Agonist-Specific, High-Affinity Binding Epitopes Are Contributed by an Arginine in the N-Terminus of the Human Oxytocin Receptor†

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Received November 28, 2001; Revised Manuscript Received February 18, 2002

ABSTRACT: The effects of the peptide hormone oxytocin (OT) are mediated by the oxytocin receptor, which is a member of the G-protein-coupled receptor family. Defining differences between the binding of agonists and antagonists to the OTR, at the molecular level, is of fundamental importance to understanding OTR activation and to rational drug design. Previous reports have indicated that the N-terminus of the OTR is required for OT binding. The aim of this study was to identify which individual residues within the N-terminal domain of the human OTR provided these OT binding epitopes. A series of truncated OTRs and mutant receptor constructs with systematic alanine substitution were characterized with respect to their pharmacological profile and intracellular signaling capability. Although a number of residues within the OTR will be required for optimal OT−OTR interaction, our data establish that Arg^{34} within the N-terminal domain contributes to high-affinity OT binding. Removal of Arg^{34} by truncation or substitution resulted in a 2000-fold decrease in OT affinity. In addition, we show that the arginyl at this locus is required for high-affinity binding of agonists in general. However, the importance of Arg^{34} is restricted to agonist interaction with the OTR, as it was not required for binding peptide antagonist or non-peptide antagonist. It is noteworthy that the corresponding Arg in the related rat V_{1a} vasopressin receptor is also required for high-affinity agonist binding. This study defines, at the molecular level, the role of the N-terminus of the OTR in high-affinity agonist binding and identifies a key residue for this function.

The peptide hormone oxytocin (OT)[1] stimulates contraction of the uterus at parturition and also contracts mammalian myoepithelium during lactation. This effect of OT on uterine contraction has resulted in OT being employed routinely in clinics to augment labor (*1*, *2*). The physiological effects of OT are mediated by a specific oxytocin receptor (OTR) expressed by target tissues. For example, increased responsiveness of the uterus to OT near to term is paralleled by an increase in the abundance of OTRs expressed by the myometrium (*3*, *4*). The OTR is a member of the G-protein-coupled receptor (GPCR) family and exhibits the characteristic structural motifs common to these receptors including seven transmembrane helices. To date, only one OTR subtype has been cloned from a range of species (*5*). This implies that the wide range of physiological effects of OT (*6*) are mediated by a single GPCR.

Pre-term labor is a serious problem and is a major cause of neonatal mortality. This was the stimulus for the development of a series of OTR antagonists to control pre-term labor. The peptide Atosiban (d[D-Tyr(Et)^2,Thr^4,Orn^8]OT) has been reported to suppress episodes of pre-term labor and to prolong uterine quiescence in human patients (*7*). More recently, non-peptide antagonists have also been reported (*8*, *9*) which have the advantage over peptides of greater metabolic stability in vivo.

Although OT, peptide antagonists, and non-peptide antagonists exhibit competitive binding to the OTR, they are fundamentally different in that the agonist induces the natural hormone response whereas the antagonists do not. Understanding the structural differences, at the molecular level, between the agonist−receptor complex and the antagonist−receptor complex will provide insight into hormone action and provide a basis for the rational design of drugs. Several groups have utilized computer modeling, supplemented by binding data from mutant or chimeric receptors, to reveal possible intermolecular contacts in the OT−OTR complex. Initially it was reported that the OT binding site was predominantly buried deep within the bundle of the transmembrane helices plus the first extracellular loop (*10*); however, chimeric receptor studies indicated that the OT binding platform required a more extensive involvement of the extracellular domains (*11*). Subsequent structural refinements to the OT−OTR interaction model (*12*) reveal the hormone binding site to be formed by the extracellular ends of transmembrane helices (TM) III−VII plus the first and second extracellular loops.

† This paper is dedicated to the memory of Ph.D. student Victoria J. Wesley. This study was supported by grants from the BBSRC to M.W.

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[1] Abbreviations: AVP, [arginine^8]vasopressin; GPCR, G-protein-coupled receptor; InsP, inositol phosphate; $InsP_3$, inositol trisphosphate; ITR, isotocin receptor; MTR, mesotocin receptor; OT, oxytocin; OTA, d$(CH_2)_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr$(NH_2)^9$vasotocin; OTR, oxytocin receptor; PhAc, phenylacetyl; TGOT, [Thr^4,Gly^7]OT; TM, transmembrane helix; V_{1a}R, V_{1a} vasopressin receptor; VTR, vasotocin receptor. Truncation nomenclature: numbers refer to the positions in the human OTR (hOTR) sequence of the residues deleted; hence, [Δ2−35]OTR is the hOTR with residues 2−35 inclusive deleted.

10.1021/bi015990v CCC: $22.00
Published on Web 03/27/2002

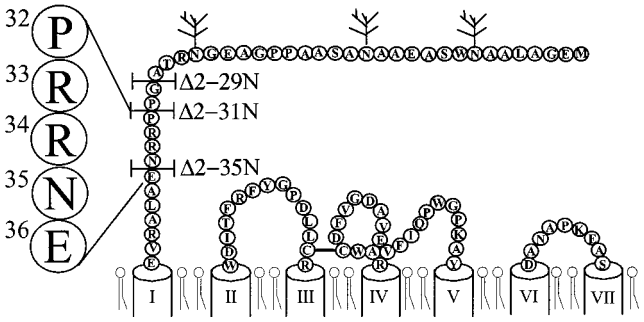

FIGURE 1: Extracellular domains of the hOTR and engineered constructs. Only the extracellular face of the receptor is illustrated, with the top of the transmembrane domains represented by cylinders I−VII. Putative glycosylation sites are indicated by the branched structures. Truncations of the N-terminus are indicated by bars labeled [Δ2−29]OTR, [Δ2−31]OTR, and [Δ2−35]OTR, respectively, where the numbers refer to the positions in the sequence of the residues deleted. Residues mutated to Ala within an N-terminal subdomain (Pro^{32}−Glu^{36}) are also shown enlarged.

Recently, it has been shown experimentally that the N-terminus of the receptor is required for high-affinity agonist binding to the OTR (*13*). In this study, we characterize in detail this role of the N-terminus of the receptor in ligand binding. Individual residues within the N-terminus which are required for agonist binding are identified by utilizing truncated receptor constructs and alanine-scanning mutagenesis. Our data reveal that, within this domain, Arg^{34} is required for high-affinity agonist binding. Furthermore, the contribution of this arginyl is agonist-specific as it is not required for binding either peptide or non-peptide antagonists.

EXPERIMENTAL PROCEDURES

Materials. AVP, OT, and Thr^4Gly^7OT (TGOT) were from Sigma. The cyclic antagonist 1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-*O*-methyltyrosine, 4-threonine, 8-ornithine, 9-tyrosinamide vasotocin [d$(CH_2)_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr$(NH_2)^9$vasotocin; OTA], and PhAc*D*-Tyr(Me)^{2}Arg^{6}Tyr$(NH_2)^9$AVP were from Bachem (U.K.). L-368,-899 was a kind gift from Dr. Douglas J. Pettibone (Merck Research Laboratories, West Point, PA). All materials for tissue culture were supplied by Gibco BRL (Paisley, U.K.). Restriction enzyme *Bsp*120I was obtained from MBI Fermentas (Sunderland, U.K.).

Truncated and Mutant Receptor Constructs. All the OTR constructs used in this study were engineered using cDNA for the OTR from human. Truncations of the N-terminus of the hOTR were made using a PCR approach (*14*). Truncation oligonucleotides were 5′-C-TAG-GGA-TTC-**GCC-ACC**-*ATG*-GGA-CCC−CCG-CGG-CGC-AAC-GAG-GCC-3′ and 5′-C-TAG-GGA-TCC-**GCC-ACC**-*ATG*-CCG-CGG-CGC-AAC-GAG-GCC-C-3′ for the [Δ2−29]OTR and [Δ2−31]-OTR truncations, respectively (Figure 1). Each primer contained a *Bam*HI restriction site (underlined), a Kozak consensus sequence (shown in boldface type), and an ATG start site (shown in italics) followed by the hOTR sequence. The PCR cycling conditions were as follows: denaturing, 94 °C (1 min), annealing, 60−65 °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 human OTR coding sequence in pBluescript SK II vector (Stratagene) utilizing unique *Bam*HI and *Bsp*120I sites. Truncated hOTR constructs were subcloned into the mammalian expression vector pcDNA3.1(−) (Invitrogen) utilizing *Xba*I and *Kpn*I restriction sites. A unique *Xho*I restriction site was engineered into the wild-type hOTR cDNA sequence using PCR to facilitate site-directed mutagenesis. The sense oligonucleotide was 5′-G-GCC-CTG-G**C**T-CG**A**-GTG-GAG-GTG-G-3′ and contained two base changes (indicated in boldface) which created a unique *Xho*I restriction site (underlined) without altering the amino acid sequence. A *Bsp*120I digest of the PCR fragment was subcloned into the pcDNA3.1(−) vector containing the wild-type hOTR. The mutant hOTR constructs [P32A]OTR, [R33A]OTR, [R34A]OTR, [N35A]OTR, and [E36A]OTR were engineered by PCR using the following antisense oligonucleotides: 5′-CAC-CTC-CAC-TCG-AGC-CAG-GGC-CTC-GTT-GCG-CCG-CG**C**-GGG-TCC-GGC-3′, 5′-CAC-CTC-CAC-TCG-AGC-CAG-GGC-CTC-GTT-GCG-C**GC**-CGG-GGG-TCC-3′, 5′-CAC-CTC-CAC-TCG-AGC-CAG-GGC-CTC-GTT-G**GC**-CCG-CGG-GGG-TCC-GGC-3′, 5′-CAC-CTC-CAC-TCG-AGC-CAG-GGC-CTC-G**GC**-GCG-CCG-CGG-3′, and 5′-CAC-CTC-CAC-TCG-AGC-CAG-GGC-C**GC**-GTT-GCG-CCG-3′, respectively. Each contained the unique *Xho*I restriction site (underlined) and the appropriate base change(s) for each mutation (indicated in boldface). The PCR products were subcloned into the human OTR sequence in pcDNA3.1(−) using unique *Bam*HI and *Xho*I restriction sites. The mutant rat V_{1a} vasopressin receptor (rV_{1a}R) construct [R46A]V_{1a}R was engineered using PCR with sense and antisense oligonucleotides. The sense primer was 5′-GGG-GAA-GGT-GAC-AGC-CCC-TTA-GGG-GAC-GTA-**GC**C-AAT-GAG-GAG-CTG-GCC-3′ which incorporated the appropriate base changes for the Arg^{46}→Ala^{46} mutation (shown in boldface). A *Bam*HI/*Sda*I digest of this PCR fragment was subcloned into the pcDNA3-V_{1a}R vector. All truncated and mutant receptor constructs were confirmed by automated fluorescent sequencing (Alta Bioscience, University of Birmingham, Birmingham, U.K.).

Cell Culture and Transfection. HEK 293T cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (2 mM), D-glucose (4500 mg/L), and sodium pyruvate (1 mM) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 μg/mL) 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 with 10 μg of DNA/dish.

Radioligand Binding Assays. Membranes from transfected HEK 293T cells were prepared as described (*15*), and the protein concentration was determined using the BCA protein assay kit (Pierce Chemical Co.) with BSA as standard. Radioligand binding assays were performed essentially as described previously (*16*, *17*). For hOTR constructs, either the natural agonist [Tyr^2-2,6-^{3}H]OT ([^{3}H]OT) (44 Ci/mmol; DuPont NEN, UK) or the OTR-selective peptide antagonist [Tyr^9-^{125}I]OTA ([^{125}I]OTA) (2200 Ci/mmol; DuPont NEN, UK) (*18*) was employed as tracer ligand. For the rV_{1a}R, the natural agonist [Phe^3-3,4,5-^{3}H]AVP (64.2 Ci/mmol; DuPont NEN, UK) or the V_{1a}R-selective peptide antagonist [Phe^3-3,4,5-^{3}H]d$(CH_2)_5$Tyr(Me)^{2}AVP (99 Ci/mmol; DuPont NEN, UK) (*19*) was tracer ligand. Competition binding assays (final volume of 500 μL) containing radioligand (0.3−1.2 nM [^{3}H]-

Table 1: Pharmacological Profile of Truncated hOTRs[a]

ligand	binding affinities, K_d (nM)			
	hOTR	[Δ2−29]OTR	[Δ2−31]OTR	[Δ2−35]OTR
OT	1.4 ± 0.5	2.2 ± 0.7	3.2 ± 1.1	2500 ± 380
OTA	1.7 ± 0.5	0.8 ± 0.1	1.9 ± 0.6	0.6 ± 0.2
L-368,899	13 ± 2.0	18 ± 4.1	12 ± 1.4	6.9 ± 1.1
cell surface expression (fmol/mg of protein)	1100 ± 80	110 ± 24	154 ± 15	165 ± 45

[a] Truncated hOTRs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) were calculated from IC_{50} values and corrected for radioligand occupancy as described under Experimental Procedures. Data shown are the mean ± SEM ($n = 3$) of three replicates.

OT, 2−75 pM [^{125}I]OTA, 0.3−0.7 nM [^{3}H]AVP, 0.3−1.1 nM [^{3}H]d(CH_2)$_5$Tyr(Me)^{2}AVP), cell membranes (80−800 μg), and competing ligand (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, and dissolved in tissue solubilizer (Soluene-350, Packard), and the radioactivity was quantified by liquid scintillation spectroscopy using HiSafe 3 (Wallac) as cocktail. Nonspecific binding was determined in parallel incubations using OTA (1 μM) or d(CH_2)$_5$Tyr(Me)^{2}AVP (1 μM) for hOTR and rV_{1a}R, respectively. 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 (*20*) using the radioligand affinity (K_d) which was experimentally determined for each individual construct.

Whole Cell Oxytocin Receptor Binding Assay. Cell surface expression was determined by whole cell binding assays (*21*). Briefly, HEK 293T cells were seeded at a density of 1 × 10^5 cells/well in poly(D-lysine)-coated 12 well plates and transfected after 24 h using Transfast (Promega). After 36 h, cells were washed twice with PBS, after which each well received 0.5 mL of binding buffer [20 mM HEPES, 10 mM $Mg(CH_3COO)_2$, 1 mM EGTA, 2% (w/v) BSA, pH 7.4] containing 20−60 pM [^{125}I]OTA in the presence (nonspecific binding) or absence (total binding) of 1 μM OTA. Plates were incubated for 90 min at 37 °C before removal of the medium by aspiration. After the cells were washed 3 times with ice-cold PBS, 0.5 mL of 0.1 M NaOH was added to each well and incubated for 15 min at 37 °C to extract radioactivity. Samples from each well were transferred to scintillation vials containing 10 mL of HiSafe 3 scintillant for liquid scintillation counting.

Measurement of Agonist-Induced Inositol Phosphate Accumulation. HEK 293T cells were seeded at a density of 2.5 × 10^5 cells/well in poly(D-lysine)-coated 12 well plates and transfected after 24 h using Transfast (Promega). The assay for accumulation of inositol phosphates induced by OT or AVP was based on that described previously (*22*). Briefly, 24 h post-transfection, medium was replaced with inositol-free DMEM (custom synthesis, Invitrogen) containing 2 μCi/mL myo-[2-^{3}H]inositol (Tocris Cookson) and 1% (v/v) FCS for 24 h. Cells were then washed with PBS and incubated with medium containing 10 mM LiCl for 30 min, after which OT or 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 with 10 mL of water and glycerophosphoinositol with 10 mL of 60 mM NH_4COOH containing 0.1 M HCOOH, a mixed inositol fraction containing mono-, bis-, and trisphosphates (InsP−$InsP_3$) was eluted with 10 mL of 850 mM NH_4COOH containing 0.1 M HCOOH. EC_{50} values were determined by nonlinear regression after fitting of logistic sigmoidal curves to experimental data.

RESULTS

Role of the N-Terminus of the hOTR in Ligand Binding. It has recently been shown that the N-terminus of the hOTR is required for high-affinity binding of OT but not antagonists (*11*, *13*). The aim of this study was to define the nature of this requirement, at the molecular level, by identifying which individual residue(s) within the hOTR N-terminus provide(s) these agonist-specific binding epitopes. A series of truncated hOTR constructs was generated which had increasing portions of the N-terminus deleted. Three constructs were engineered which were truncated at Ala^{29}, Pro^{31}, or Asn^{35} as indicated in Figure 1. These constructs retained the initiation methionine and were termed [Δ2−29]OTR, [Δ2−31]OTR, and [Δ2−35]OTR, respectively, where the numbering refers to the amino acid residues deleted.

Each of the truncated hOTRs was expressed in HEK 293T cells and the pharmacological profile compared to wild-type receptor. Three different classes of ligand were employed to probe the ligand binding site of the hOTR: (i) the natural agonist OT; (ii) the cyclic peptide antagonist OTA (*18*); and (iii) a non-peptide antagonist, L-368,899 (*23*). Competition radioligand binding curves were determined for each of these different classes of ligand using [^{125}I]OTA as tracer (data not shown). The K_d values are presented in Table 1, corrected for radioligand occupancy. Truncation of the N-terminus in the [Δ2−29]OTR and [Δ2−31]OTR constructs had no effect on the binding of the natural agonist OT, the peptide antagonist, or the non-peptide antagonist (Table 1). In contrast, further deletion of the N-terminus in the construct [Δ2−35]OTR resulted in a 2000-fold decrease in the affinity for OT (Table 1). This disruption of binding was agonist-specific, as the affinity of both classes of antagonists for [Δ2−35]OTR was similar to wild-type hOTR (Table 1). These data indicated that an epitope required for high-affinity OT binding is located in the distal segment of the N-terminus of the hOTR between Pro^{32} and Asn^{35}.

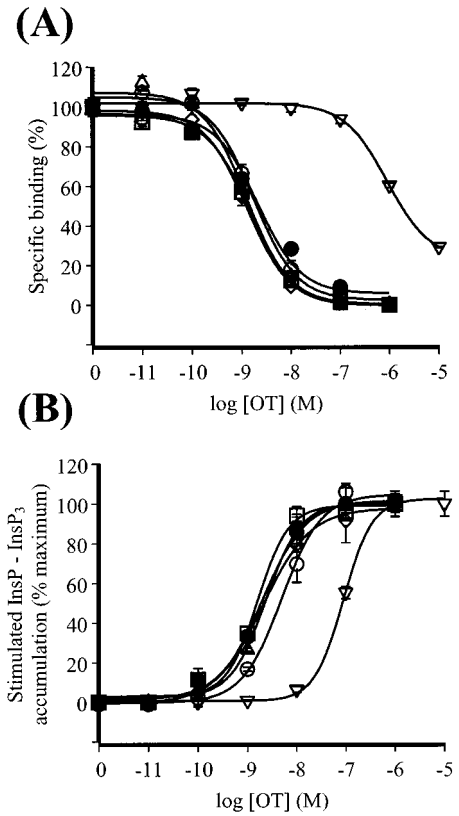

FIGURE 2: Pharmacological characterization of mutant hOTRs. (A) Competition radioligand binding studies with OT were performed using a membrane preparation of HEK 293T cells transiently transfected with either wild-type hOTR (●), P32A (□), R33A (△), R34A (▽), N35A (○), or E36A (◇). Values are expressed as percent specific binding where nonspecific binding was defined by OTA (1 μM). A theoretical Langmuir binding isotherm has been fitted to the experimental data as described under Experimental Procedures. (B) Intracellular signaling by mutant hOTR constructs. OT-induced accumulation of InsP−InsP$_3$ in HEK 293T cells transfected with either wild-type hOTR (●), P32A (□), R33A (△), R34A (▽), N35A (○), or E36A (◇). Data shown in (A) and (B) are the means ± SEM of three separate experiments each performed in triplicate. Values are expressed as percent maximum stimulation induced by OT at the stated concentrations.

Identification of Critical Agonist-Specific Binding Contact(s) in the Distal N-Terminus of the hOTR. Alanine-scanning mutagenesis was used to identify individual residue(s) in the distal N-terminus of the hOTR which are a prerequisite for high-affinity OT binding. A series of five mutant receptors was engineered, each of which contained a single alanine substitution in the region of interest [P32A]-OTR, [R33A]OTR, [R34A]OTR, [N35A]OTR, and [E36A]-OTR. The pharmacological characteristics of each construct were determined after expression in HEK 293T cells (Figure 2) and the binding affinities are presented in Table 2. Wild-type and mutant constructs were expressed at a similar level of approximately 0.5−1.5 pmol/mg of protein.

With the exception of [R34A]OTR, all the mutant hOTRs possessed a pharmacological profile similar to wild-type hOTR (Table 2). In marked contrast, the affinity of OT for [R34A]OTR decreased 2000-fold compared to the wild-type receptor (Figure 2, Table 2). However, the affinity of both the peptide and the non-peptide antagonists for [R34A]OTR was only marginally affected, being approximately 2-fold lower than wild-type hOTR (Table 2). Consequently, the pharmacological characteristics of [R34A]OTR were very similar to those of the truncated construct [Δ2−35]OTR. These results established that Arg^{34} within the distal N-terminal segment of the hOTR is important for high-affinity OT binding to its receptor.

Table 2: Pharmacological Profile of Mutant hOTRs[a]

	binding affinities, K_d (nM)			stimulation of InsP−InsP$_3$	
construct	OT	OTA	L-368,899	EC_{50} (nM)	E_{max} (*x*-fold)
hOTR	1.4 ± 0.5	1.7 ± 0.5	13 ± 2.0	2.0 ± 0.5	6.6 ± 0.7
P32A	1.1 ± 0.4	0.7 ± 0.1	15 ± 2.5	2.5 ± 1.3	5.3 ± 0.1
R33A	0.8 ± 0.2	0.6 ± 0.1	15 ± 3.8	2.4 ± 0.7	6.0 ± 0.7
R34A	2800 ± 1100	3.5 ± 0.8	27 ± 6.6	105 ± 15	3.5 ± 0.8
N35A	1.2 ± 0.3	0.4 ± 0.1	21 ± 2.8	3.8 ± 0.8	4.3 ± 1.0
E36A	0.8 ± 0.2	0.6 ± 0.1	25 ± 1.1	3.1 ± 1.9	3.9 ± 0.6

[a] Mutant hOTRs were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) were calculated from IC_{50} values and corrected for radioligand occupancy as described under Experimental Procedures. Data shown are the mean ± SEM (n = 3) of three replicates. E_{max} values are expressed as fold-stimulation over basal induced by OT (1 μM). Basal values (mean ± SEM) were 498 ± 148, 478 ± 106, 479 ± 157, 672 ± 245, 538 ± 193, and 512 ± 166 dpm for wild-type hOTR, P32A, R33A, R34A, N35A, and E36A mutant receptors, respectively. All values shown are the means ± SEM of three separate experiments each performed in triplicate.

Arg^{34} Is Important for Binding Agonists in General. Data presented above established that Arg^{34} was critical for OT binding to the hOTR. It was important to ascertain if this phenomenon was unique to the natural agonist OT, or if an arginyl at this locus was a prerequisite for high-affinity agonist binding in general. TGOT and AVP are analogues of OT and are also agonists at the hOTR (*24*, *25*). The affinity of both TGOT and AVP for [R34A]OTR was markedly reduced compared to wild-type hOTR values. The affinity of TGOT binding decreased from 19 ± 2.1 nM for wild-type hOTR to 2400 ± 940 nM for [R34A]OTR (mean K_d value ± SEM, n = 3). Likewise, the K_d value for AVP binding to wild-type hOTR and [R34A]OTR was 9.2 ± 0.8 and 2200 ± 490 nM (mean ± SEM, n = 3), respectively. Consequently, the importance of Arg^{34} was not merely restricted to OT binding but extended to agonists in general.

Role of the Distal N-Terminus of the hOTR in Intracellular Signaling. Once the importance of the distal N-terminus of the hOTR for agonist recognition had been established, it was important to investigate the role of this region in intracellular signaling. Consequently, the OT-induced accumulation of InsPs mediated by the mutant hOTR constructs was investigated and compared to wild-type hOTR (Figure 2B, Table 2). With the exception of [R34A]OTR, all the mutant constructs displayed EC_{50} values similar to wild-type hOTR (Table 2). In marked contrast, [R34A]OTR exhibited a 50-fold increase in the EC_{50} value compared to wild-type (Figure 2B, Table 2). The maximum InsP response induced by OT (1 μM) at [R34A]OTR was also reduced compared to wild-type (Table 2), consistent with the decreased affinity of this construct for the hormone (Table 2). A reduction in the maximum response was also observed with [N35A]OTR and [E36A]OTR, albeit to a lesser extent (Table 2).

It was not possible to characterize the signaling capability of hOTR constructs with N-terminal truncations. A possible reason for this is low receptor abundance, so this was investigated. Cell surface expression of the three truncated receptors [Δ2−29]OTR, [Δ2−31]OTR, and [Δ2−35]OTR

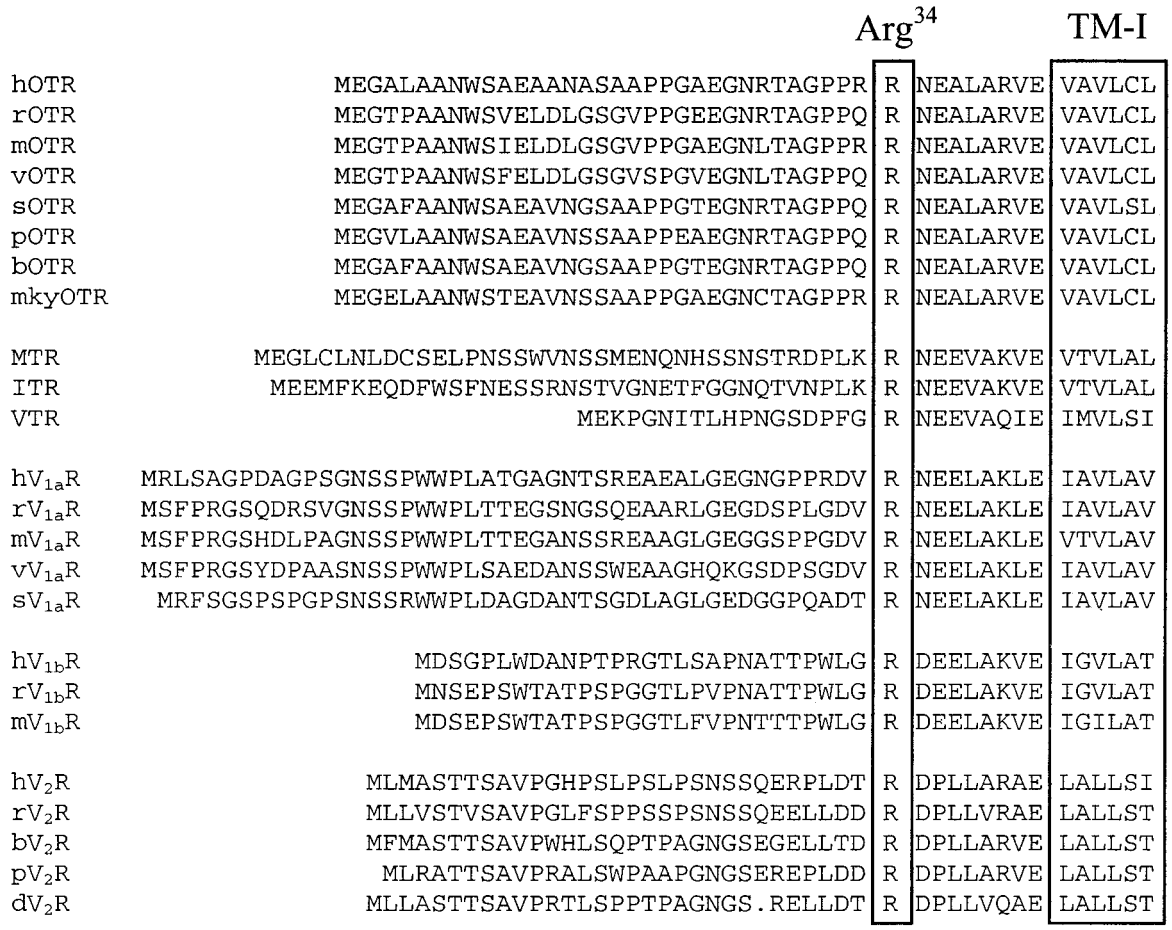

FIGURE 3: Sequence alignment of the N-terminus of OTRs and structurally related GPCRs cloned from different species. The position of the top of the first transmembrane domain (TM-I) is boxed. The species of origin is indicated by a single-letter code preceding the receptor subtype: h, human; r, rat; m, mouse; v, vole; s, sheep; p, pig; b, cow; mky, rhesus monkey; d, dog. Also shown is the N-terminal sequence of an amphibian mesotocin receptor (MTR), an isotocin receptor (ITR), and vasotocin receptor (VTR) from teleost fish and vasopressin receptor subtypes V_{1a}, V_{1b}, and V_2. The conserved arginyl in all of the aligned sequences, equivalent to Arg^{34} in the human OTR, is indicated by a box and is labeled. Sequences cited were obtained from SwisProt PDB and GenEMBL.

was determined using whole cell binding assays and compared to that of wild-type hOTR. To ensure accurate comparison, all the truncated constructs and the wild-type hOTR were transfected and analyzed in parallel (Table 1). This revealed that cell surface expression of the truncated constructs was only 10−15% of wild-type hOTR expression (Table 1). The binding assay employed was inherently more sensitive than the assay for inositol phosphates. Consequently, the very low abundance of the truncated receptor constructs [Δ2−29]OTR, [Δ2−31]OTR, and [Δ2−35]OTR could be quantified and pharmacologically characterized, but agonist-induced second messenger generation was not detectable.

The Corresponding Arginyl of $rV_{1a}R$ Fulfills the Same Role as Arg^{34} in the hOTR. An arginyl corresponding to Arg^{34} in the hOTR is present at this locus in all members of the neurohypophysial peptide hormone receptor family of GPCRs cloned to date (Figure 3). This conservation of structure is perhaps suggestive of a conservation of function with respect to agonist binding. To investigate this further, the conserved arginyl was mutated in another member of this receptor family. For the $V_{1a}R$ from rat, Arg^{46} corresponds to Arg^{34} in the hOTR. The construct $[R46A]V_{1a}R$ was engineered and pharmacologically characterized after expression in HEK 293T cells. Both wild-type $rV_{1a}R$ and $[R46A]V_{1a}R$ were expressed at 1−2 pmol/mg of protein. Mutation of Arg^{46} of the $rV_{1a}R$ caused a 1300-fold decrease in agonist binding affinity but had no effect on antagonist binding (Table 3). Furthermore, intracellular signaling by both [R34A]OTR (Table 2) and $[R46A]V_{1a}R$ (Table 3) was impaired compared to the wild-type receptors.

Table 3: Pharmacological Profile of Wild-Type and Mutant $rV_{1a}R$[a]

	binding affinities, K_d (nM)		stimulation of InsP−InsP$_3$	
construct	AVP	antagonist	EC_{50} (nM)	E_{max} (fold)
$rV_{1a}R$	1.0 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	5.5 ± 1.8
R46A	1300 ± 190	0.3 ± 0.1	26 ± 3.0	5.2 ± 0.9

[a] Wild-type $rV_{1a}R$ and $[R46A]V_{1a}R$ were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) for AVP and the peptide antagonist PhAc*D*-Tyr(Me)^{2}Arg^{6}Tyr(NH$_2$)^{9}AVP were calculated from IC_{50} values and corrected for radioligand occupancy as described under Experimental Procedures. Data shown are the mean ± SEM (n = 3) of three replicates. E_{max} values are expressed as fold-stimulation over basal induced by AVP (1 μM). Basal values (mean ± SEM) were 1200 ± 265 and 938 ± 85 dpm for wild-type $rV_{1a}R$ and $[R46A]V_{1a}R$, respectively. All values shown are the means ± SEM of at least three separate experiments each performed in triplicate.

DISCUSSION

The primary event in activation of a receptor is the binding of the hormone to the receptor protein. Given the central

importance of this phenomenon to cell signaling, a great deal of research effort has been aimed at defining the molecular contacts which exist between hormones and their respective receptor proteins. In addition to the natural hormone, many analogues, both agonists and antagonists, have been developed for therapeutic intervention. Understanding the structural differences, at the molecular level, between the agonist−receptor complex and the antagonist−receptor complex will provide insight into hormone action and provide a basis for the rational design of drugs.

It has been shown recently that the N-terminus of the OTR is required for high-affinity agonist binding but that this segment does not contribute to antagonist binding (*13*). In this study, the agonist-specific binding contact within this domain has been identified. Three different classes of ligand were employed as pharmacological probes of the binding site: (i) peptide agonist, including the natural agonist OT; (ii) peptide antagonist, an analogue of OT; and (iii) non-peptide antagonist, structurally unrelated to OT (*23*). Characterization of a series of N-terminally truncated hOTR constructs established that the agonist-specific binding was actually provided by the short sequence $Pro^{32}-Asn^{35}$ in the distal N-terminus (Figure 1). Consequently, [Δ2−31]OTR exhibited wild-type OT affinity whereas the affinity of OT binding to [Δ2−35]OTR was 2000-fold lower (Table 1). In contrast, [Δ2−35]OTR bound both peptide and non-peptide antagonists with wild-type affinity. This retention of high-affinity antagonist binding allowed radiolabeled antagonist to be used as a tracer to accurately quantify changes in the affinity of OT and its analogues. In contrast to the OTR, the reverse situation has been reported for the NK_1 receptor, in that extracellular antagonist-specific binding epitopes have been described. The non-peptide antagonist CP 96345 bound to the NK_1 receptor using epitopes that were not utilized by the natural agonist substance P (*26*), and mutations in extracellular loop 2 impaired binding of an analogue of CP 96345 without affecting substance P (*27*).

Detailed investigation of the hOTR, using alanine-scanning mutagenesis, revealed that the agonist-specific binding epitope within the distal N-terminus was provided by a single residue, Arg^{34}. Consequently, the loss of high-affinity OT binding observed by truncating the N-terminus of the hOTR by 34 residues in the [Δ2−35]OTR construct was reproduced by a single arginyl to alanyl substitution in the construct [R34A]OTR (Tables 1 and 2). The importance of Arg^{34} was not unique to OT binding, but it was restricted to agonists. The agonists TGOT and AVP are analogues of OT, being nonapeptides, amidated at the C-terminus, and with a disulfide bond between Cys^1 and Cys^6. However, they differ from OT at positions 4 and 7 (TGOT) and at positions 3 and 8 (AVP). Despite these sequence differences, Arg^{34} was required for high-affinity binding of all three agonists. Molecular modeling of OT docked to the hOTR indicates that the hormone interacts with TMIII−TMVII plus the first and second extracellular loops (*12*). The N-terminus of the hOTR was not modeled, as it was thought previously that this region was unstructured for GPCRs in general. However, the recently published crystal structure of rhodopsin (*28*) established that the N-terminus is globular and forms a compact, glycosylated unit which overlays the extracellular loops (*29*). Furthermore, the strand of the N-terminus of rhodopsin, corresponding to that containing Arg^{34} of the hOTR, is positioned along the top of the transmembrane helical bundle and occupies the space between TMI and TMII (*28*). This arrangement would position the guanidinium group of Arg^{34} in an ideal position to form intramolecular contacts between the N-terminus and key residues in other regions of the hOTR, particularly the extracellular loops. This conformational information from rhodopsin also suggests a plausible mechanism for the role of Arg^{34} in the structurally related hOTR, in which intramolecular contacts made by Arg^{34} promote a receptor conformation with high affinity for agonists. In contrast, antagonist binding (peptide and non-peptide) is not sensitive to the conformational consequences of these Arg^{34} interactions. It is also possible that Arg^{34} could have a direct interaction with the agonist. A direct interaction between the agonist and the N-terminus has been reported for some GPCRs. For example, the LH receptor has an extended N-terminus which binds LH with high affinity even when it is expressed in the absence of any transmembrane domain (*30*). Likewise, a peptide corresponding to residues 1−47 of the cholecystokinin-A receptor (CCK_A-R) N-terminus has been reported to bind the octapeptide agonist CCK8 (*31*, *32*). Both the CCK_A-R and the hOTR have arginyl at position 34. However, intermolecular NOEs from a ^1H NMR spectroscopy study of a complex between the ligand and receptor fragment revealed that, in contrast to the hOTR, Arg^{34} of the CCK_A-R does not appear to have a role in agonist binding (*31*).

An arginyl corresponding to Arg^{34} in the hOTR is conserved at this locus in all OTRs cloned to date (Figure 3). The hOTR is a member of the neurohypophysial peptide hormone receptor subfamily of GPCRs which includes receptors for OT, mesotocin, isotocin, vasotocin, and the three subtypes of vasopressin receptor. Placental mammals have OT, but virtually all vertebrate species possess an OT-like peptide. Amphibians, reptiles, birds, and marsupials possess the evolutionary precursor of OT, mesotocin ([Ile^3]-OT), and the more primitive bony fish possess isotocin ([Ser^4,Ile^8]OT) (*33*). Interestingly, the Arg identified in this study as being important for agonist binding to the hOTR is also conserved in the receptors for mesotocin and isotocin (Figure 3). The vasotocin receptor (VTR) and the three subtypes of vasopressin receptor are structurally related to the hOTR, and these also possess an Arg at the same locus as Arg^{34} in the hOTR (Figure 3). This conservation of an arginyl in the distal N-terminus of all these related receptors raised the possibility that the role of this residue in agonist binding may also be conserved throughout the neurohypophysial peptide hormone receptor subfamily of GPCRs. There was some experimental support for this, as it had been reported that the N-terminus of both the VTR and the $V_{1a}R$ was required for agonist binding (*34*, *14*). The role of Arg^{46} of the $rV_{1a}R$ was investigated as this residue corresponds to Arg^{34} of the hOTR. In common with [R34A]OTR, [R46A]-$V_{1a}R$ possessed wild-type antagonist binding but exhibited a decrease in agonist binding and impaired second-messenger generation (Tables 2 and 3). These observations support our hypothesis that the conserved Arg fulfills a common role throughout this subfamily of GPCRs.

This study also characterized a nonglycosylated hOTR for the first time. A common structural feature of GPCRs is that the N-terminus is glycosylated (*17*, *35*). The hOTR possesses three consensus *N*-glycosylation sites, all within the N-

terminus, located at Asn^{8}, Asn^{15}, and Asn^{26}. An assessment of the role of glycosylation at each individual locus using site-directed mutagenesis concluded that the posttranslational modification did not influence ligand affinity or receptor trafficking (*36*). However, the fully nonglycosylated receptor was not characterized. Each of the truncated constructs used in the present study, [Δ2−29]OTR, [Δ2−31]OTR, and [Δ2−35]OTR, was devoid of glycosylation sites, and all exhibited severely impaired cell surface expression compared to wild-type hOTR (Table 1). These data indicate that glycosylation of the hOTR N-terminus is important for efficient receptor trafficking or stability of the receptor in the plasma membrane. This conclusion is supported by studies on the $V_{1a}R$ which is closely related pharmacologically and structurally to the hOTR (*25*). The function of the nonglycosylated $V_{1a}R$ was perturbed in a manner similar to the truncated hOTRs in the current study, with the receptor pharmacology unchanged but cell surface expression decreased 80% compared to wild-type $V_{1a}R$ (*17*).

In conclusion, previous studies have shown that the N-terminus of the hOTR is required for agonist binding. Optimal agonist−OTR interaction requires a number of key residues located at various positions throughout the receptor protein (10−12). This study identifies Arg^{34}, in the distal segment of the N-terminal domain, as an important residue for contributing epitopes required for high-affinity agonist binding. The crystal structure of rhodopsin indicates that Arg^{34} is ideally positioned to do this by interacting with other elements of the extracellular face of the hOTR or directly with the ligand. Furthermore, the importance of arginyl at this position is restricted to agonist−hOTR complex formation as Arg^{34} is not required for antagonist binding, neither peptide nor non-peptide.

ACKNOWLEDGMENT

We are grateful to Dr. Tadashi Kimura (University of Osaka, Japan) for the gift of the human OTR clone and to Dr. Douglas J. Pettibone (Merck Research Laboratories, West Point, PA) for supplying L-368,899.

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BI015990V