Identification of an extracellular segment of the oxytocin receptor providing agonist-specific binding epitopes

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The effects of the peptide hormone oxytocin are mediated by oxytocin receptors (OTRs) expressed by the target tissue. The OTR is a member of the large family of G-protein-coupled receptors. Defining differences between the interaction of agonists and antagonists with the OTR at the molecular level is of fundamental importance, and is addressed in this study. Using truncated and chimaeric receptor constructs, we establish that a small 12-residue segment in the distal portion of the N-terminus of the human OTR provides important epitopes which are required for agonist binding. In contrast, this segment does not contribute to the binding site for antagonists, whether peptide or non-peptide. It does, however, have a role in agonist-induced OTR signalling. Oxytocin is also an agonist at the vasopressin

V_{1a} receptor (V_{1a}R). A chimaeric receptor (V_{1a}R^N-OTR) was engineered in which the N-terminus of the OTR was substituted by the corresponding, but unrelated, sequence from the N-terminus of the V_{1a}R. We show that the V_{1a}R N-terminus present in V_{1a}R^N-OTR fully restored both agonist binding and intracellular signalling to a dysfunctional truncated OTR construct. The N-terminal segment does not, however, contribute to receptor-selective agonism between the OTR and the V_{1a}R. Our data establish a key role for the distal N-terminus of the OTR in providing agonist-specific binding epitopes.

Key words: cell signalling, non-peptide, peptide, vasopressin.

INTRODUCTION

The peptide hormone oxytocin (OT) stimulates contraction of the uterine myometrium at parturition and contracts the mammary myoepithelium during lactation. As pregnancy approaches term, the sensitivity of the uterus to OT increases [1]. This increased responsiveness results from an increase in the number of oxytocin receptors (OTRs) expressed by the myometrium at parturition [1,2]. The increased receptor number is paralleled by an increase in transcription. For example, in humans, levels of the OTR mRNA are more than 300-fold higher at term than in the non-pregnant myometrium [3]. As a result, OT is administered extensively in the clinic to induce or augment labour. The corollary to this is that peptide [4], and more recently non-peptide [5], OTR antagonists have been developed to block OTRs and thereby prevent premature birth. Consequently, understanding ligand recognition by the OTR at the molecular level is of fundamental importance and may aid future drug design. Furthermore, as peptide agonists can be converted into antagonists by relatively minor structural modifications [6], defining ligand–receptor interactions that are unique to agonists will aid our understanding of the mechanisms underlying receptor activation.

The OTR is a G-protein-coupled receptor and exhibits the structural features typical of this protein family, including seven transmembrane domains [7]. Characterization of the architecture of the ligand-binding site of the OTR is aided by the availability of three different classes of ligand that can be used as probes: (i) peptide agonists, including the natural agonist OT, (ii) peptide antagonists, and (iii) non-peptide antagonists.

In the present study, we have employed a series of truncated receptors in order to identify an extracellular segment, located in

the distal N-terminus of the OTR, which provides agonist-specific binding epitopes and is required for receptor activation. Furthermore, as many OT analogues also bind to V_{1a} vasopressin receptors (V_{1a}Rs), we were able to use a V_{1a}R-OTR chimaeric receptor construct to establish that this segment does not contribute to the OTR/V_{1a}R receptor-specificity exhibited by some agonists.

EXPERIMENTAL

Materials

All materials for tissue culture were supplied by Gibco BRL (Paisley, Scotland, U.K.). The cyclic antagonist 1-(β -mercapto- β , β -cyclopentamethylene propionic acid), 2-(*O*-methyl-D-tyrosine, 4-threonine, 8-ornithine, 9-tyrosinamide) vasotocin {[d(CH₂)₅Tyr(Me)²,Thr⁴,Orn⁸,Tyr(NH₂)⁹]vasotocin; OTA} was from Bachem. [Arg⁸]Vasopressin (AVP), OT, [Thr⁴,Gly⁷]OT (TGOT) and [Phe²,Orn⁸]vasotocin (POVT) were from Sigma. Oxypressin ([Phe³]OT) was synthesized and generously provided by Professor Maurice Manning (Medical College of Ohio, Toledo, OH, U.S.A.). L-368,899 was kindly donated by Dr Douglas J. Pettibone (Merck Sharp & Dohme Research Laboratories, West Point, PA, U.S.A.).

Construction of truncated OTRs and chimaera

Truncations of the N-terminus of the OTR were carried out using a PCR approach. Truncation oligonucleotides were 5'-C-TAG-GGA-TTC-GCC-ACC-ATG-GAG-GGC-AAC-CGC-ACC-GCC-GG-3' and 5'-G-GGG-GGA-TCC-GCC-ACC-ATG-GAG-GCC-CTG-GCG-CGC-GTG-GAG-G-3' for the

Abbreviations used: AVP, [Arg⁸]vasopressin; OT, oxytocin; OTA, [d(CH₂)₅Tyr(Me)²,Thr⁴,Orn⁸,Tyr(NH₂)⁹]vasotocin; OTR, oxytocin receptor; POVT, [Phe²,Orn⁸]vasotocin; TGOT, [Thr⁴,Gly⁷]OT; V_{1a}R, vasopressin V_{1a} receptor; V_{1a}R^N-OTR, chimaeric receptor with the N-terminal domain of the OTR replaced by the N-terminus of the V_{1a}R. Truncation nomenclature: numbers refer to the positions in the human OTR sequence of the residues deleted; hence [Δ 2–23]OTR is the OTR with residues 2–23 inclusive deleted.

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Figure 1 Extracellular domains of the OTR, 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 labelled [Δ2–23]OTR and [Δ2–35]OTR, where the numbers refer to the positions in the sequence of the residues deleted. The N-terminus of the OTR was also replaced, from the position marked, by the corresponding $V_{1a}R$ sequence (shown below the main Figure) to generate the $V_{1a}R^N$ -OTR chimaeric receptor.

truncations [Δ2–23]OTR (i.e. human OTR sequence with residues 2–23 inclusive deleted) and [Δ2–35]OTR respectively (see Figure 1). Each primer contained a *Bam*HI restriction site (underlined), a Kozak consensus sequence [8] (shown in bold) and an ATG start site (shown in italics), followed by the OTR sequence. The PCR cycling conditions were: denaturing, 94 °C (1 min); annealing, 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 human OTR coding sequence in pBluescript SK II vector (Stratagene) utilizing unique *Bam*HI and *Apa*I sites. Truncated OTR constructs were subcloned into the mammalian expression vector pcDNA3.1(–) (Invitrogen) utilizing *Xba*I and *Kpn*I restriction sites.

A $V_{1a}R^N$ -OTR chimaera was made by amplification (as described above) of the rat $V_{1a}R$ N-terminus by PCR using 5′-CTC-CTC-**CCG-CGG**-TAC-GTC-CCC-CAG-CGG-GCT-GTC-ACC-3′ as the antisense oligonucleotide. This primer contained five base changes in the $V_{1a}R$ sequence (indicated in bold) which created a unique *Sac*II restriction site (underlined) without altering the amino acid sequence. A *Bam*HI/*Sac*II digest of this PCR fragment was subcloned into pcDNA3.1(–) OTR. This generated a chimaeric receptor construct in which the OTR N-terminal sequence was substituted by an in-frame $V_{1a}R$ N-terminal sequence. All truncations and chimaeric 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 supplemented with 10 % (v/v) foetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), glutamine (2 mM) and sodium pyruvate (1 mM) in humidified 5 % CO₂ in air at 37 °C. Cells were seeded at a density of approx. 5×10^5 cells/100 mm dish and transfected after 48 h using a calcium phosphate precipitation protocol [9] with 10 µg of DNA/dish.

Radioligand binding assays

Membranes from appropriately transfected HEK 293T cells were prepared as described [10] and the protein concentration determined using a BCA protein assay kit (Pierce Chemical Co.) with BSA as standard. Radioligand binding assays were performed essentially as described previously [11,12] using either the natural agonist [*Tyr*^{2-2,6-3}H]OT (35 Ci/mmol; DuPont NEN) or the OTR-selective peptide antagonist [*Tyr*⁹⁻¹²⁵I]OTA (2200 Ci/mmol; DuPont NEN) [13] as tracer ligand. Competition binding assays (final volume of 500 µl) containing radioligand (0.7–1.1 nM), cell membranes (100–300 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 radioactivity was quantified by liquid scintillation spectroscopy using HiSafe 3 (Packard) as cocktail. Non-specific binding was determined in parallel incubations containing 1 µM of unlabelled OT or OTA as appropriate. Binding data were analysed by non-linear regression to fit theoretical Langmuir binding isotherms to the experimental data using the Fig. P program (Biosoft). Individual IC₅₀ values obtained for competing ligands were corrected for radioligand occupancy according to the method of Cheng and Prusoff [14] using the radioligand affinity (*K_d*) determined experimentally for each construct.

Measurement of agonist-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 the OT-induced accumulation of inositol phosphates was based on that described previously [15,16]. Briefly, at 24 h post-transfection, medium was replaced with serum-free, inositol-free, Dulbecco’s modified Eagles medium containing 2.5 µCi/ml *myo*-[2-³H]inositol (Tocris Cookson Ltd, Bristol, U.K.) for 48 h. Cells were then washed with PBS and incubated with medium containing 10 mM LiCl for 30 min, after

Table 1 Pharmacological profiles of truncated and chimaeric OTRs

Truncated and chimaeric receptors were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (*K_d*) and the concentration of receptor sites (*B_{max}*) were calculated from IC₅₀ values and corrected for radioligand occupancy as described in the Experimental section. Data are means ± S.E.M. (*n* = 3) of three replicates.

Receptor	<i>K_d</i> (nM)			<i>B_{max}</i> (pmol/mg of protein)
	OT	OTA	L-368,899	
OTR	1.4 ± 0.5	1.7 ± 0.5	13.0 ± 2.0	0.5 ± 0.2
[Δ2–23]OTR	1.1 ± 0.3	1.4 ± 0.6	7.0 ± 1.1	0.3 ± 0.1
[Δ2–35]OTR	2500 ± 380	0.6 ± 0.2	6.9 ± 1.1	1.7 ± 0.35
$V_{1a}R^N$ -OTR	0.9 ± 0.3	0.4 ± 0.2	2.0 ± 0.3	0.2 ± 0.1

which OT or test ligand 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 5% (w/v) HClO_4 , 1 mM EDTA and 1 mg/ml phytic acid hydrolysate. After neutralization, samples were loaded on to Bio-Rad AG1-X8 columns (formate form). Following the elution of inositol (10 ml of water) and glycerophosphoinositol (10 ml of 25 mM ammonium formate), a mixed inositol fraction containing mono-, bis-, tris- and tetrakis-phosphates was eluted with 10 ml of 1.25 M ammonium formate containing 0.1 M formic acid.

RESULTS

Role of the N-terminus of the OTR in ligand binding

The role of the N-terminus of the OTR in ligand recognition was investigated by generating receptor constructs with sequential N-terminal deletions. Mutant receptors were engineered which were truncated at Ala²³ and Asn³⁵, as indicated in Figure 1. The initiation methionine was retained in each case. These constructs were termed $[\Delta 2-23]\text{OTR}$ and $[\Delta 2-35]\text{OTR}$ respectively, where the numbering refers to the amino acid residues deleted. The truncated OTRs were expressed in HEK 293T cells and the pharmacological profiles compared with that of the wild-type receptor. The wild-type receptor and $[\Delta 2-23]\text{OTR}$ construct were expressed at approx. 0.2–0.5 pmol/mg of protein, whereas expression of $[\Delta 2-35]\text{OTR}$ was slightly greater, being approx. 3-fold higher than that of the wild-type receptor (Table 1).

Three different classes of ligand were utilized to probe the ligand-binding site of the OTR: (i) the natural agonist OT; (ii) the peptide antagonist OTA, which is structurally related to the agonist OT and, like OT, possesses an intramolecular disulphide bond between residues 1 and 6 [13]; and (iii) the camphor-based tolylpiperazine non-peptide antagonist L-368,899, which has no structural similarity to OT [17]. Competition radioligand binding curves were determined for each of these different classes of ligand (Figure 2). The K_d values are presented in Table 1, corrected for radioligand occupancy. Truncation of the N-terminus as far as Ala²³ had little or no effect on the binding of any of the ligands employed. The affinity of the natural agonist OT and of the peptide antagonist OTA was unchanged (Figure 2; Table 1). The affinity of the non-peptide antagonist L-368,899 was increased slightly (2-fold) by removal of this proximal section of the N-terminus.

Removal of the distal segment of the N-terminus of the OTR by truncation to Asn³⁵ ($[\Delta 2-35]\text{OTR}$) resulted in a marked decrease in the affinity exhibited for OT, with the K_d increasing approx. 2000-fold. In contrast, the affinity of both the peptide and non-peptide antagonists for $[\Delta 2-35]\text{OTR}$ was only slightly affected, being 2–3-fold higher than with the wild-type OTR (Table 1). This retention of high-affinity antagonist binding by the distally truncated OTR construct was important practically, as it allowed the altered binding characteristics for OT to be determined quantitatively using the antagonist [¹²⁵I]OTA as a radiotracer.

The distal segment of the OTR N-terminus is important for binding agonists in general

The data presented above established the importance of a short segment within the distal part of the N-terminus of the OTR for high-affinity binding of OT, but not of OTA or L-368,899. This observation suggested two possible interpretations regarding ligand recognition by the receptor. Either the role of this segment is restricted to recognizing the unique structure of OT or, alternatively, the intermolecular contacts that it provides con-

Figure 2 Pharmacological characterization of truncated and chimaeric receptors

Competition radioligand binding studies with OT (A), OTA (B) and L-368,899 (C) were performed using a membrane preparation of HEK 293T cells transiently transfected with wild-type OTR (●), $[\Delta 2-23]\text{OTR}$ (□), $[\Delta 2-35]\text{OTR}$ (△) or $V_{1a}R^N\text{-OTR}$ (▽). Data are means $\pm$ S.E.M. of three separate experiments, each performed in triplicate. Values are expressed as percentage specific binding, where non-specific binding was defined by either OT or OTA (1 μM). A theoretical Langmuir binding isotherm has been fitted to the experimental data, as described in the Experimental section.

stitute part of an agonist-specific binding site. The structure of the cyclic nonapeptide OT is not very tolerant to change with retention of agonism [18]. Nevertheless, a few substituted analogues of OT have been described which are agonists. The Gln and Pro residues at positions 4 and 7 respectively can be substituted to generate the agonist [Thr⁴,Gly⁷]OT (TGOT) [19], and the residues Ile³ and Leu⁸ are substituted by Phe³ and Arg⁸ in the naturally occurring agonist analogue AVP.

Table 2 Agonist binding profiles of truncated and chimaeric OTRs

Truncated and chimaeric receptors were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (K_d) and the concentration of receptor sites (B_{max}) were calculated from IC_{50} values and corrected for radioligand occupancy as described in the Experimental section. Data are means $\pm$ S.E.M. ($n = 3$) of three replicates. ND, not determined.

Ligand	K_d (nM)				
	OTR	$[\Delta 2-23]$ OTR	$[\Delta 2-35]$ OTR	$V_{1a}R^N$ -OTR	$V_{1a}R$
OT	1.4 ± 0.5	1.1 ± 0.3	2500 ± 380	0.9 ± 0.3	1400 ± 320
TGOT	19 ± 2.1	25 ± 8.7	4000 ± 1400	37 ± 16	$> 10\,000$
AVP	9.2 ± 0.8	13 ± 0.9	830 ± 130	7.4 ± 3.2	1.0 ± 0.1
Oxypressin	7.1 ± 0.84	ND	ND	11 ± 4.3	270 ± 90
POVT	46 ± 5.4	ND	ND	39 ± 15	23 ± 4.0

Figure 3 Characterization of agonist binding to truncated and chimaeric OTR constructs

Competition radioligand binding studies were performed using membrane preparations of HEK 293T cells transiently transfected with wild-type OTR (●), $[\Delta 2-23]$ OTR (□), $[\Delta 2-35]$ OTR (△) or $V_{1a}R^N$ -OTR (▽), using the agonists TGOT (A), AVP, (B), oxypressin (OP) (C) and POVT (D). Data are means $\pm$ S.E.M. of three separate experiments, each performed in triplicate. Values are expressed as percentage specific binding, where non-specific binding was defined by either OT or OTA ($1 \mu\text{M}$). A theoretical Langmuir binding isotherm has been fitted to the experimental data, as described in the Experimental section.

The $[\Delta 2-23]$ OTR construct exhibited wild-type affinity for both TGOT and AVP (Table 2; Figure 3A and 3B). In contrast, the affinity of both of these agonists for the $[\Delta 2-35]$ OTR construct was decreased dramatically compared with the wild-type (Table 2; Figure 3A and 3B). As the binding data for TGOT and AVP mirrored the behaviour of OT, it could be concluded that the distal N-terminal segment of the OTR is not required solely for

OT binding, but is a pre-requisite for high-affinity agonist binding in general.

Intracellular signalling also requires the distal N-terminal segment

The ability of the wild-type OTR and the truncated constructs to generate an intracellular signal in response to OT was investi-

Figure 4 Coupling of truncated and chimaeric receptor constructs

Shown is the OT-induced accumulation of inositol mono-, bis-, tris- and tetrakis-phosphates (InsP–InsP₄) in HEK 293T cells transfected with wild-type hOTR (●), [Δ2–23]OTR (□), [Δ2–35]OTR (△) or V_{1a}R^N-OTR (▽). Data are means ± S.E.M. of a representative experiment performed in triplicate three times. Values are expressed as a percentage of the maximum stimulation induced by OT at the stated concentrations

gated. From the dose–response curves presented in Figure 4, it can be seen that the [Δ2–23]OTR construct was fully competent with respect to the OT-induced accumulation of inositol phosphates. This was also reflected in the calculated EC₅₀ values, which were 2.4 ± 0.9 nM and 2.5 ± 1.0 nM for wild-type OTR and [Δ2–23]OTR respectively. In contrast, the [Δ2–35]OTR construct was completely devoid of any signalling ability (Figure 4). It was noteworthy that, even at a concentration of 10 μM, OT did not induce any second messenger generation (results not shown). Given that OT binding to [Δ2–35]OTR exhibited a K_d of 2.5 μM, it can be calculated that 10 μM OT would occupy 80 % of the receptors [20]. Lack of intracellular signalling even at this high level of receptor occupancy indicates not only that the distal segment of the N-terminus is crucial for agonist binding, but also that it is involved in enabling the OTR to adopt the active conformation for G-protein coupling.

The pharmacological characteristics of the wild-type OTR are fully restored to [Δ2–35]OTR by the V_{1a}R N-terminus

AVP binds with high affinity to the OTR (Figure 3; Table 2) and is a partial agonist (Figure 5A). However, *in vivo* the effects of AVP are usually mediated by specific vasopressin receptors. The V_{1a}R is responsible for the majority of the physiological actions of AVP, with the notable exception of anti-diuresis, which is mediated by the V₂ subtype. Interestingly, OT is a low-affinity, full agonist at the V_{1a}R (Figure 5B) and has been reported to excite sympathetic preganglionic neurons in neonate rat spinal cord by acting through V_{1a}Rs, not OTRs [21]. Consequently, the OTR and the V_{1a}R have overlapping pharmacology. Nevertheless, receptor-selective agonists have been developed. For example, TGOT [19] and POVT [22] are selective for the OTR and the V_{1a}R respectively (Figures 5A and 5B). Given that a segment of the N-terminus of the OTR which is essential for high-affinity agonist binding has been defined above, it was of interest to investigate if this extracellular segment also provides the molecular basis for these receptor-selective agonists. It is noteworthy in this respect that the sequence of the agonist-binding segment of the OTR between residues 24 and 35 is well conserved across species (Figure 6). The corresponding domain of the V_{1a}R from different species is also conserved. However, there is no identity between the OTR and V_{1a}R conserved sequences (Figure 6).

Figure 5 Intracellular signalling generated by receptor-specific agonists at wild-type OTR, wild-type V_{1a}R and V_{1a}R^N-OTR

Shown is the agonist-induced accumulation of inositol mono-, bis-, tris- and tetrakis-phosphates (InsP–InsP₄) in cells expressing OTR (A), V_{1a}R (B) or V_{1a}R^N-OTR (C). Receptor-selective agonists used were OT (□), TGOT (○), AVP (■) and POVT (●). Data are means ± S.E.M. of three experiments performed in triplicate. Values are expressed as a percentage of the maximum stimulation induced by either OT (for OTR and V_{1a}R^N-OTR) or AVP (for V_{1a}R).

A chimaeric receptor was engineered in which the N-terminus of the OTR was replaced by the corresponding sequence from the V_{1a}R, and is referred to as V_{1a}R^N-OTR. The presence of the V_{1a}R N-terminus fully restored both high-affinity agonist binding and intracellular signalling capability to [Δ2–35]OTR, notwithstanding the lack of identity between the OTR and V_{1a}R N-terminal segments. Despite the presence of the V_{1a}R N-terminus, this chimaera exhibited wild-type OTR affinity for the receptor-selective ligands OT, TGOT and oxytocin (Table 2; Figures 2A, 3A and 3C). A slightly elevated affinity for OTA and L-368,899 was noted (Table 1). Consequently, the pharmacology of the V_{1a}R^N-OTR was essentially the same as that of the wild-type OTR, and not the V_{1a}R. Furthermore, the V_{1a}R^N-OTR did not

						TM I
Human	OTR	MEG	al	AA	NS	MEG
Sheep	OTR	MEG	af	AA	NS	MEG
Mouse	OTR	MEG	tp	AA	NS	MEG
Pig	OTR	MEG	vl	AA	NS	MEG
Bovine	OTR	MEG	af	AA	NS	MEG
Marsupial	OTR	MEG	el	AA	NS	MEG
Rat	OTR	MEG	tp	AA	NS	MEG
Human	V _{1a} R	Mrl	sag	pda	gpgs	NSS
Sheep	V _{1a} R	Mrf	sgs	pdp	gpgs	NSS
Mouse	V _{1a} R	Msf	prg	shd	lpag	NSS
Rat	V _{1a} R	Msf	prg	sqd	rsvg	NSS
V _{1a} R ^N -OTR		Msf	prg	sqd	rsvg	NSS

Figure 6 Comparison of the N-terminal sequences of the OTR and the V_{1a}R cloned from different species

The amino acid sequences of the OTR and the V_{1a}R cloned from various species are aligned. The sequence of the V_{1a}R^N-OTR chimaera is also shown. Residues that are conserved within the OTR sequences, or within the V_{1a}R sequences, from different species are in bold type. The position of the top of transmembrane domain I (TM I) is indicated by the box.

Table 3 Agonist-induced signalling by the V_{1a}R^N-OTR chimaera, the OTR and the V_{1a}R

Shown are EC₅₀ values for the agonist-induced accumulation of inositol mono-, bis-, tris- and tetrakis-phosphates in cells expressing the OTR, V_{1a}R or V_{1a}R^N-OTR. Values are means ± S.E.M. of three separate experiments each performed in triplicate.

Ligand	EC ₅₀ for inositol phosphate accumulation (nM)		
	OTR	V _{1a} R	V _{1a} R ^N -OTR
OT	2.4 ± 0.6	102 ± 35	3.1 ± 0.7
AVP	30 ± 12	0.4 ± 0.2	57 ± 11
TGOT	14 ± 4.0	> 10000	9.9 ± 3.0
POVT	21 ± 1.0	0.4 ± 0.1	29 ± 8.6

exhibit some sort of hybrid pharmacological profile containing elements of both OTR and V_{1a}R pharmacology. The intracellular signalling characteristics of the V_{1a}R^N-OTR chimaera were compared with those of the wild-type OTR and V_{1a}R. From the dose-response curves for inositol phosphate accumulation (Figure 5) and the derived EC₅₀ values (Table 3), it can be seen that intracellular signalling by the V_{1a}R^N-OTR was the same in all respects as that of the wild-type OTR. These signalling data paralleled the binding data (Table 2) and established that, although the distal N-terminal segment of the OTR is crucial for agonist binding and receptor activation, it does not contribute to receptor-specific agonism.

DISCUSSION

The physiological role played by OT in birth has resulted in the hormone being employed routinely in clinics world-wide to augment labour [3,23]. It is perhaps a logical progression from this, that in recent years pharmaceutical research has shifted to the design of OTR antagonists which could be used to suppress pre-term labour. This has resulted in the development of the peptide antagonist d[D-Tyr(Et)²,Thr⁴,Orn⁸]OT (Atosiban), which has been reported to be effective in the treatment of acute episodes of pre-term labour and in prolonging uterine quiescence in human patients [24]. In order to overcome some of the inherent problems of administering a peptide drug, including lack of stability and oral bioavailability, an increasing number of

non-peptide antagonists of differing chemical classes have also been developed [5,25]. Given the interest in targeting the OTR for therapeutic intervention using agonist and antagonist analogues, it is of fundamental importance that the architecture of the ligand-binding site is defined at the molecular level. Furthermore, any differences that exist between the agonist-OTR interaction and the antagonist-OTR interaction will have ramifications with respect to understanding the conformational requirements for receptor activation, not just for binding. It has been suggested from molecular modelling that the binding site for OT is completely buried within the hydrophobic helical bundle of the OTR transmembrane domains [26]. Other studies have shown that it is the extracellular domains (including the N-terminus), rather than the transmembrane domains, that are important [27]. In the present study, we have refined this proposal further by identifying a small extracellular segment of 12 amino acids in the distal portion of the OTR N-terminus (residues 24–35) that are required for agonist binding, but have no role in the binding of peptide antagonists or non-peptide antagonists. This preservation of high-affinity binding of antagonists, of unrelated chemical structure, was important for several reasons: (i) it established that the loss of agonist binding subsequent to ablation of the N-terminus was not merely due to a non-specific perturbation of the conformation of the receptor; (ii) it established that there was no information within the N-terminal sequence for correct folding of the OTR, or for targeting it to the plasma membrane; (iii) it allowed a radiolabelled antagonist to be used as tracer in competition radioligand binding assays, in order to accurately quantify the observed changes in agonist binding characteristics; and (iv) it revealed that the binding epitopes provided by the distal N-terminal segment were exclusively agonist-specific. It remains to be determined whether the importance of the N-terminal segment stems from a direct interaction with the ligand, or whether it establishes intra-receptor contacts that are necessary for maintaining the appropriate OTR conformation for agonist recognition. The OTR has three putative N-glycosylation sites located in the N-terminus (Figure 1). However, it is known that the oligosaccharide moieties are not involved in either ligand binding or signal transduction, as disruption of the N-glycosylation consensus sites by Asn → Asp site-directed mutagenesis did not affect OTR function [28]. Segments within the N-terminus have been implicated in the binding sites of other G-protein-coupled receptors which bind

peptide ligands, including the neurokinin NK₁ and NK₂ receptors [29,30], the angiotensin II AT₁ receptor [31] and the kappa opiate receptor [32]. It should be noted, however, that, with the exception of the neurokinin receptors, the role of the N-terminus in ligand binding to these G-protein-coupled receptors was not restricted to agonists. In contrast with our study, which addressed both peptide and non-peptide antagonists, the NK₁ and NK₂ receptor studies only investigated effects on non-peptide antagonist binding [29,30].

There is a degree of overlap between the pharmacological profiles of the OTR and the V_{1a}R. From the data presented in Figure 5, it can be seen that OT is a full agonist at the V_{1a}R and that AVP is a partial agonist at the OTR. It has also been reported that the effects of OT can sometimes be mediated by the V_{1a}R rather than the OTR [21]. In addition, the OTR peptide antagonist Atosiban is equipotent at the human V_{1a}R [33,34], and although the non-peptide antagonist L-371,257 is OTR-selective in humans, it is actually V_{1a}R-selective in rats [35]. Nevertheless, selective agonists have been developed. TGOT is OTR-selective (Figures 5A and 5B), and POVT is a potent full agonist at the V_{1a}R (Figure 5B) but a weak, low-efficacy partial agonist at the human OTR (Figure 5A). Although POVT is more efficacious at the V_{1a}R in terms of second messenger generation, it is not very receptor-selective with respect to binding, as the affinity of POVT for the OTR and the V_{1a}R is 46 nM and 23 nM respectively (Table 2). We engineered the chimaeric receptor V_{1a}R^N-OTR, which has the N-terminus of the OTR substituted by the unrelated sequence of the V_{1a}R N-terminus. In marked contrast with the [Δ2–35]OTR truncation, this V_{1a}R^N-OTR construct exhibited the pharmacology and coupling capability of the wild-type OTR. Consequently, although the residues introduced into the chimaera were different from those of the OTR, they were nevertheless able to provide fully functional binding epitopes for agonists, and facilitated the adoption of the agonist-induced active conformation. Furthermore, the V_{1a}R^N-OTR chimaera allowed us to address whether the distal N-terminal OTR segment not only provided agonist-specific binding epitopes, but also contributed to receptor-specific agonist recognition. As the V_{1a}R^N-OTR chimaera exhibited wild-type OTR pharmacology and intracellular signalling, it was concluded that the N-terminus does not select between receptor-specific agonists such as TGOT and POVT.

In summary, using truncated and chimaeric constructs, we have identified a short segment in the distal portion of the OTR N-terminus which is required for high-affinity agonist binding, but has no role in antagonist binding or in discriminating between receptor-selective agonists.

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