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 V1a receptor (V1a,R). A chimaeric receptor (V1a,Rx-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 V1a,R. We show that the V1a,R N-terminus present in V1a,Rx-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 V1a,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 V1a vasopressin receptors (V1a,Rs), we were able to use a V1a,R-OTR chimaeric receptor construct to establish that this segment does not contribute to the OTR/V1a,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-(/3-mercaptopropionyl-3-butyryl-cyclopentamethylene propionic acid), 2-(O-methyl-3-threonine, 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 [Phe6,Orn10]vasotocin (POVT) were from Sigma. Oxypressin ([Phe4]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 (Merrick 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'-TAG-GGA-TTC-GCC-ACC-ATG-GAG-GGC-AAC-CGC-ACC-GCC-GG-3' and 5'-G-GGG-GGA-TCC-GCC-GGC-CAG-GAAG-GGC-CTG-GGC-GCG-CTG-GGC-GCG-GC-GG-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, [Phe6,Orn10]vasotocin; TGOT, [Thr4,Gly7]OT; V1a,R, vasopressin V1a receptor; V1a,Rx-OTR, chimaeric receptor with the N-terminal domain of the OTR replaced by the N-terminus of the V1a,R. Truncation nomenclature: numbers refer to the positions in the human OTR sequence of the residues deleted; hence [Δ2–23]OTR is the OTR with residues 2–23 inclusive deleted.

1 To whom correspondence should be addressed (e-mail M.Wheatley@bham.ac.uk).
This generated a chimaeric receptor construct in which the OTR without altering the amino acid sequence. A Table 1 Pharmacological profiles of truncated and chimaeric OTRs contained five base changes in the V

GTC-ACC-3

and [2–23]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

R sequence (shown below the main Figure) to generate the V

R-OTR chimaeric receptor.

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, respectively (see Figure 1). Each primer contained a BamHI restriction site (underlined), a Kozak consensus sequence [8] (shown in bold) and an ATG start site (shown in italics) following the translation 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 BstX I and Sac II restriction sites.

A V

R-OTR chimaera was made by amplification (as described above) of the rat V

R N-terminus by PCR using 5'-CTC-TCG-GGG-TAC-GTC-CCC-CAG-CG-GCT-GTC-ACC-3' as the antisense oligonucleotide. This primer contained five base changes in the V

R sequence (indicated in bold) which created a unique SacII restriction site (underlined) without altering the amino acid sequence. A BamHI/SacII digest of this PCR fragment was subcloned into pcDNA3.1(−) (Invitrogen) utilizing XbaI and KpnI restriction sites.

Table 1 Pharmacological profiles of truncated and chimaeric OTRs

Truncated and chimaeric receptors were expressed in HEK 293T cells and characterized pharmacologically. Dissociation constants (Kd) and the concentration of receptor sites (Bmax) 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.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTR</td>
<td>1.4 ± 0.5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>[Δ2–23]OTR</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>[Δ2–35]OTR</td>
<td>2500 ± 380</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>
| V

R-OTR | 0.9 ± 0.3 | 0.4 ± 0.2 | 2.0 ± 0.3 | 0.2 ± 0.1 |

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), glucose (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 × 10⁶ 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 [Tyro-2,6-3H]OT (35 Ci/mmol; DuPont NEN) or the OTR-selective peptide antagonist [Tyro-131I]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 (Kd) determined experimentally for each construct.

Measurement of agonist-induced inositol phosphate production

HEK 293T cells were seeded at a density of 2.5 × 10⁵ 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
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) HClO₄, 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 [A²–2³]OTR and [A²–3⁵]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 [A²–2³]OTR construct were expressed at approx. 0.2–0.5 pmol/mg of protein, whereas expression of [A²–3⁵]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 ([A²–3⁵]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 [A²–3⁵]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 radiotracr.

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 constitute 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 Ile⁴ and Leu⁸ are substituted by Phe⁴ 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 ($B_{\text{max}}$) were calculated from IC$_{50}$ values and corrected for radioligand occupancy as described in the Experimental section. Data are means ± S.E.M. ($n$ = 3) of three replicates. ND, not determined.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>OTR</th>
<th>$\Delta_2$–23OTR</th>
<th>$\Delta_2$–35OTR</th>
<th>$V_{1aRN}$–OTR</th>
<th>$V_{1a}$R</th>
</tr>
</thead>
<tbody>
<tr>
<td>OT</td>
<td>1.4 ± 0.5</td>
<td>1.1 ± 0.3</td>
<td>2500 ± 380</td>
<td>0.9 ± 0.3</td>
<td>1400 ± 320</td>
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<tr>
<td>TGOT</td>
<td>19 ± 2.1</td>
<td>25 ± 8.7</td>
<td>4000 ± 1400</td>
<td>37 ± 16</td>
<td>&gt; 10000</td>
</tr>
<tr>
<td>AVP</td>
<td>9.2 ± 0.8</td>
<td>13 ± 0.9</td>
<td>830 ± 130</td>
<td>7.4 ± 3.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Oxypressin</td>
<td>7.1 ± 0.84</td>
<td>ND</td>
<td>ND</td>
<td>11 ± 4.3</td>
<td>270 ± 90</td>
</tr>
<tr>
<td>POVT</td>
<td>46 ± 5.4</td>
<td>ND</td>
<td>ND</td>
<td>39 ± 15</td>
<td>23 ± 4.0</td>
</tr>
</tbody>
</table>

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 (●), $\Delta_2$–23OTR (□), $\Delta_2$–35OTR (▲) or $V_{1aRN}$–OTR (▽), using the agonists TGOT (A), AVP (B), Oxypressin (OP) (C) and POVT (D). Data are means ± 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.

The $\Delta_2$–23OTR 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 $\Delta_2$–35OTR 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-
Agonist binding to the oxytocin receptor

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 OTR (●), [Δ2–23]OTR (○), [Δ2–35]OTR (∆) or V₁₅₃OTR (*). 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 Kᵦ 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 V₁₅₃R 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 V₁₅₃R 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 V₁₅₃R (Figure 5B) and has been reported to excite sympathetic preganglionic neurons in neonate rat spinal cord by acting through V₁₅₃Rs, not OTRs [21]. Consequently, the OTR and the V₁₅₃R have overlapping pharmacology. Nevertheless, receptor-selective agonists have been developed. For example, TGOT [19] and POVT [22] are selective for the OTR and the V₁₅₃R 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 V₁₅₃R from different species is also conserved. However, there is no identity between the OTR and V₁₅₃R conserved sequences (Figure 6).

Figure 5 Intracellular signalling generated by receptor-specific agonists at wild-type OTR, wild-type V₁₅₃R and V₁₅₃R²OTR

Shown is the agonist-induced accumulation of inositol mono-, bis-, tris- and tetrakis-phosphates (InsP–InsP₄) in cells expressing OTR (●), V₁₅₃R (○) or V₁₅₃R²OTR (*). 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 V₁₅₃R²OTR) or AVP (for V₁₅₃R).

A chimaeric receptor was engineered in which the N-terminus of the OTR was replaced by the corresponding sequence from the V₁₅₃R, and is referred to as V₁₅₃R²OTR. The presence of the V₁₅₃R 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 V₁₅₃R N-terminal segments. Despite the presence of the V₁₅₃R N-terminus, this chimaera exhibited wild-type OTR affinity for the receptor-selective ligands OT, TGOT and oxypressin (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 V₁₅₃R²OTR was essentially the same as that of the wild-type OTR, and not the V₁₅₃R. Furthermore, the V₁₅₃R²OTR did not
Lack of stability and oral bioavailability, an increasing number of inherent problems of administering a peptide drug, including in human patients [24]. In order to overcome some of the episodes of pre-term labour and in prolonging uterine quiescence has been reported to be effective in the treatment of acute labour [3,23]. It is perhaps a logical progression from the hormone being employed routinely in clinics world-wide to augment labour [3,23], that in recent years pharmaceutical research has shifted to establishing that, although the distal N-terminal segment of the OTR is crucial for agonist binding and receptor activation, 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 binding, 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. 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 segment 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

**Table 3** Agonist-induced signalling by the V₈R⁻OTR chimaera, the OTR and the V₈R

<table>
<thead>
<tr>
<th>Ligand</th>
<th>OTR</th>
<th>V₈R</th>
<th>V₈R⁻OTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>OT</td>
<td>2.4 ± 0.6</td>
<td>102 ± 35</td>
<td>31 ± 0.7</td>
</tr>
<tr>
<td>AVP</td>
<td>30 ± 12</td>
<td>0.4 ± 0.2</td>
<td>57 ± 11</td>
</tr>
<tr>
<td>TGOT</td>
<td>14 ± 4</td>
<td>&gt; 10000</td>
<td>9.9 ± 3.0</td>
</tr>
<tr>
<td>POVT</td>
<td>21 ± 1.0</td>
<td>0.4 ± 0.1</td>
<td>29 ± 0.6</td>
</tr>
</tbody>
</table>

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 neuropeptide 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 neuropeptide 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$_2$R. From the data presented in Figure 5, it can be seen that OT is a full agonist at the V$_1$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$_1$R rather than the OTR [21]. In addition, the OTR peptide antagonist Atosiban is equipotent at the human V$_1$R [33,34], and although the non-peptide antagonist L-371,257 is OTR-selective [32,35], the angiotensin II AT1 receptor [36] is not very receptor-selective with respect to binding, as the N-terminus which is required for high-affinity agonist binding, 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.

We are grateful to Dr Tadashi Kimura (University of Osaka, Japan) for the gift of the human OTR clone, to Professor Maurice Manning (University of Ohio, Toledo, OH, U.S.A.) for generously supplying us with oxypressin, and to Dr Douglas J. Pettibone (Merck Sharp & Dohme Research Laboratories, West Point, PA, U.S.A.) for supplying L-368,899, a potent orally active oxytocin antagonist for potential use in preterm labor.

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Received 9 November 2000/7 December 2000; accepted 19 December 2000