-receptor activity (22). The aim of this study was to use the V1aR to systematically investigate the function of extracellular charged residues which are highly conserved throughout a sub-family of peptide GPCRs. Using a combination of mutagenesis and molecular modelling, our results indicate that specific conserved charged residues in ECL1, ECL2 and ECL3 fulfill important roles in ligand binding, receptor activation, domain conformation and cell-surface expression.

**EXPERIMENTAL PROCEDURES**

**Materials** — AVP was purchased from Sigma (Poole, UK). The cyclic peptide antagonist (CA) 1-(β-mercapto-β-cyclopentamethylenepropionic acid), 2-(O-methyl)tyrosine AVP (d(CH2)2Tyr(Me)2AVP) and linear peptide antagonist (LA) phenacyl (PhAc)-D-Tyr(Me)2ArgεTyr(NH2)2AVP were from Bachem (St. Helens, UK). SR 49059 was a gift from Sanofi Recherche (Toulouse, France). Cell culture media, buffers and supplements were purchased from Gibco (Uxbridge, UK). Restriction enzymes were obtained from MBI Fermentas (Sunderland, UK).


The constructs [E332A]V1aR and [D330A]V1aR were made for the required Ala substitution and unique ClaI restriction site (underlined). The PCR products were subcloned into pcDNA3-[E332A]V1aR as template. The [E332A]V1aR restriction site was created (shown in italics). A KpnI/EcoRI digest of this PCR fragment was subcloned into the V1aR. The PCR products were subcloned into pcDNA3-[E332A]V1aR utilizing unique HindIII and Sdal restriction sites.

The mutations [E193A]V1aR, [E195A]V1aR [K201A]V1aR, and [R216A]V1aR were made using antisense oligonucleotides 5'-GGC-GCG-GGT-ACC-CGA-TGA-GAT-GGA-ATT-CTC-A-3'. This primer contained five base changes in the V1aR sequence (shown in bold) which created the Ala mutation (shown in italics). These primers were subcloned into the V1aR. The constructs [D323A]V1aR and [D330A]V1aR were made by PCR with pcDNA3-[E332A]V1aR restriction site (underlined). The PCR products were subcloned into pcDNA3-[E332A]V1aR utilizing unique Sdal and ClaI restriction sites. All receptor constructs were confirmed by automated fluorescent sequencing (University of Birmingham, Birmingham, UK).

Cell culture and transfection — HEK 293T cells were routinely cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) in a humidified 5% (v/v) CO2 in air at 37 °C. Cells were seeded at a density of approximately 5 x 10^5 cells/100 mm dish and transfected after 48 h using a calcium phosphate precipitation protocol with 10 µg DNA/dish (16).

Radioisologand binding assays — A washed cell membrane preparation of HEK 293T cells, transfected with the appropriate receptor construct, was prepared as previously described (23) and the protein concentration determined using the BCA protein assay kit (Pierce Chemical Co., Attenhall, Birmingham, UK).
UK) using bovine serum albumin as standard. Radioligand binding assays were performed as previously described (24) using either the natural agonist [Phe\(^{3}\)-3,4,5,\(^{-3}\)H]AVP (0.5–1.5 nM), (64.2 Ci/mmol; PerkinElmer, Beaconsfield, UK) or the \(V_{1a}\)-R-selective peptide antagonist PhAc-D-[Tyr(Me)\(^{5}\)]AVP (0.5–1.5 nM) (99 Ci/mmol; DuPont NEN, UK) (25) as tracer ligand. Binding data were analyzed by non-linear regression to fit theoretical Langmuir binding isotherms to the experimental data using PRISM Graphpad (Graphpad Software Inc., San Diego, CA). Individual IC\(_{50}\) values obtained for competing ligands were corrected for radioligand occupancy as described (26) using the radioligand affinity (\(K_{d}\)) experimentally determined for each construct.

**Whole cell vasopressin \(V_{1a}\) receptor binding** — HEK 293T cells were plated onto 12-well plates at a density of 2.5 x 10\(^5\) cells/well in poly D-lysine-coated twelve well plates and transfected after 24 h using Transfast™ (Promega Corp., Southampton, UK). After 36 h, each well received 0.5 ml of binding buffer (described above) containing 2 % (w/v) BSA, 1-2 nM \(V_{1a}\)-R-selective peptide antagonist PhAc-D-Tyr(Me)\(^{5}\)Arg\(^{6}\)(3,4\(^{-3}\)H)Pro\(^{7}\)(3,5\(^{-3}\)H)Tyr\(^{8}\)NH\(_2\)-AVP (22 Ci/mmol; custom synthesis Phoenix Pharmaceuticals, INC. Belmont, CA) as tracer ligand in the presence (non-specific) or absence (total) of 1 \(\mu\)M LA. Plates were incubated for 90 min at 37 °C, before removal of the medium by aspiration. After three rinses with ice cold PBS, 0.5 ml of 0.1 M NaOH was added to each well to extract radioactivity. After 15 min incubation at 37 °C, the fluid from the plates was transferred to scintillation vials containing 10 ml HiSafe3 scintillant cocktail for counting. Cell-surface expression values were corrected for radioligand occupancy as described (26) using the radioligand affinity (\(K_{d}\)) experimentally determined for each construct.

**Determination of cell-surface expression using ELISA** — All receptor constructs incorporated an HA epitope tag in the N-terminus which enabled cell surface expression to be determined by ELISA (27). Briefly, HEK 293T cells were seeded at a density of 1 x 10\(^5\) cells/well in poly D-lysine-coated 12-well plates and transfected after 24 h using Transfast™ (Promega Corp., Southampton, UK). After 36 h, cells were fixed with 3.7 % (v/v) formaldehyde in TBS (20 mM TRIS, pH 7.5, 150 mM NaCl) for 15 min at 37 °C, then washed three times with TBS. Non-specific binding was blocked with 1 % (w/v) BSA in TBS for 45 min. Anti-HA primary antibody (HA-7; Sigma, Poole, UK) was diluted to 1:1000 in TBS containing 1 % (w/v) BSA for 60 min at room temperature with occasional shaking, followed by three gentle washes with TBS. Cells were briefly re-blocked with 1 % (w/v) BSA in TBS for 15 min, prior to incubation with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse; Bio-Rad Laboratories, Hemel Hempstead, UK) diluted to 1:3000 in 1 % (w/v) BSA/TBS for 60 min with occasional shaking. Cells were washed three times with TBS before a colorimetric alkaline phosphate substrate (Bio-Rad Laboratories) was added and incubated at 37 °C for 30 min. A 100 \(\mu\)l aliquot from each well was mixed with an equal volume of 0.4 M NaOH prior to measuring absorbance at 405 nm. Results were normalized against a wild-type control processed in parallel. Non-transfected cells were used to determine background. All experiments were performed in quadruplet.

**AVP-induced inositol phosphates production** — HEK 293T cells were seeded at a density of 2.5 x 10\(^5\) cells/well in poly D-lysine-coated twelve well plates and transfected after 24 h using Transfast™ (Promega). AVP-induced accumulation of inositol phosphates (InsPs) was assayed as described previously (12). Briefly, following pre-labeling of transfected cells with 2 \(\mu\)Ci/ml myo-[2\(^{-3}\)H]inositol (22.0 Ci/mmol; Perkin Elmer, Beaconsfield, UK) in inositol-free DMEM containing 1 % (v/v) FCS, a mixed fraction containing mono-, bis, and trisphosphates (InsP - InsP\(_3\)) was collected following stimulation by AVP, at the concentrations indicated, in the presence of 10 mM LiCl.

**Molecular modelling of the \(V_{1a}\)R** — The \(V_{1a}\)R sequence was aligned against the sequence corresponding to the crystal structure coordinates of bRho using CLUSTALW (28). The alignment was then used to generate homology models using MODELLER v6.2 (29). A collection of 200 model structures were generated and ranked based on an objective function score provided by MODELLER6v2. From this ensemble, a single structure was selected for further analysis. Further refinement of the homology model was achieved through molecular dynamics (MD) simulations of the receptor embedded in a hydrated 1,2-
dipalmitoyl-sn-glycero-3-phosphocholine bilayer. MD simulations were carried out using the GROMOS96 force-field parameters, with minor modifications, as implemented in GROMACS (30). Partial charges for the heavy atoms of Lys and Arg side-chains were determined using the 6-31G basis set as implemented in GAMESS US.

RESULTS

Role of charged residues in the 1st extracellular loop (ECLI) of the V1aR — The sequences of the extracellular loops, plus the extracellular boundaries, of the neurohypophysial peptide hormone sub-family of GPCRs are aligned in Fig. 1A. Within ECLI (including the extracellular regions of TMII and TMIII), there are five conserved charged residues: Asp112, Arg116, Arg118, Asp121, and Arg125 (Fig. 1B, residue number based on rV1aR). Residues Arg116, Arg121, and Arg125 are absolutely conserved across all VPR/OTRs cloned to date (Fig. 1A). Asp112 is also conserved with the exception of the human V2R (Lys) and the VTR (Glu). Arg118 is only conserved in V1aRs, although positively charged residues are present at this locus in all V1Rs (Fig. 1A).

To assess the importance of these conserved residues in V1aR function, each residue was substituted individually by Ala then pharmacologically characterized using the natural agonist AVP and three different structural classes of antagonist: (i) cyclic peptide antagonist (CA), [d(CH3)2Tyr(Me)]AVP (25); (ii) linear peptide antagonist (LA) ([PhAc-D-Tyr(Me)2Arg9Tyr(NH2)2]AVP, (31)) and (iii), non-peptide antagonist (SR 49059; (32)). The $K_d$ values are presented in Table I, corrected for radioligand occupancy. Mutating Arg116, Arg118, Asp121 or Arg125 to Ala had only a slight effect on the binding of the agonist AVP or the three different classes of antagonist (Table I, Fig. 2). [D112A]V1aR was also essentially wild-type although the $K_d$ for LA was slightly (5-fold) increased. Furthermore, the mutations [D112A]V1aR, [R118A]V1aR, [D121A]V1aR had little effect on signaling, with EC$_{50}$ values for AVP-stimulated inositol phosphates (InsP$_3$-InsP$_2$) accumulation comparable to wild-type V1aR (Table I). In contrast, [R116A]V1aR and [R125A]V1aR had a marked effect on signaling, increasing the EC$_{50}$ value 70-fold and 16-fold respectively compared to wild-type (Figs. 3A and 3B).

A positive charge is required at residue-116 in ECLI — The charge requirements of residue-116 were investigated further by engineering [R116D]V1aR, [R116E]V1aR (incorporating a negative charge) and [R116K]V1aR (maintaining a positive charge). A negative charge was not tolerated at this position, as the affinity of AVP decreased 1600–fold and 730–fold for [R116D]V1aR and [R116E]V1aR respectively, compared to wild-type V1aR (Fig 2A). In contrast, the binding affinities of the three different antagonists to [R116D]V1aR, [R116E]V1aR were relatively unchanged, although a small decrease (~6-fold) was observed for CA (Table I). Incorporating a negative charge at this locus also perturbed receptor activation, increasing the EC$_{50}$ value for [R116D]V1aR and [R116E]V1aR by 53-fold and 23-fold respectively (Fig. 3A). In contrast, maintaining a positive charge at this position ([R116K]V1aR) resulted in a receptor which exhibited essentially wild-type binding (Fig. 2A and Table I) and signaling (Fig. 3A).

A positive charge is required at residue-125 in ECLI — The charge requirements of residue-125 were investigated further. Retaining a positive charge ([R125K]V1aR) resulted in a wild-type receptor profile (Table I). In contrast, introduction of a negative charge at this locus ([R125D]V1aR) ablated specific binding of the radio-tracers (agonist and antagonist) and impaired signaling, with a marked decrease in AVP potency compared to wild-type V1aR (Fig. 3B). Molecular modelling of the V1aR indicated that Arg125 orients into the lipid bilayer (Fig. 4A), with the side-chain methylene groups interacting with the lipid hydrocarbon tails and the guanidinium group interacting with the lipid phosphate head groups and solvent. These contacts are preserved in [R125K]V1aR, consistent with the wild-type characteristics of this mutant receptor. In contrast, molecular dynamics of [R125D]V1aR revealed a re-ordering of the phospholipids in this region resulting from mutual repulsion between the negatively-charged lipid phosphate head group and the carboxyl of the Asp side-chain. This re-ordering of the lipids increased solvent accessibility at the extracellular end of TMIII and TMIV (Fig. 4A).

Species-specific and receptor subtype-specific differences at position-112 in ECLI — An Asp is highly conserved at residue-112 throughout this family of GPCRs, with the exception of the
VTR and the human V2R which possess Glu and Lys respectively (Fig. 1). Pharmacological differences arising from this sequence variation were assessed. Conservative substitution ([D112E]V1aR) resulted in wild-type binding and intracellular signaling, with only a small change in affinity for the CA antagonist (3-fold). Reversing the charge in [D112K]V1aR also slightly decreased the affinity of CA (5-fold) and reduced the affinity of the linear antagonist LA 8-fold (Table I) but was otherwise wild-type (Fig. 3C). However, in marked contrast to [D112K]V1aR, the construct [D112R]V1aR exhibited low affinity for AVP (Fig. 2B) and perturbed signaling (Fig. 3C). These effects were not due to a non-specific disruption of the receptor tertiary fold as the affinity of the three classes of antagonist was unchanged (Table I).

Asp112 is located at the membrane/solvent interface at the extracellular end of TMII. Molecular modelling shows that when residue-112 is Glu or Lys they occupy a similar position to Asp112, consistent with the near wild-type profile observed with these constructs. However, the increased side-chain length of Arg112 compared to Lys112, positions the positively-charged guanidinium moiety of Arg112 3.0 Å from the carboxyl group of Glu54(1.35) (top of TMI), resulting in a charge-charge interaction between these two residues (Fig. 4B). A comparable interaction between the amine of Lys112 and Glu54 is far less likely as the functional groups are further apart (4.8 Å). Furthermore, the guanidinium of Arg has higher partial charges on its heavy atoms compared to the amine of Lys, which increases the potential of the Arg guanidinium to establish ionic interactions compared to the amine of Lys. In addition, the planar nature of the guanidinium group may aid directive interactions.

**Role of charged residues in the 2nd extracellular loop (ECL2) of the V1aR —** The ECL2 domain (including the extracellular borders of TMIV and TMV) of the V1aR contains five charged residues Glu193, Glu195, Lys201, Asp204 and Arg216 (Fig. 1B). Sequence analysis of ECL2 revealed that: (i) charged residues are well-conserved at loci corresponding to Glu195, Asp204 and Arg216 throughout the vertebrate VPR/OTR family, whereas Glu193 and Lys201 are found only in the V1aR subtype, (ii) Asp204 is absolutely conserved with the single exception of the chick VTR which has a Glu (33), (iii) a negative charge (usually a Glu but an Asp in V1R) is conserved at residue-195 with the exception of the human V2R which has Asn and (iv) a positive charge (Arg/Lys) is conserved at position-216 but is replaced by a Pro in the sheep V1aR (Fig. 1A).

To assess the functional importance of these conserved charged residues, each residue was mutated individually to Ala and the pharmacological characteristics compared to wild-type V1aR (Table I). With the exception of [D204A]V1aR, all the mutant constructs exhibited binding and signaling characteristics similar to wild-type (Table I). In marked contrast, [D204A]V1aR possessed a marked decrease in AVP affinity (2300-fold; Fig. 2C and Table I) and impaired signaling (Fig. 3D). The affinity of [D204A]V1aR for the cyclic and non-peptide antagonists remained unchanged (Table I) indicating that the receptor protein was folded appropriately, nevertheless the Kd for LA was increased 20-fold relative to wild-type (Table I). The charge requirements at position-204 were investigated. Retaining a negative charge ([D204E]V1aR) resulted in wild-type ligand binding and signaling (Figs. 2C and 3D, Table I), whereas reversing the charge ([D204R]V1aR) markedly decreased both AVP affinity (Fig 2C) and signaling (Fig. 3D) and to a lesser extent LA and CA affinity (24-fold and 6-fold respectively; Table I). The binding of the non-peptide antagonist to [D204R]V1aR was wild-type. **Investigating a possible interaction between Asp204 and Arg125 or Arg46 —** Mutagenesis of Asp204 or Arg125 generated similar effects. Consequently, substitution by Ala ([D204A]V1aR and [R125A]V1aR) decreased the potency of AVP signaling by approximately 20-fold compared to wild-type V1aR (Figs. 3D and 3B, respectively) and reversing the charge ([D204R]V1aR and [R125D]V1aR) increased the EC50 100-fold compared to wild-type V1aR. Although Arg125 (top of TMIII) and Asp204 (ECL2) are in different domains, they are located at opposite ends of the highly-conserved disulfide bond, and therefore in close proximity and spatially constrained. It was possible that a mutual charge interaction existed between these two residues which was required for receptor activation. However, the double reciprocal mutant [R125D/D204R]V1aR, did not bind [3H]tracer ligands, did not signal when challenged with AVP (>10 µM) and was poorly expressed (Table I). Consequently, these data do not support a mutual interaction between Arg125 and Asp204.
We have established previously that a single residue (Arg$^{46}$) located within the distal N-terminus of the V$_{1a}$R is critical for binding AVP but not peptide or non-peptide antagonists (14) and that reversing the charge at this locus ([R46D]V$_{1a}$R or [R46E]V$_{1a}$R) impaired receptor function (34) in a similar manner to that observed for [D204R]V$_{1a}$R in this study. Given that high affinity agonist binding required both Arg$^{46}$ and Asp$^{204}$, it was feasible that a direct intra-molecular ionic interaction between Arg$^{46}$ and Asp$^{204}$ may contribute to high-affinity agonist binding and receptor activation. However, the double reciprocal mutant [R46D/D204R]V$_{1a}$R bound AVP with very low affinity ($K_i = 2500$ nM), a similar affinity to [R46D]V$_{1a}$R or [D204R]V$_{1a}$R (Table I), and the signaling capability of [R46D/D204R]V$_{1a}$R was also severely compromised. The overall tertiary fold of the receptor was nevertheless good as the non-peptide antagonist bound with wild-type affinity and the peptide antagonists CA and LA also bound with high affinity, albeit less than wild-type (Table I). Cell-surface expression of [R46D/D204R]V$_{1a}$R was only c.20% of wild-type. These data do not provide evidence for a direct interaction between Arg$^{46}$ and Asp$^{204}$.

Molecular modelling indicated that Asp$^{204}$ lies at the center of a pocket defined by residues Lys$^{128(3.29)}$ (TMIII), Gln$^{131(3.32)}$ (TMIII), Trp$^{206}$ (ECL2), Phe$^{283(6.51)}$ (TMVI) and Gln$^{287(6.55)}$ (TMVI). Asp$^{204}$ forms a salt bridge with Lys$^{128(3.29)}$ and hydrogen bonds with Gln$^{131(3.32)}$, both in TMIII (Fig. 4C). These interactions with Lys$^{128(3.29)}$ and Gln$^{131(3.32)}$ are preserved in the conservative substitution [D204E]V$_{1a}$R (not shown), consistent with the wild-type pharmacological profile (Table I). Removal of the negative charge at this locus ([D204A]V$_{1a}$R), resulted in a decrease in both AVP affinity and signaling potency (Fig. 3D, Table I). MD simulation of [D204A]V$_{1a}$R revealed that removal of the negative charge breaks the wild-type contacts between ECL2 and TMIII (Fig. 4D). This leads to a partial unfolding of the β-hairpin within ECL2 (Fig. 4E). In addition, the side-chain of Lys$^{128}$ rotates away from its wild-type position and orientates towards TMVI (Fig. 4D). A similar perturbation was observed with the construct [D204R]V$_{1a}$R, again leading to reorganization of ECL2. However, the introduction of an Arg at residue-204 also created an alternative hydrogen bonding network involving new interactions between Arg$^{204}$ in ECL2 and residues in TMII (Gln$^{104(2.57)}$, Gln$^{108(2.61)}$) and TMVII (Ala$^{272(7.42)}$, Ser$^{273(7.43)}$) (Fig. 4F).

Role of charged residues in ECL3 of the V$_{1a}$R — ECL3 (including the extracellular borders of TMVI and TMVII) of the V$_{1a}$R contains three conserved charged residues Asp$^{323}$, Asp$^{330}$ and Glu$^{332}$ (Fig. 1B). Analysis of sequence alignments of ECL3 of the vertebrate VPR/OTR family revealed that: (i) an Asp is completely conserved at the locus corresponding to Asp$^{323}$, (ii) a negative charge (usually an Asp) is conserved at residue-330 in V$_{1a}$Rs, V$_{1b}$Rs and VTRs but is replaced by Pro in OTRs and V$_{2}$Rs, (iii) a Glu is conserved at the locus corresponding to Glu$^{332}$ with the exception of V$_{1b}$Rs which possess a Thr (Fig. 1A). To determine the functional importance of these residues, each residue was mutated individually to Ala. Pharmacological characterization established that [D323A]V$_{1a}$R, [D330A]V$_{1a}$R and [E332A]V$_{1a}$R were almost identical to wild-type V$_{1a}$R with respect to binding all four classes of ligand (Table I) and intracellular signalling (Table I), indicating that the conserved charged residues in ECL3 had little or no role in these functions. It was however noteworthy that [D323A]V$_{1a}$R exhibited reduced cell-surface expression (c.50%) compared to wild-type V$_{1a}$R (Table I).

**DISCUSSION**

The aim of this study was to use the V$_{1a}$R to systematically investigate the function of extracellular charged residues which are highly conserved throughout a sub-family of peptide GPCRs. Within the ECL domains of the V$_{1a}$R, the charged residues were sub-divided into two groups (i) those that are conserved in all members of the sub-family and (ii) those that are conserved within a specific subtype. Thirteen conserved charged residues were identified in the ECL domains and associated TM boundaries, with five in ECL1, five in ECL2 and three in ECL3. Ala substitution within ECL1 had little effect on ligand binding. However, [R116A]V$_{1a}$R and [R125A]V$_{1a}$R exhibited impaired intracellular signaling (70-fold and 16-fold respectively) indicating a role in receptor activation. Although [R125A]V$_{1a}$R was expressed at 56% of wild-type, this was unlikely to be responsible for the impaired signaling of [R125A]V$_{1a}$R, as [D323A]V$_{1a}$R was expressed at 52% of wild-type but retained essentially wild-type signaling capability (Table I). A positive
charge is essential at residue-116, as retaining a positive charge ([R116K]V1αR) preserved wild-type signaling whereas reversing the charge ([R116D]V1αR, [R116E]V1αR) not only compromised signaling but also profoundly decreased agonist affinity. This loss of AVP binding was agonist-specific and not due to aberrant assembly of the receptor as the binding of antagonists (peptide and non-peptide) was unaffected. Consequently, Arg125 is required to stabilize the active R* conformation of the V1αR and is absolutely conserved throughout the vertebrate neurohypophysial hormone sub-family of GPCRs cloned to-date (Fig. 1A).

Arg125 is located near to the extracellular end of TMIII, immediately adjacent to the conserved disulfide bond, where it interacts with lipids. This Arg-lipid interaction has been referred to as ‘snorkeling’ (35). The absolute conservation of this Arg throughout the neurohypophysial peptide hormone receptor family (Fig. 1A) implies functional importance. This is supported by a report that the naturally-occurring mutation R113W in the human V2R (which corresponds to Arg125 in the V1αR) causes the receptor dysfunction responsible for nephrogenic diabetes insipidus (NDI) in some patients (36). Furthermore, an alignment of 717 sequences of Family A GPCRs which bind peptide ligands, revealed that a positively charged residue is conserved at this position in 85% of receptors and that Asp and Glu are excluded (GPCR database http://www.gpcr.org). This is indicative of a generic role for this residue in signaling by peptide-GPCRs, a notion supported by mutagenesis studies on the CXCR2 and angiotensin II type-1 receptors (37, 38). It is now well-established that relative movement between TMIII, TMVI and TMVII is central to the R® transition of GPCRs (39). The location of Arg125 in the extracellular extremity of TMIII may allow it to act as a structural support for TMIII during receptor activation. Reversing the charge at this locus in [R125D]V1αR was very detrimental due to charge-charge repulsion between the side-chain carboxyl and the membrane lipid phosphate headgroups. This repulsion resulted in re-ordering of the surrounding phospholipids, increased solvent accessibility at the extracellular end of TMIII/TMIV and altered local conformation which could have ramifications along the length of TMIII. These conformational changes observed in silico would explain why Asp/Glu are excluded from this locus in peptide-binding GPCRs. In marked contrast, GPCRs for biogenic amines actually favor a negatively charged residue at the position corresponding to Arg125. Analysis of an alignment of 371 sequences of amine-GPCRs from different species revealed that approximately 70% have Glu/Asp at this locus (GPCR database http://www.gpcr.org) with exclusion of Arg/Lys. Exceptions to this trend are the H1 histamine receptor and trace amine receptors which do possess a positive charge. It is possible that the charge difference at this single locus between peptide-GPCRs and amine-GPCRs may reflect differences in the binding mode between these ligands. Biogenic amines access a binding site enclosed within the TM bundle whereas peptides bind to extracellular domains plus TM helices. If Glu/Asp at the top of TMIII in amine-GPCRs (corresponding to Arg125 in V1αR) increases solvent penetration into the TM bundle (analogous to the mutant [R125D]V1αR) it may facilitate ligand access to the binding site. Support for such a mechanism is perhaps provided by [3H]propylbenzilycholine mustard ([3H]PrBCM) labeling of the M1 mChR. In addition to alkylating Asp112 in TMIII (the ‘classical’ amine counter-ion), [3H]PrBCM also labeled Asp99 (corresponding to Arg125 in the V1αR) (40). Furthermore, mutation of this Asp99 to Asn moderately decreased the affinity of a range of ligands and strongly decreased both alkylation by [3H]PrBCM and agonist-induced second messenger generation (41).

Asp112 in the V1αR is conserved throughout the neurohypophysial hormone receptor family with the exception of the human V2R and the VTR which possess Lys and Glu respectively. It has been reported that this locus is important for binding some V2R-selective agonists (42). The substitutions [D112A]V1αR, [D112E]V1αR and [D112K]V1αR had little effect on receptor function, introducing Arg112 however, impaired agonist binding and signaling but had little effect on any antagonist binding. Although Arg and Lys are superficially similar, [D112K]V1αR and [D112R]V1αR exhibited very different affinity for AVP. Molecular modeling revealed that Arg112 formed a stable ionic interaction with Glu54(1.35) at the top of TM1 which was absent with the shorter side-chain of Lys112 (and also absent in wild-type and [D112E]V1αR). Glu54(1.35) has recently been
identified as a key residue for high affinity agonist binding and signaling (15), consequently an inappropriate interaction with Arg\(^{125}\) may prevent Glu\(^{124(1.35)}\) adopting an optimal conformation for AVP binding and signaling.

ECL2 is usually the longest ECL in GPCRs and in bRho forms a \(\beta\)-hairpin which projects into the binding crevice allowing the \(\beta4\)-strand to contact retinal (1, 2). There is also evidence that this ECL2 fold is not restricted to bRho and occurs in other GPCRs (43). Of the five conserved charged residues substituted by Ala in ECL2 of the V\(_{1a}\)R, only [D204A]V\(_{1a}\)R had a marked effect on receptor function, with a profound decrease in AVP affinity, decreased LA affinity and impaired signaling potency. Molecular modeling revealed that Asp\(^{204}\) provides interactions between ECL2 and TMII by hydrogen bonding with Gln\(^{131(3.32)}\) and forming a salt bridge with Lys\(^{128(3.29)}\). Substitution by Ala in [D204A]V\(_{1a}\)R disrupted this contact network resulting in partial unfolding of ECL2 and re-arrangement of the Lys\(^{128(3.29)}\) side-chain. Substitution of Lys\(^{128(3.29)}\) or Gln\(^{131(3.32)}\) by Ala also disrupted AVP binding and signaling (8), consistent with the proposed role for Asp\(^{204}\). The reduction in affinity of both AVP and LA was similar following either removal of the negative charge ([D204A]V\(_{1a}\)R) or charge reversal ([D204R]V\(_{1a}\)R) (Table I). Although [D204R]V\(_{1a}\)R and [D204A]V\(_{1a}\)R had the same affinity for AVP, the decrease in potency of AVP-stimulated InsP\(_3\) production was greater with Arg\(^{204}\) than with Ala\(^{204}\) (Table I), suggesting that Arg\(^{204}\) stabilized the receptor ground-state. Molecular modeling indicated that for both of these constructs the interactions between ECL2 and TMII were disrupted in a similar manner leading to reorganization of ECL2. However, the introduction of the longer side-chain of Arg\(^{204}\) also created new interactions between ECL2-TMII (Gln\(^{104(2.57)}\), Gln\(^{108(2.61)}\), and ECL2-TMVI (Ala\(^{272(7.42)}\), Ser\(^{273(7.43)}\)) which reduced the R\(\rightarrow\)R* transition. Interestingly, Ser\(^{273(7.43)}\) corresponds to the retinal attachment site in bRho and this locus has been implicated in activation of other GPCRs (44, 45). In addition, Ala substitution of both of the TMII contacts, Gln\(^{104(2.57)}\) and Gln\(^{108(2.61)}\), has been reported previously to perturb both ligand binding and intracellular signaling (8). Consequently, the inappropriate new contacts established by Arg\(^{204}\) are with residues required for receptor activation and explain the perturbed pharmacological profile observed with [D204R]V\(_{1a}\)R. In contrast, the conservative substitution [D204E]V\(_{1a}\)R, which occurs naturally in a chick VTR (33), maintains the normal ECL2-TMIII contacts and exhibits wild-type characteristics. Our investigations establish the importance of an acidic residue at position-204 and provide an explanation for the absolute conservation of Asp(Glu) at this locus throughout a sub-family of GPCRs. In addition, our study also provides a feasible mechanism for the naturally-occurring ‘loss-of-function’ mutation D191G in the human V\(_2\)R (corresponding to Asp\(^{204}\) in the V\(_{1a}\)R) which has been identified as a cause of NDI in some families (46). Asp\(^{204}\) is juxtaposed to the disulfide bridge (Cys\(^{205}\)), conserved in the majority of GPCRs, and is therefore under positional restraint. Interestingly, the residue corresponding to Asp\(^{204}\) has been reported to be functionally important in other GPCRs. For example, Met\(^{46}\) of the cholecystokinin-A receptor is required for interaction with CCK (47) and the mutation I185A affected CXCR4 co-receptor activity for some HIV strains (48).

The functional importance of Asp\(^{204}\) for agonist binding and signaling by V\(_{1a}\)R is a property shared by Arg\(^{125}\) (this study) and also Arg\(^{46}\) in the N-terminus (14). It was therefore possible that a charge-charge interaction between Arg\(^{125}\)-Asp\(^{204}\) or Arg\(^{46}\)-Asp\(^{204}\) was required for high affinity agonist binding. Interaction between these two charge-pairs was theoretically possible as Arg\(^{125}\)-Asp\(^{204}\) are located at opposite ends of the same disulfide bond and in bRho the N-terminus has been shown to make multiple contacts with ECL2 (1, 2). However, the double reciprocal mutants [R125D/D204R]V\(_{1a}\)R and [R46D/D204R]V\(_{1a}\)R were both severely compromised, therefore our data do not support a direct interaction of Asp\(^{204}\) with either Arg\(^{125}\) or Arg\(^{46}\).

Although ECL3 charged residues are important for peptide ligands binding to some GPCRs (49-51), substitution of the three conserved charged residues in ECL3 of the V\(_{1a}\)R did not affect either ligand binding or activation of the receptor. However, the mutant [D323A]V\(_{1a}\)R did exhibit decreased cell-surface expression (c.50 % of wild-type). It is noteworthy that Asp\(^{213}\) is the only ECL3 charged residue absolutely conserved throughout the vertebrate neurohypophysial hormone receptors cloned to-date, suggesting that it may fulfill an important role in maintaining cell-surface expression that is common to all members.
of this family. Our data do not support the suggestion (52) that ECL3 acidic residues might be implicated in binding AVP and AVT.

In conclusion, we have shown that key charged residues located throughout the extracellular face of the V1aR are required for normal receptor function, identifying Arg116 (ECL1), Arg125 (top of TMIII) and Asp204 (ECL2) as important for high affinity agonist binding and/or receptor activation and Asp323 (ECL3) as important for cell-surface expression. Consistent with their fundamental role in receptor function, these charged residues are highly conserved throughout the neurohypophysial hormone receptor sub-family of GPCRs.

REFERENCES


FOOTNOTES

1 The abbreviations used are: AVP, [arginine]vasopressin; AVT, vasotocin; bRho, bovine rhodopsin; CA, cyclic peptide antagonist; CCK, cholecystokinin; ECL, extracellular loop; ELISA, enzyme-linked immunosorbent assay; GPCR, G-protein-coupled receptor; HA, hemagglutinin; InsP, inositol phosphate;
Inositol trisphosphate; LA, linear peptide antagonist; NDI, nephrogenic diabetes insipidus; OT, oxytocin; OTR, oxytocin receptor; PhAc, phenylacetyl; TM, transmembrane helix; V1aR, V1a vasopressin receptor; V1bR, V1b vasopressin receptor; V2R, V2 vasopressin receptor; VTR, vasotocin receptor.

Residues in the TMs are referred to by residue number and the nomenclature of Ballosteros and Weinstein (53). This work was supported by grants to M.W. from the Wellcome Trust, the BBSRC and Ferring Research. We are grateful to Dr. Claudine Serradeil-Le Gal (Sanofi Recherche, France) for providing a sample of SR 49059. SRH current address – Novartis Pharma AG, WSJ-386.9.59, CH-4002 Basel, Switzerland.

FIGURE LEGENDS

FIG. 1. The extracellular face of neurohypophysial hormone receptors. A, Sequence alignment of the extracellular loop regions of vasopressin and oxytocin receptors cloned from different species. The sequences of the extracellular loop regions (ECL1, ECL2 and ECL3) of the V1aR, OTR, V1bR and V2R from different species have been aligned. The species of origin is indicated by a single letter code preceding the receptor subtype: r, rat; m, mouse; v, vole; s, sheep; h, human; p, pig; b, cow; mky, rhesus monkey; d, dog. Also shown is the sequence of the vasotocin and isotocin receptors from teleost fish and an amphibian mesotocin receptor. Conserved charged residues within these domains which were investigated in this study are boxed and numbered according to the rV1a sequence. Sequences cited were from SwissProt PDB and GenEMBL. B, Schematic diagram of the extracellular face of the rV1aR. Residues shown as white text on a black circle are the conserved charged residues investigated in this study.

FIG. 2. Pharmacological characterization of ECL mutant receptors. Radioligand binding studies with AVP as competing ligand were performed using a membrane preparation of HEK 293T cells transiently transfected with: A, wild-type (Arg116) V1aR, (○); [R116A]V1aR, (●); [R116E]V1aR, (▲); [R116D]V1aR, (●) and [R116K]V1aR, (◆). B, wild-type (Asp204) V1aR, (○); [D112A]V1aR, (●); [D112E]V1aR, (▲); [D112K]V1aR, (●) and [D112R]V1aR, (◆). C, wild-type (Asp53) V1aR, (○); [D204A]V1aR, (●); [D204E]V1aR, (▲) and [D204R]V1aR, (■). Data are the mean ± SEM of three separate experiments each performed in triplicate using [3H]AVP (0.5–1.5 nM) or [3H]CA (0.5–1.5 nM) as tracer. Values are expressed as percent specific binding where non-specific binding was defined by d(CH2)5Tyr(Me)2AVP (1 μM). A theoretical Langmuir binding isotherm has been fitted to the experimental data as described in “Experimental Procedures”.

FIG. 3. Intracellular signaling by ECL mutant receptors. AVP-induced accumulation of mono-, bis-, and trisphosphates in HEK 293T cells transiently transfected with: A, wild-type (Arg116) V1aR, (○); [R116A]V1aR, (●); [R116E]V1aR, (▲); [R116D]V1aR, (●) and [R116K]V1aR, (◆). B, wild-type (Arg125) V1aR, (○); [R125A]V1aR, (●); [R125D]V1aR, (▲) and [R125K]V1aR, (◆). C, wild-type (Asp112) V1aR, (○); [D125A]V1aR, (●); [D125E]V1aR, (▲) and [D125K]V1aR, (◆). D, wild-type (Asp204) V1aR, (○); [D204A]V1aR, (●); [D204E]V1aR, (▲) and [D204R]V1aR, (■). Data are the mean ± SEM of three separate experiments each performed in triplicate. Values are stimulation induced by AVP at the stated concentrations expressed as percent maximum.

FIG. 4. Molecular modelling of wild-type and mutant receptors. Panel A: The overlapped positions of solvent molecules are presented for wild-type V1aR (yellow) and [R125D]V1aR (blue) simulations. It can be seen that the mutation [R125D]V1aR increased the solvent accessible surface as a result of re-ordering of the phospholipids surrounding the extracellular end of TMIII. Panel B: The mutant [D112R]V1aR enabled inappropriate hydrogen bonds (dotted green lines) to be formed between this residue and Glu54 in TM1. Panel C: In the wild-type V1aR, Asp204 in ECL2 hydrogen bonds (dotted green lines) with Lys128 and Gln131 in TMIII. These contacts are broken in the mutant [D204A]V1aR (Panel D) which results in
partial unfolding of the β-hairpin structure of ECL2. Panel E: the partially unfolded ECL2 β-hairpin structure of [D204A]V_{1a}R (blue) is revealed when superimposed onto that of wild-type V_{1a}R (yellow). Panel F: The mutant [D204R]V_{1a}R inappropriately hydrogen bonds (dotted green) with residues in TMVII and TMII. See text for details.
TABLE I - Pharmacological profile of mutant V₁₉₅Rs.


<table>
<thead>
<tr>
<th>Receptor Constructs</th>
<th>Binding affinities $K_d$ (nM)</th>
<th>Stimulation of InsP – InsP$_3$</th>
<th>Cell-surface expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVP</td>
<td>CA</td>
<td>LA</td>
</tr>
<tr>
<td>WT</td>
<td>1.0</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>ECL1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp$^{112}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.2</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>E</td>
<td>2.3</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>K</td>
<td>2.0</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>R</td>
<td>390</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Arg$^{116}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.4</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>D</td>
<td>1600</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>E</td>
<td>730</td>
<td>4.7</td>
<td>0.5</td>
</tr>
<tr>
<td>K</td>
<td>3.8</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>R118A</td>
<td>2.6</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>D121A</td>
<td>0.8</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Arg$^{125}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.4</td>
<td>1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>K</td>
<td>1.1</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>ECL2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E193A</td>
<td>1.9</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>E195A</td>
<td>1.3</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Asp$^{204}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2300</td>
<td>2.0</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>1.6</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>R</td>
<td>2600</td>
<td>4.4</td>
<td>12</td>
</tr>
<tr>
<td>K201A</td>
<td>1.4</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>R216A</td>
<td>1.2</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>R46D$^*$</td>
<td>1800</td>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>R46D/D204R</td>
<td>2500</td>
<td>5.7</td>
<td>15</td>
</tr>
<tr>
<td><strong>ECL3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D323A</td>
<td>1.2</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>D330A</td>
<td>1.8</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>E332A</td>
<td>2.3</td>
<td>0.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>
FIG. 1.

A)

<table>
<thead>
<tr>
<th></th>
<th>ECL1</th>
<th>ECL2</th>
<th>ECL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>112</td>
<td>114</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>125</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>216</td>
<td>323</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>332</td>
<td></td>
</tr>
</tbody>
</table>

B)

![Diagram of receptor structure](http://www.jbc.org/)
FIG. 2.
FIG. 3.
Charged extracellular residues, conserved throughout a G-protein-coupled receptor family, are required for ligand binding, receptor activation and cell-surface expression
Stuart R. Hawtin, John Simms, Matthew Conner, Zoe Lawson, Rosemary A. Parslow, Julie Trim, Andrew Sheppard and Mark Wheatley

J. Biol. Chem. published online September 21, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M607639200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts