Critical Role of a Subdomain of the N-Terminus of the V_{1a} Vasopressin Receptor for Binding Agonists but Not Antagonists; Functional Rescue by the Oxytocin Receptor N-Terminus†

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ABSTRACT: A fundamental issue in molecular pharmacology is to define how agonist:receptor interaction differs from that of antagonist:receptor. The V_{1a} receptor ($V_{1a}R$) is a member of a family of related G-protein-coupled receptors that are activated by the neurohypophysial peptide hormone arginine-vasopressin (AVP). Here we define a short subdomain of the N-terminus of the $V_{1a}R$ from Glu^{37} to Asn^{47} that is an absolute requirement for binding AVP and other agonists. In marked contrast to the situation for agonists, deleting this segment has little or no effect on the binding of either peptide or non-peptide antagonists. In addition, we established that this subdomain was crucial for receptor activation and second messenger generation. The oxytocin receptor (OTR) also binds AVP with high affinity but exhibits a different pharmacological profile to the $V_{1a}R$. Substitution of the N-terminus of the $V_{1a}R$ with the corresponding sequence from the OTR generated a chimeric receptor (OTR^N-$V_{1a}R$). The presence of the OTR N-terminus recovered high affinity agonist binding such that the OTR^N-$V_{1a}R$ possessed almost wild-type $V_{1a}R$ pharmacology and signaling. Consequently, a domain within the N-terminus is required for agonist binding but it does not provide the molecular discriminator for subtype-selective agonist recognition. Cotransfection and peptide mimetic studies demonstrated that this N-terminal subdomain had to be contiguous with the receptor polypeptide to be functional. This study establishes that a segment of the $V_{1a}R$ N-terminus has a pivotal role in the mechanism of agonist binding and provides molecular insight into key differences between the interaction of agonists and antagonists with a peptide receptor family.

G-protein-coupled receptors (GPCRs)[1] are a large family of structurally related proteins which mediate their effects by coupling to G-proteins. Several hundred GPCRs have now been cloned. Despite being activated by a wide variety of stimuli from photons to glycoproteins, these receptors exhibit primary sequence homology and a conserved tertiary structure comprising a bundle of seven transmembrane domains (*1*). Analysis of these sequences has revealed that GPCRs can be further categorized into the rhodopsin/β-adrenergic receptor family, the secretin receptor family and metabotropic glutamate receptors (*2*−*4*). The largest and most extensively characterized of these is the rhodopsin/β-adrenergic receptor family.

The neurohypophysial hormones [$arginine^8$]vasopressin (AVP) and oxytocin (OT) are structurally homologous. Both are nonapeptides, possess an intramolecular disulfide bond and have sequences which differ at just two positions. Nevertheless, these neuropeptides have discrete physiological roles. AVP exhibits a plethora of responses in addition to the well-characterized vasopressor and antidiuretic actions (*5*) and OT stimulates uterine contraction at parturition and lactation (*6*). The physiological effects of both of these hormones are mediated by receptors which belong to the rhodopsin/β-adrenergic receptor family. Four different receptor subtypes have been defined and cloned. The V_{1a} vasopressin receptor ($V_{1a}R$), the V_{1b} vasopressin receptor ($V_{1b}R$), and the OT receptor (OTR) stimulate phosphoinositidase C, whereas the V_2 vasopressin receptor (V_2R) stimulates adenylyl cyclase (*7*, *8*). The $V_{1a}R$ is widely distributed and mediates nearly all of the actions of AVP with the notable exceptions of antidiuresis (V_2R) and ACTH secretion ($V_{1b}R$). All four receptor subtypes exhibit different, but related, pharmacological profiles (*9*).

The primary event in the activation of a receptor by a neurotransmitter or hormone is the binding of the natural agonist to its receptor. The use of synthetic analogues to mimic this event (agonists), or to prevent its occurrence (antagonists), is of fundamental importance to a wide range of therapeutic strategies. For this reason, the identification of the ligand-binding site in GPCRs has been the subject of

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[1] Abbreviations: AVP, [$arginine^8$]vasopressin; AVT, [$arginine^8$]-vasotocin; GPCR, G-protein-coupled receptor; InsP, inositol phosphate; $InsP_4$, inositol tetrakisphosphate; OP, oxypressin (Phe^3OT); OT, oxytocin; OTR, oxytocin receptor; OTR^N-$V_{1a}R$, chimeric receptor with the N-terminal domain of the $V_{1a}R$ replaced by the N-terminus of the OTR.; PhAc, phenylacetyl; POVT, [Phe^2,Orn^8]vasotocin; TGOT, [Thr^4,Gly^7]-OT.; $V_{1a}R$, vasopressin V_{1a} receptor; $V_{1b}R$, vasopressin V_{1b} receptor; V_2R, vasopressin V_2 receptor. Truncation/deletion nomenclature: numbers refer to the positions in the sequence of the residues deleted, hence [Δ37−47]$V_{1a}R$ is the $V_{1a}R$ with residues 37−47 inclusive deleted.

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extensive research in recent years (*2*, *10*). Although the binding of agonists and antagonists to the receptor is usually competitive, there is obviously a critical difference in that occupancy by agonists generates an intracellular signal whereas binding antagonists does not. Defining the differences at the molecular level between the receptor:agonist complex and receptor:antagonist complex is central to our understanding of GPCR activation and to the rational design of receptor-specific agonists and antagonists. The V_{1a}R is particularly useful for addressing this aspect of GPCRs as an extensive range of potent and well-characterized ligands is available for probing the binding site (*9*). Furthermore, these ligands can be characterized into four different classes: (i) peptide agonists, (ii) cyclic peptide antagonists possessing a disulfide bond, a 20-membered ring and a short peptide tail, (iii) linear peptide antagonists, and (iv) non-peptide antagonists. In addition, the V_{1a}R and the OTR are homologous and possess related, but discrete, pharmacology which has resulted in the development of a series of agonists with different V_{1a}R/OTR selectivities. This makes it possible not only to investigate agonist:receptor binding but also agonist selectivity.

In this study, we have engineered a series of truncated, deleted, and chimeric receptors which has allowed us to identify a short subdomain of the N-terminus which is critical for both agonist binding and receptor activation. In contrast, this domain does not constitute part of the binding site for antagonists, whether peptide or non-peptide. Interestingly, substitution of this domain with the corresponding sequence from the OTR (OTR^N-V_{1a}R) recovered almost wild-type V_{1a}R pharmacology. However, coexpression with an N-terminus construct, or incubation with a peptide mimetic corresponding to this domain of the V_{1a}R, could not bestow agonist recognition on a binding-deficient truncation. Our data establish that a short segment of the N-terminus is not only critical for high affinity agonist binding but that this same extracellular subdomain has an important role in the receptors ability to adopt the agonist-induced active conformation state.

EXPERIMENTAL PROCEDURES

Materials. AVP, OT, [Thr^4,Gly^7]oxytocin (TGOT), and [arginine^8]vasotocin (AVT) were purchased from Sigma. The cyclic antagonist 1-(β-mercapto-β,β-cyclopentamethylene-propionic acid), 2-(*O*-methyl)tyrosine AVP [d$(CH_2)_5$Tyr-$(Me)^2$]AVP was from Bachem (U.K.). The linear antagonist PhAc*D*-Tyr$(Me)^2$Arg^{6}Tyr$(NH_2)^9$AVP was synthesized and purified in our laboratory as described previously (*11*). Phe^3OT (OP, oxypressin) and [Phe^2,Orn^8]vasotocin (POVT) were synthesized and provided by Prof. Maurice Manning (Toledo, OH). SR 49059 was obtained from Sanofi Recherche (Toulouse, France). Cell culture media, buffers, and supplements were purchased from Gibco (Uxbridge, U.K.).

Truncated, Deleted, and Chimeric Receptor Constructs. Progressive truncations of the N-terminus of the V_{1a}R were made using a PCR approach (*12*). Truncation oligonucleotides were 5′-GGG-GGG-CCC-GGA-TCC-**GCC-ACC**-*ATG*-GAG-GGC-TCC-AAC-GGC-AGT-CAG-G-3′, 5′-GGG-GGG-CCC-GGA-TCC-**GCC-ACC**-*ATG*-GAA-GGT-GAC-AGC-CCG-CTG-GGG-3′ and 5′G-GGG-GGG-CCC-GGA-TCC-**GCC-ACC**-*ATG*-GAG-GAG-CTG-GCC-AAA-CTG-GAA-ATC-GC-3′ for the [Δ2−23]V_{1a}R, [Δ2−36]V_{1a}R, [Δ2−47]V_{1a}R truncations, respectively (Figure 1). Each primer contained an *Apa*I and *Bam*HI restriction sites (underlined), Kozak consensus sequence [shown in bold (*13*)] and an ATG start site (shown in italics) followed by the V_{1a}R sequence. The PCR cycling conditions were denaturing, 94 °C (1 min); annealing, 50−60 °C (2 min); extension, 72 °C (1 min) for 30 cycles followed by extension at 72 °C (7 min). The PCR products were subcloned into the rat V_{1a}R coding sequence in pBluescript KS II vector (Stratagene) utilizing unique *Apa*I and *Bbu*I sites. Truncated V_{1a}R constructs were subcloned into the mammalian expression vector pcDNA3 (Invitrogen) utilizing *Bam*HI and *Eco*RI restriction sites. The OTR^N-V_{1a}R chimera was made by amplification (as described above) of the human OTR N-terminus by PCR using 5′-CTC-GTT-GCG-TAC-**G**GG-GGG-TCC-GGC-GGT-GCG-GTT-GCC-3′ as the antisense oligonucleotide. This primer contained a single base change in the OTR sequence (indicated in bold) which created a unique *Pfl*23II restriction site (underlined) without altering the amino acid sequence. A *Bam*HI/*Pfl23*II digest of this PCR fragment was subcloned into the pcDNA3-V_{1a}R vector. This generated a chimeric receptor construct in which the V_{1a}R N-terminal sequence was substituted by an in-frame OTR N-terminal sequence. The [Δ37−47]V_{1a}R deletion was constructed using the antisense primer 5′-CAC-TGC-**T**AG-CAC-AGC-GAT-TTC-CAG-**C**TT-**A**GC-CAG-CTC-CTC-CCC-AAG-CCT-GGC-TGC-CTC-CTG-ACT-GCC-3′. This contained three base changes (in bold) to the V_{1a}R sequence which created unique *Nhe*I and *Blp*I restriction sites (underlined) but did not change the amino acid sequence. A *Hin*dIII/*Nhe*I cassette was subsequently subcloned into the pcDNA3-V_{1a}R vector. The [1−86]V_{1a}R construct was engineered using PCR with the oligonucleotide 5′-CCC-CCG-AAT-TC*A*-CAT-GCG-GGA-TGT-CTT-GC-3′ as antisense primer. This incorporated a stop codon (italics) and an *Eco*RI restriction site (underlined). A *Hin*dIII/*Eco*RI cassette was subcloned into the expression vector. All receptor constructs were confirmed by automated fluorescent sequencing (Alta Bioscience, University of Birmingham, U.K.).

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, penicillin (100 IU/mL), streptomycin (100 μg/mL), glutamine (2 mM), and sodium pyruvate (1 mM) in humidified 5% (v/v) CO_2 in air at 37 °C. Cells were seeded at a density of approximately 5×10^5 cells/100 mm dish and transfected after 48 h using a calcium phosphate precipitation protocol (*14*) with 10 μg of DNA/dish.

Radioligand Binding Assays. A washed cell membrane preparation of HEK 293T cells, transfected with the appropriate receptor construct, was prepared as previously described (*15*), and the protein concentration determined using the BCA protein assay kit (Pierce Chemical Co.) with bovine serum albumin as standard. Radioligand binding assays were performed as previously described (*16*) using either the natural agonist [Phe^3-3,4,5-^{3}H]AVP (64.2 Ci/mmol; DuPont NEN, U.K.) or the V_{1a}R-selective peptide antagonist [Phe^3-3,4,5 -^{3}H] d$(CH_2)_5$Tyr$(Me)^2$AVP (30.8 Ci/mmol; DuPont NEN, U.K.) (*17*) as tracer ligand. Competition binding assays (final volume of 500 μL) containing radioligand (0.3−1.1 nM), cell membranes (50−300 μg), and competing ligand

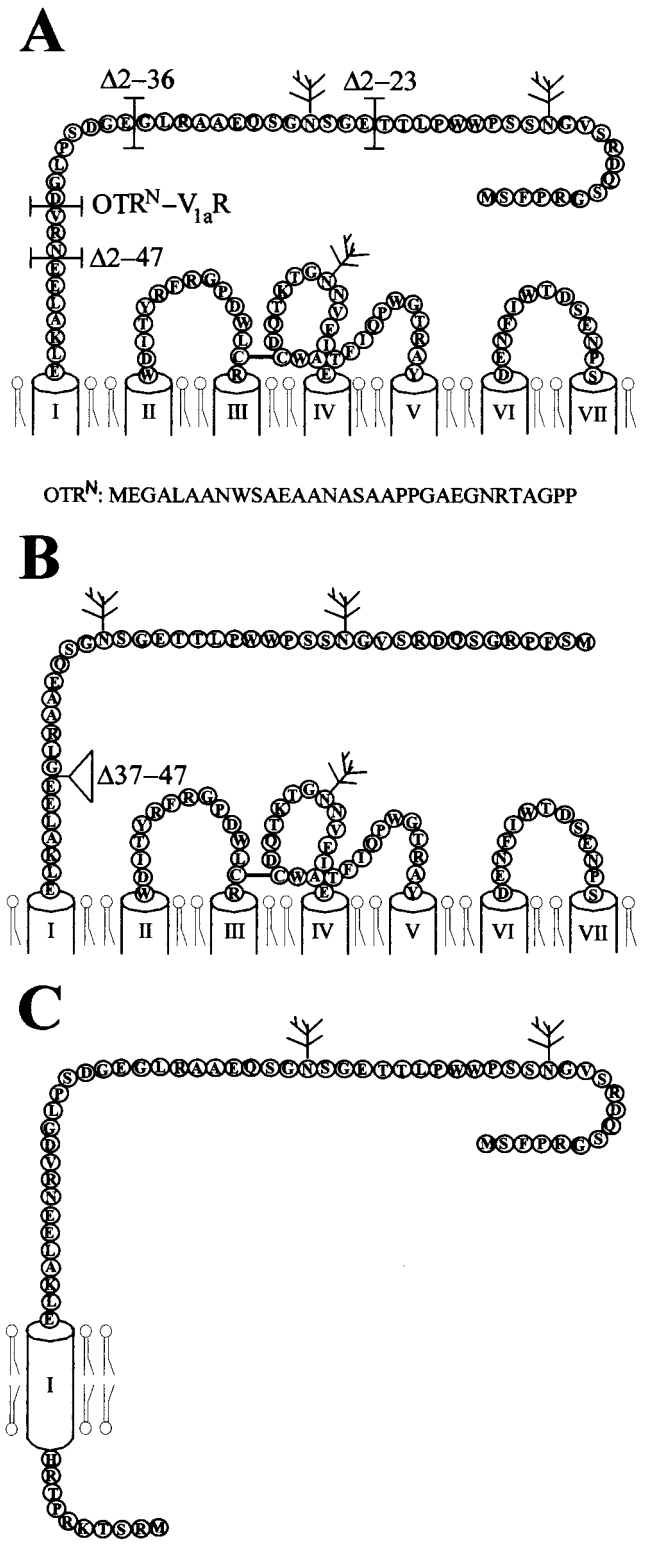


FIGURE 1: Extracellular domains of the $V_{1a}R$ and engineered constructs. (A) Only the extracellular face of the receptor is illustrated, with the top of the transmembrane domains represented by cylinders I−VII. Putative glycosylation sites are indicated by the branched structures. Truncations of the N-terminus are indicated by bars labeled Δ2−23, Δ2−36, and Δ2−47 respectively, where the numbers refer to the positions in the sequence of the residues deleted. The N-terminus of the $V_{1a}R$ was also replaced, from the position marked, by the corresponding OTR sequence (shown below the main figure) to generate the OTR^N-$V_{1a}R$ chimeric receptor. Panel B; as for panel A but showing the [Δ37−47]$V_{1a}R$ construct. Panel C shows the [1−86]$V_{1a}R$ construct with the TM1 domain represented schematically as a cylinder for clarity.

(at the concentrations indicated) were incubated at 30 °C for 90 min to establish equilibrium. Membranes were sedimented by centrifugation (12000*g*, 10 min) to separate bound from free ligand, washed, dissolved in tissue solubilizer (Soluene-350, Packard) and radioactivity quantified by liquid scintillation spectroscopy using HiSafe 3 (Packard) as cocktail. Nonspecific binding was determined in parallel incubations using 10 μM unlabeled AVP or $d(CH_2)_5Tyr(Me)^2AVP$ as appropriate. Binding data were analyzed by nonlinear regression to fit theoretical Langmuir binding isotherms to the experimental data using the Fig. P program (Biosoft). Individual IC_{50} values obtained for competing ligands were corrected for radioligand occupancy according to Cheng and Prusoff (*18*) using the radioligand affinity (K_d) experimentally determined for each construct.

AVP-Induced Inositol Phosphate Production. HEK 293T cells were seeded at a density of 2.5×10^5 cells/well in poly D-lysine-coated six well plates and transfected as above. The assay for AVP-induced accumulation of inositol phosphates was based on that described previously (*19*, *20*). Briefly, 24 h posttransfection, medium was replaced with serum-free, inositol-free, DMEM containing 2.5 μCi/mL myo-[2-^{3}H]-inositol (22.3 Ci/mmol; Tocris Cookson) for 48 h. Cells were then washed with PBS, incubated with medium containing 10 mM LiCl for 30 min, after which AVP was added at the concentrations indicated. Incubations were terminated after 30 min by washing in PBS followed by the addition of 0.5 mL/well of 5% (w/v) $HClO_4$, 1 mM EDTA, and 1 mg/mL phytic acid hydrolysate. After neutralization, samples were loaded onto Bio-Rad AG1-X8 columns (formate form). Following the elution of inositol (10 mL of water) and glycerophosphoinositol (10 mL of 25 mM NH_4COOH), a mixed inositol fraction containing mono-, bis-, tris-, and tetrakisphosphates ($InsP-InsP_4$) was eluted with 10 mL of 1.25 M NH_4COOH containing 0.1 M HCOOH, mixed with 10 mL of UltimaFlo AF scintillation cocktail (Packard) and radioactivity quantified by liquid scintillation spectroscopy.

Synthesis of the Peptide Mimetic. The peptide mimetic corresponding to residues 25−47 of the $V_{1a}R$ was synthesized by Alta Bioscience (University of Birmingham, U.K.) on a 0.2 mmol scale using *N*α-9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and conventional solid-phase methodology. The peptide was carboxy-amidated and purified to homogeneity by semipreparative reversed-phase HPLC with a Vydac C_{18} column (*21*, *22*). The purity and identity of the peptide was confirmed using a Micromass LC-TOF electrospray mass spectrometer. This established that the synthesized peptide had a mass of 2284 Da, which was the mass predicted for the required sequence.

RESULTS

Role of the N-Terminal Domain of the $V_{1a}R$ in Ligand Binding. To address the role of the N-terminal domain of the $V_{1a}R$ in ligand recognition, a series of receptor constructs was made with progressively greater N-terminal deletions. Three mutant receptors were engineered which were truncated at either Thr^{23}, Gly^{36}, or Asn^{47} as indicated in Figure 1A. The initiation methionine was retained in each case. These constructs were termed [Δ2−23]$V_{1a}R$, [Δ2−36]$V_{1a}R$, and [Δ2−47]$V_{1a}R$, respectively, where the numbering refers to the amino acid residues deleted.

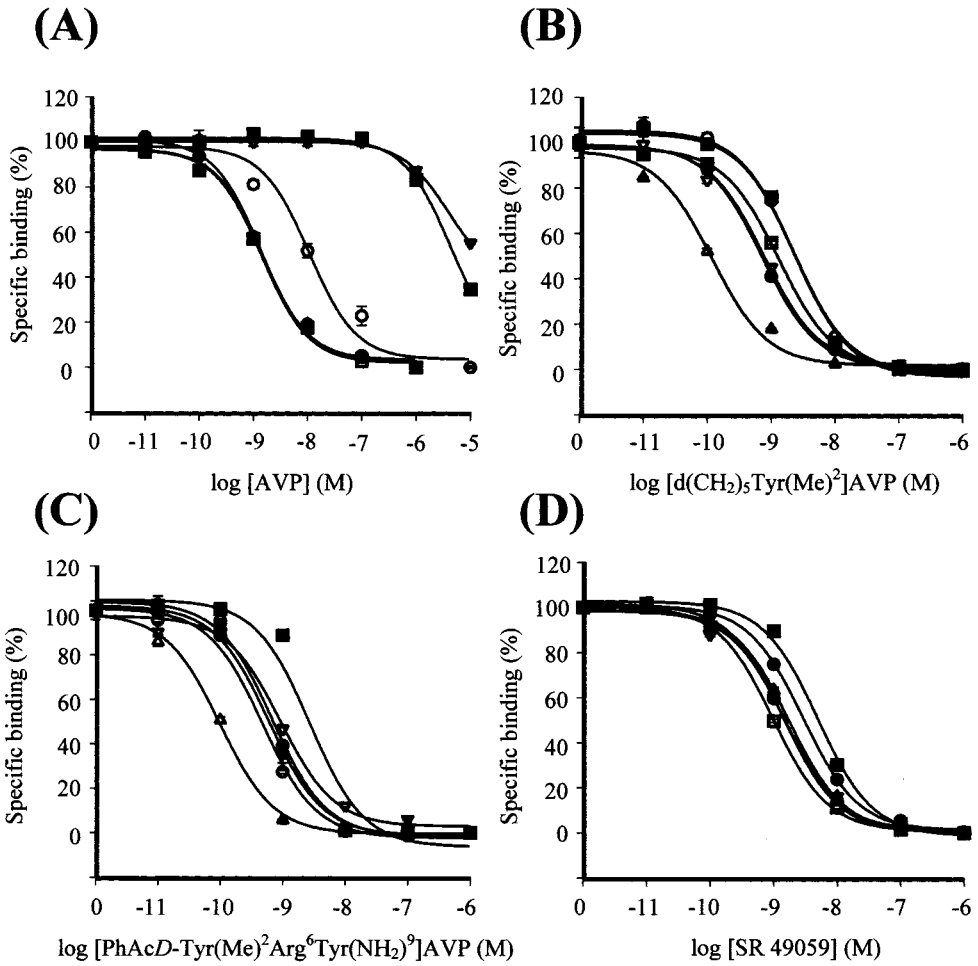


FIGURE 2: Pharmacological characterization of truncated and chimeric receptors. Competition radioligand binding studies with AVP (A), $d(CH_2)_5Tyr(Me)^2AVP$ (B), PhAc*D*-$Tyr(Me)^2Arg^6Tyr(NH_2)^9AVP$ (panel C) and SR 49059 (panel D) were performed using a membrane preparation of HEK 293T cells transiently transfected with either wild-type $V_{1a}R$, (●); $[\Delta 2-23]V_{1a}R$, (□); $[\Delta 2-36]V_{1a}R$, (△); $[\Delta 2-47]$-$V_{1a}R$, (▽); $[\Delta 37-47]V_{1a}R$, (■); OTR^N-$V_{1a}R$, (○). Data are the mean ± SEM of three separate experiments each performed in triplicate. Values are expressed as percent specific binding where nonspecific binding was defined by $d(CH_2)_5Tyr(Me)^2AVP$ (10 μM). A theoretical Langmuir binding isotherm has been fitted to the experimental data as described in Experimental Procedures.

Table 1: Pharmacological Profile of Truncated, Deleted, and Chimeric Vasopressin Receptors[a]

ligand	binding affinities K_d (nM)					
	$V_{1a}R$	$[\Delta 2-23]V_{1a}R$	$[\Delta 2-36]V_{1a}R$	$[\Delta 2-47]V_{1a}R$	$[\Delta 37-47]V_{1a}R$	OTR^N-$V_{1a}R$
AVP	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.4	1800 ± 130	5500 ± 2400	7.5 ± 2.2
[PhAc*D*-$Tyr(Me)^2Arg^6Tyr(NH_2)^9$]AVP	0.5 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	1.2 ± 0.4	2.3 ± 0.5	0.7 ± 0.1
$d(CH_2)_5Tyr(Me)^2AVP$	0.7 ± 0.3	1.2 ± 0.5	0.1 ± 0.1	0.6 ± 0.2	2.7 ± 0.2	2.4 ± 0.8
SR 49059	1.9 ± 0.3	0.5 ± 0.1	0.8 ± 0.1	1.1 ± 0.4	4.9 ± 0.5	1.3 ± 0.1
receptor expression (B_{max}) (pmoles/mg protein)	1.1 ± 0.4	1.0 ± 0.1	1.4 ± 0.1	1.7 ± 1.0	1.9 ± 0.4	2.2 ± 0.2

[a] Truncated, deleted and chimeric V_{1a}Rs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) and concentration of receptor sites (B_{max}) were calculated from IC_{50} values and corrected for radioligand occupancy as described in Experimental Procedures. Data shown are the mean ± SEM ($n = 3$) of three replicates.

The diversity of structural classes available for probing the ligand binding pocket is greater for V_{1a}Rs than for most GPCRs with peptide ligands. The natural agonist AVP has an intramolecular disulfide bond between Cys^1 and Cys^6 which forms a 20-membered ring with a tripeptide tail. This bond is retained in the peptide $d(CH_2)_5Tyr(Me)^2AVP$ which is a cyclic antagonist (*17*). In contrast, the high affinity antagonist [PhAc*D*-$Tyr(Me)^2Arg^6Tyr(NH_2)^9$]AVP is linear as it has phenylacetyl (PhAc) and arginyl residues at positions 1 and 6 respectively (*23*, *24*). Recent reports of non-peptide antagonists, such as SR 49059 (*25*), have provided a fourth class of ligand.

The pharmacological characteristics of the truncated receptor constructs were investigated and compared to wild-type receptors, following expression in HEK 293T cells. Competition radioligand binding curves were determined using the four different classes of ligand (Figure 2). The K_d values are presented in Table 1, corrected for radioligand occupancy. The wild-type receptor and truncated constructs were all expressed at the same level of approximately 1−2 pmol/mg protein (Table 1).

Truncation of the N-terminus as far as Gly^{36} (i.e., constructs $[\Delta 2-23]V_{1a}R$ and $[\Delta 2-36]V_{1a}R$) had no effect on the binding of agonists or non-peptide antagonist. Likewise,

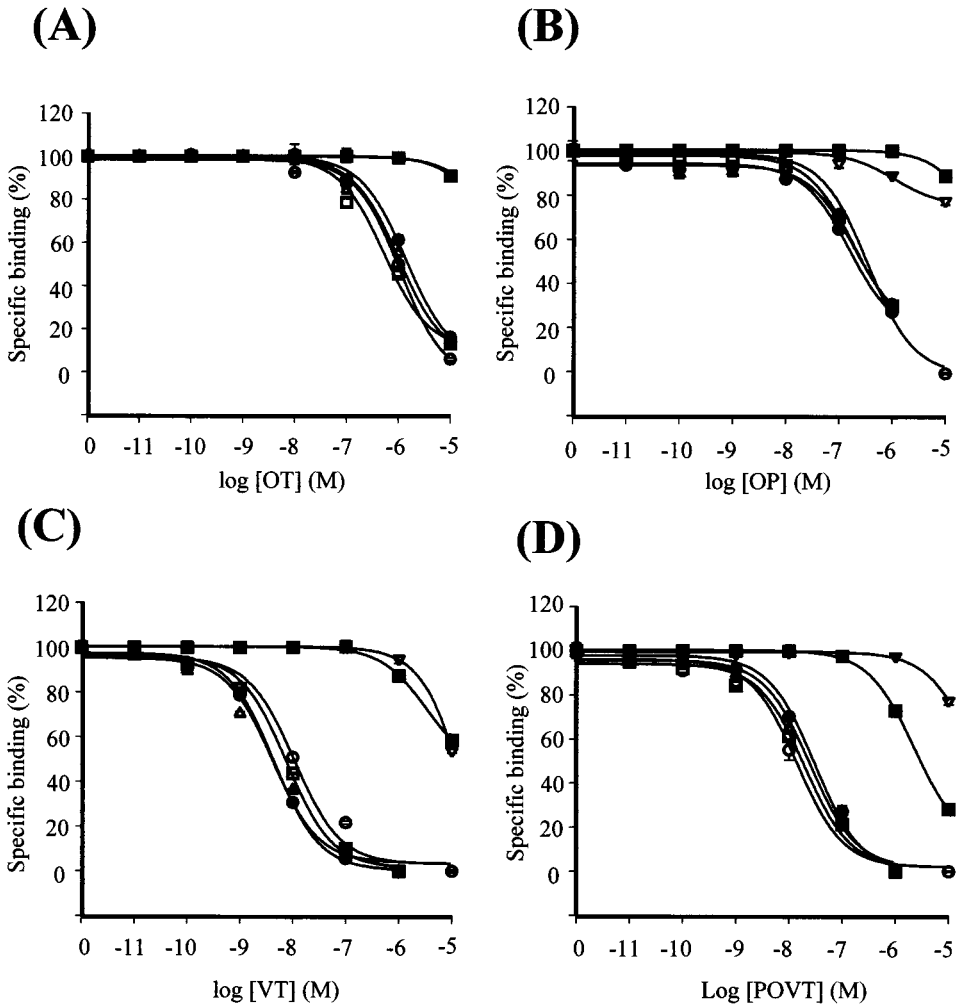


FIGURE 3: Comparison of agonists binding to the various $V_{1a}R$ constructs. Competition radioligand binding studies were performed using a membrane preparation of HEK 293T cells transiently transfected with either wild-type $V_{1a}R$, (●); [Δ2−23]$V_{1a}R$, (□); [Δ2−36]$V_{1a}R$, (△); [Δ2−47]$V_{1a}R$, (▽); [Δ37−47]$V_{1a}R$, (■); OTR^N-$V_{1a}R$, (○) with the agonists OT (A), OP, (B), AVT (C), and POVT (D). Data are the mean ± SEM of three separate experiments each performed in triplicate. Values are expressed as percent specific binding where nonspecific binding was defined by $d(CH_2)_5Tyr(Me)^2AVP$ (10 μM). A theoretical Langmuir binding isotherm has been fitted to the experimental data as described in Experimental Procedures.

the binding of peptide antagonists was largely unaffected, although a slight increase (5-fold) in the affinity of peptide antagonists compared to wild-type was observed when the N-terminus was truncated from Glu^{24} to Gly^{36} (Figure 2 and Table 1, compare [Δ2−36]$V_{1a}R$ to [Δ2−23]$V_{1a}R$ and wild-type $V_{1a}R$). However, further truncations of the N-terminus to Asn^{47} ([Δ2−47]$V_{1a}R$) resulted in a profound decrease in agonist affinity with the K_d for AVP increasing approximately 2000-fold. In marked contrast, the affinity of the three antagonists for the [Δ2−47]$V_{1a}R$ was not markedly different from wild-type (Table 1). This retention of high-affinity antagonist binding by the truncated $V_{1a}R$ constructs was important practically as it allowed the altered binding characteristics of agonists to be determined using the antagonist $[^3H]d(CH_2)_5Tyr(Me)^2AVP$ as tracer.

To verify the functional importance of the segment of the N-terminus from Glu^{37}−Asn^{47}, we engineered the construct [Δ37−47]$V_{1a}R$. This construct was similar to the wild-type $V_{1a}R$ in that it had an N-terminus with two glycosylation sites but it lacked residues Glu^{37}−Asn^{47} (Figure 1B). Characterization of [Δ37−47]$V_{1a}R$ revealed that it displayed essentially the same pharmacological properties as the [Δ2−47]$V_{1a}R$ construct, with very low affinity for AVP (Figure 2, Table 1). The binding affinity of each class of antagonist to [Δ37−47]$V_{1a}R$ was slightly decreased (2−5-fold), probably reflecting the formation of some unfavorable contacts upon deletion of residues 37−47.

Identification of an N-Terminal Subdomain Critical for High Affinity Agonist Binding. Data presented above established the importance of a short subdomain within the N-terminus of the $V_{1a}R$ for high affinity binding of AVP. It was important to ascertain if this phenomenon was unique to the natural agonist AVP, or if the presence of this segment between Glu^{37} and Asn^{47} is a prerequisite for high-affinity binding of agonists in general. The pharmacological profile of the truncated and deleted receptors was characterized using a series of agonists which possessed affinities for the wild-type $V_{1a}R$ ranging from approximately 1 to 1000 nM. The [Δ2−23]$V_{1a}R$ and [Δ2−36]$V_{1a}R$ exhibited wild-type pharmacology for these agonists. In contrast, the [Δ2−47]$V_{1a}R$ and [Δ37−47]$V_{1a}R$ had a dramatically reduced affinity for all of the agonists tested (Figure 3 and Table 2). Consequently, it can be concluded that although the critical role of this subdomain in high affinity binding is not restricted to AVP, it is restricted to agonists.

Table 2: Agonist Binding Profile of Truncated, Deleted, and Chimeric Vasopressin Receptors[a]

	binding affinities K_d (nM)						
ligand	$V_{1a}R$	[Δ2−23]$V_{1a}R$	[Δ2−36]$V_{1a}R$	[Δ2−47]$V_{1a}R$	[Δ37−47]$V_{1a}R$	OTR^N-$V_{1a}R$	OTR
OT	1400 ± 320	590 ± 170	830 ± 130	>10 μM	>10 μM	850 ± 170	1.4 ± 0.5
OP	270 ± 90	350 ± 130	360 ± 100	>10 μM	>10 μM	280 ± 53	7.0 ± 0.8
TGOT	>10 μM	>10 μM	>10 μM	>10 μM	>10 μM	>10 μM	19 ± 2
AVP	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.4	1800 ± 130	5500 ± 2400	7.5 ± 2.2	9.2 ± 0.8
AVT	3.7 ± 1.3	1.8 ± 0.3	1.8 ± 0.3	>10 μM	3900 ± 2300	7.7 ± 0.5	1.0 ± 0.1
POVT	23 ± 4	28 ± 10	20 ± 1.8	>10 μM	3900 ± 660	11.4 ± 3.8	46 ± 5

[a] Truncated, deleted, and chimeric V_{1a}Rs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) and concentration of receptor sites (B_{max}) were calculated from IC_{50} values and corrected for radioligand occupancy as described in Experimental Procedures. Data shown are the mean ± SEM ($n = 3$) of three replicates.

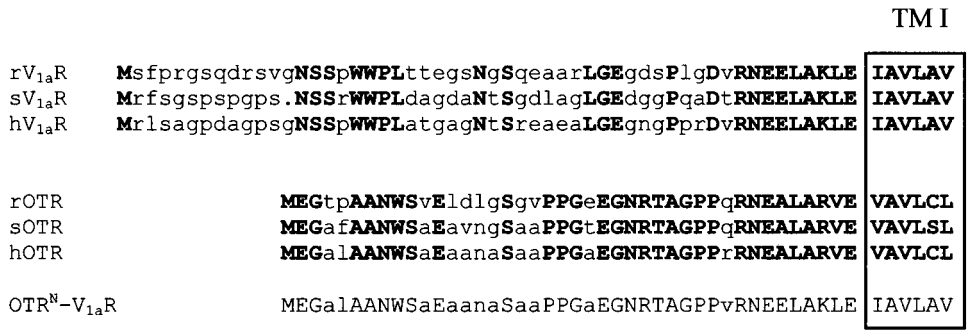
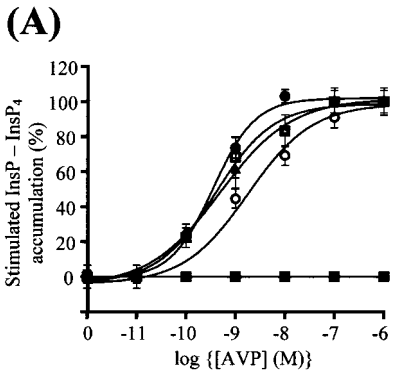


FIGURE 4: Comparison of the sequence of the N-terminus of vasopressin and oxytocin receptors cloned from different species. The sequences of the N-terminus of the V_{1a}R from rat [rV_{1a}R (*54*−*56*)], sheep [sV_{1a}R (*57*)], and human [hV_{1a}R (*58*)] and the OTR also from rat [rOTR (*59*)], sheep [sOTR (*60*)], and human [hOTR (*61*)] are aligned. The corresponding sequence of the OTR^N-V_{1a}R chimera is also shown. Residues that are conserved in the V_{1a}R or OTR sequences from different species are in bold type. The position of the top of transmembrane domain I (TM I) is indicated by the box.

Functional Rescue Studies Using the V_{1a}R N-Terminus. To investigate if the N-terminus of the V_{1a}R could recover high affinity AVP binding to the functionally impaired [Δ2−47]V_{1a}R, we engineered [1−86]V_{1a}R. This construct contains the wild-type V_{1a}R N-terminus, the first transmembrane domain (TM1), and most of the first intracellular loop as shown in Figure 1C. [1−86]V_{1a}R alone did not bind AVP and when coexpressed with [Δ2−47]V_{1a}R, which exhibits a very low affinity for agonists (Table 2), the affinity of AVP was unchanged and AVP-induced accumulation of inositol phosphates was not observed (data not shown). The use of relatively small synthetic peptides corresponding to sequences within receptors has proven to be a useful strategy for establishing the functional importance of individual domains. Such peptides can sometimes mimic a property of the whole receptor such as ligand binding (*26*) or G-protein activation (*27*). We synthesized the peptide ^{25}GSNGSQEAARLGEGDSPLGDVRN^{47}, which incorporates the subdomain in the N-terminus required for agonist binding. This was subsequently used in radioligand binding assays with the [Δ2−47]V_{1a}R construct. The presence of the synthetic peptide mimetic (10 μM) did not recover high affinity binding of AVP (data not shown). It was concluded that the N-terminal subdomain had to be contiguous with the receptor polypeptide chain to fulfill its role in high affinity agonist binding. It could not function as a discrete, independently folded, supplementary domain.

Role of the N-Terminus in the Selectivity of Receptor-specific Agonists. The V_{1a}R and the OTR exhibit related pharmacological profiles with many analogues (agonist, antagonist; peptide and non-peptide) binding to both receptors (*9*, *28*). Indeed, AVP itself binds with high affinity to both receptors (Table 2). Agonists displaying selectivity for V_{1a}R, or OTR, have nevertheless been developed (e.g., refs *29* and *30*). The N-terminal sequence of V_{1a}Rs and OTRs from different species is compared in Figure 4. The N-terminus of the OTRs is some 12 residues shorter than that of the V_{1a}Rs. Moreover, with the exception of the juxtamembrane region, there is no homology between the N-terminus of V_{1a}Rs and OTRs. Sequence homology is apparent, however, within each receptor subtype (Figure 4). It is therefore possible that this N-terminal subdomain not only allows agonists to bind but also contributes to agonist selectivity. To address this possibility, a chimeric receptor (OTR^N-V_{1a}R) was constructed in which the N-terminus domain of the V_{1a}R was replaced by the corresponding OTR sequence. The affinity of AVP for this OTR^N-V_{1a}R chimera ($K_d = 7.5$ nM) was increased 240-fold compared to the [Δ2−47]V_{1a}R and was only slightly less than wild-type V_{1a}R (Figures 2 and 3, Tables 1 and 2). The pharmacological profile of the OTR^N-V_{1a}R chimera was then studied in greater detail using a range of analogues and found to be essentially the same as wild-type V_{1a}R (Tables 1 and 2). Consequently, OT, OP, and the OTR-selective agonist TGOT exhibited very low affinity for the chimeric receptor despite the presence of the OTR N-terminus domain. Nevertheless, the OTR N-terminus was effectively providing epitopes for ligand binding as the affinity of OT and OP for the OTR^N-V_{1a}R chimera was increased relative to the truncated receptor [Δ2−47]V_{1a}R. The presence of the OTR N-terminus also resulted in a marked recovery of binding by vasotocin (AVT) and the V_{1a}R-selective agonist POVT, relative to the truncated receptor.

Coupling Properties of the Truncated and Chimeric Receptors. For a complete understanding of the role of the N-terminal domain in V_{1a}R function, it was important not

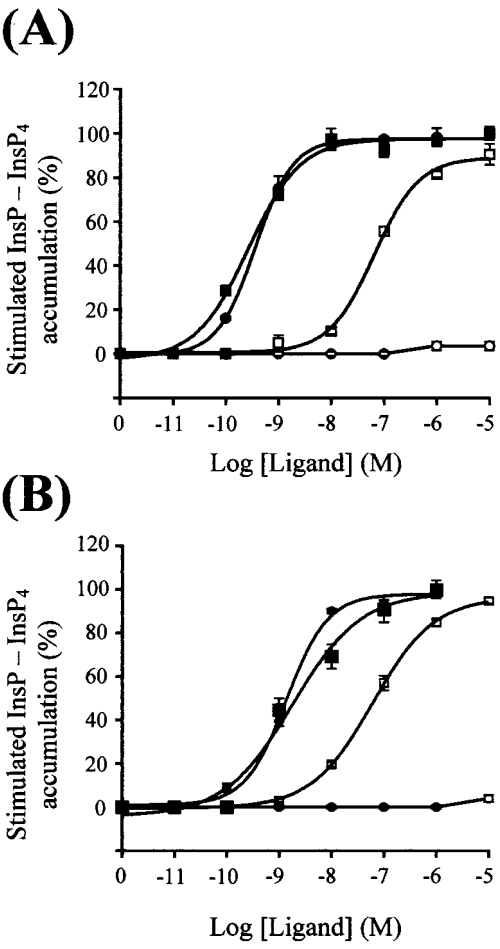


(B)

Receptor	Stimulation of InsP - $InsP_4$ {EC_{50} Values (nM)}
$V_{1a}R$	0.4 ± 0.1
[Δ2-23]$V_{1a}R$	0.4 ± 0.2
[Δ2-36]$V_{1a}R$	0.5 ± 0.2
[Δ2-47]$V_{1a}R$	> 10 μM
[Δ37-47]$V_{1a}R$	> 10 μM
OTR^N–$V_{1a}R$	1.8 ± 1.0

FIGURE 5: Comparison of functional coupling of truncated and chimeric receptor constructs. (A) AVP-induced accumulation of mono-, bis-, tris-, and tetrakisphosphates in HEK 293T cells transiently transfected with either wild-type $V_{1a}R$ (●); [Δ2−23]-$V_{1a}R$ (□); [Δ2−36]$V_{1a}R$ (△); [Δ2−47]$V_{1a}R$ (▽); [Δ37−47]$V_{1a}R$ (■), or OTR^N-$V_{1a}R$ (○). The data points for [Δ2−47]$V_{1a}R$ are occluded by the data points for [Δ37−47]$V_{1a}R$. Data are the mean ± SEM of a representative experiment performed in triplicate three times. Values are expressed as percent maximum stimulation induced by AVP at the stated concentrations. (B) EC_{50} values for AVP-induced inositol phosphates generation determined from at least three separate experiments performed in triplicate.

only to address ligand:receptor interactions but also to establish if there were any ramifications with respect to receptor-effector coupling. AVP-induced accumulation of inositol phosphates was measured, and the dose−response curves for each receptor construct is presented in Figure 5. For all of the receptor constructs, with the exception of [Δ2−47]$V_{1a}R$ and [Δ37−47]$V_{1a}R$, AVP-induced a 2.5−3.5-fold activation of phosphoinositidase C. The receptor−effector coupling of the truncated receptors [Δ2−23]$V_{1a}R$ and [Δ2−36]$V_{1a}R$ was very similar to wild-type $V_{1a}R$, with EC_{50} values for AVP-induced inositol phosphate accumulation of 0.4 nM, 0.5 nM, and 0.4 nM respectively (Figure 5). In contrast, the lack of signaling by [Δ2−47]$V_{1a}R$ and [Δ37−47]$V_{1a}R$ was very marked. Moreover, even high concentrations of AVP (10 μM) did not stimulate second messenger generation. The dose−response curve of the OTR^N-$V_{1a}R$ chimera was slightly to the right of the wild-type $V_{1a}R$, with an EC_{50} of 1.8 nM, consistent with the slightly lower affinity of AVP for this construct (K_d = 7.5 nM, Table 1). The $V_{1a}R$-selective agonist POVT and OT also stimulated inositol phosphate signaling by OTR^N-$V_{1a}R$ with EC_{50} values of 1.4 ± 0.1 and 71 ± 28 nM, respectively (Figure 6). This pattern of signaling is typical of a wild-type $V_{1a}R$. This was supported by the observation that the OT-selective agonist TGOT (10 μM) did not stimulate accumulation of inositol phosphates (Figure 6).



(C)

Ligand	Stimulation of InsP - $InsP_4$ {EC_{50} Values (nM)}	
	$V_{1a}R$	OTR^N-$V_{1a}R$
AVP	0.4 ± 0.1	1.8 ± 1.0
OT	102 ± 35	71 ± 28
POVT	0.4 ± 0.1	1.4 ± 0.1
TGOT	> 10 μM	> 10 μM

FIGURE 6: Comparison of intracellular signaling by the OTR^N-$V_{1a}R$ and wild-type $V_{1a}R$. Agonist-induced accumulation of mono-, bis-, tris- and tetrakisphosphates by wild-type $V_{1a}R$ (A) or OTR^N-$V_{1a}R$ (B). Selective ligands tested were AVP (■), POVT (●), OT (□), and TGOT (○). Data are the mean ± SEM of three separate experiments each performed in triplicate. Values are expressed as percent maximum stimulation induced by AVP at 1 μM. (C) EC_{50} values for agonist-induced inositol phosphates generation determined from at least three separate experiments performed in triplicate.

DISCUSSION

Since the GPCRs for neurotransmitters were first cloned in the mid 1980s (*31*, *32*), a key objective for investigators

has been to define the molecular architecture of the ligand-binding site. For small biogenic amines, such as acetylcholine and norepinephrine, this binding site is located in the hydrophobic core created by the receptor's transmembrane helical bundle. Evidence for this was provided by mutagenesis (*33*), microsequencing of affinity-labeled receptors (*34*), and the use of fluorescent ligands (*35*). Peptide ligands are generally far larger than catecholamines, and size considerations alone dictate that the binding site for these ligands may incorporate extracellular domains. This has been shown to be the case for several peptide receptors including the neurokinin-1 (NK_1) receptor (*36*), the angiotensin AT_1 receptor (*37*), the neuropeptide Y1 receptor (*38*), and the endothelin ET_A receptor (*39*). Indeed, for the large glycoprotein hormones such as luteinizing hormone (LH), the markedly extended N-terminal domain alone is sufficient for high affinity binding (*40*).

We have probed the ligand-binding site of the $V_{1a}R$ using mutated and chimeric receptor constructs. The efficient expression of all of the truncated receptor constructs implies that no essential information for assembly of functional $V_{1a}Rs$ is provided by the N-terminal sequence. The N-terminus contains two consensus sites for *N*-glycosylation at Asn^{14} and Asn^{27}. Oligosaccharide modification of these residues does not have a role in ligand recognition, or receptor folding, as the $[\Delta 2-36]V_{1a}R$ construct exhibited wild-type pharmacology despite lacking both of these glycosylation sites. This conclusion has been corroborated by a separate study in which we substituted both of these asparaginyl residues using site-directed mutagenesis (Hawtin and Wheatley, manuscript in preparation).

This study has allowed us to identify a short subdomain of the N-terminus of the $V_{1a}R$ which is critical for high affinity binding of all six agonists studied. The loss of agonist binding observed when this segment was ablated was not due to aberrant assembly of the receptor or to local distortion of the mature protein, as the binding of both peptide and non-peptide antagonists was largely unaffected. Moreover, this preservation of antagonist binding provided us with the means of accurately characterizing changes in agonist binding by using radioligand binding studies with [^{3}H]antagonist as tracer.

The contribution of this segment to ligand binding may be a direct contact between receptor and agonist but does not necessarily have to be so. Equally plausible is an indirect effect of the N-terminus, in which the critical subdomain interacts with juxtaposed extracellular loops to constrain or orientate them into a conformation with high affinity for agonists. With regard to this latter scenario, it is notable that both peptide mimetic studies and photoaffinity labeling have implicated the first extracellular loop of AVP receptors in ligand recognition (*26*, *41*). In addition, a single residue in this loop (Tyr^{115} in the $V_{1a}R$ and the corresponding locus Asp^{103} in the V_2R) has been shown to have a role in agonist selectivity (*42*, *43*). Although the N-terminus of the $V_{1a}R$ is important for agonist binding, the lack of [^{3}H]AVP binding to the $[1-86]V_{1a}R$ construct established that the N-terminus alone cannot recognize AVP. This contrasts to the LH receptor, where it has been shown that the N-terminus can bind hormone with high affinity in the absence of a transmembrane helical bundle (*40*). It is possible that the critical role of the receptor's N-terminus does not reflect the final docked position of AVP but merely one of a series of transitional conformational states of the agonist:receptor complex which exist between the initial "capture" of AVP and receptor activation. This would be analogous to the light-activation of rhodopsin, which generates metarhodopsin II via several conformational intermediates with different spectral properties (*44*) and different G-protein activation potential (*45*). Previous studies addressing the location of the ligand-binding site for AVP and related peptides have been contradictory. Consequently, it has been reported that the agonist is (i) completely buried in a 15−20 Å deep cleft within the transmembrane domains (*46*), (ii) bound to extracellular domains (*47*), or (iii) bound to a combination of extracellular domains and the top of the flanking transmembrane helices (*48*). Whatever the location of the final "docked" position of AVP, our data establish that interactions provided by a subdomain of the N-terminus are a prerequisite for high affinity agonist binding.

The $V_{1a}R$ is a member of a family of neurohypophysial peptide hormone receptors which possess sequence homology, bind AVP, and exhibit related pharmacological profiles. Consequently, we would predict that the corresponding N-terminal subdomain of the $V_{1b}R$, V_2R and OTR is important for agonist binding. It is noteworthy that a small segment of the N-terminus of the NK_1 receptor is important for binding substance P but not antagonists (*49*). However, our findings cannot be uniformly extrapolated to GPCRs with peptide ligands. For example, mutations within a cluster of residues in the N-terminus of the AT_1 receptor decreased the affinity of angiotensin II (AII) but also decreased the affinity of the peptide antagonist [Sar^1,Leu^8]AII (*37*). For the κ opiate receptor, the situation is reversed with mutations in the N-terminus affecting antagonist but not agonist binding (*50*). In contrast, there appears to be no role for the N-terminus in binding ligands to the melanocortin receptor (*51*) or the cholecystokinin-B receptor (*52*).

It is noteworthy that when the $[\Delta 2-47]V_{1a}R$ was challenged with 10 μM AVP, no agonist-induced increase in inositol phosphates was observed. Basic receptor theory (*53*) dictates that this concentration of AVP would have produced 85% occupancy of the receptors given that the affinity of the $[\Delta 2-47]V_{1a}R$ construct for AVP is 1.8 μM. As this high level of receptor occupancy by agonist failed to stimulate second messenger generation, it establishes that the N-terminal subdomain is not only critical for high affinity agonist binding but that it also has an important role in agonist-induced activation of the receptor.

The OTR, like the $V_{1a}R$, couples to $G_{q/11}$ and binds AVP with high affinity but has a different overall pharmacology. Substitution of the N-terminus of the $V_{1a}R$ with the corresponding sequence of the OTR generated a chimeric receptor with almost identical pharmacologically to wild-type $V_{1a}R$. Consequently, although the OTR N-terminus exhibits very little homology to the $V_{1a}R$ N-terminus, the presence of the OTR N-terminal sequence in the OTR^N-$V_{1a}R$ chimera provided the necessary epitopes for high affinity agonist binding and efficient intracellular signaling. The presence of the OTR N-terminus in OTR^N-$V_{1a}R$ did not result in a receptor exhibiting an OTR-like pharmacology or a hybrid pharmacology resembling both $V_{1a}R$ and OTR. It can therefore be concluded that although the OTR N-terminus provided the necessary epitopes to recover near wild-type

$V_{1a}R$ agonist function, the N-terminus does not contain the molecular discriminators which give rise to subtype-selective agonists such as TGOT and POVT.

In conclusion, by carefully characterizing a series of truncated $V_{1a}R$ constructs we have identified a short segment of the N-terminus ($Glu^{37}-Asn^{47}$), which is critical for high affinity binding of agonists to the $V_{1a}R$. The role of this segment in ligand recognition is restricted to agonists, as the binding of both peptide and non-peptide antagonists was largely unaffected when the subdomain was deleted. This study provides insight at the molecular level into a fundamental difference between agonist and antagonist recognition by a family of GPCRs with peptide ligands. Our data also establish a second crucial role for this same N-terminal domain in receptor-mediated intracellular signaling but exclude a role for the N-terminus discriminating between subtype-selective agonists.

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