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Agonist-specific requirement for a glutamate in transmembrane helix 1 of the oxytocin receptor

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ABSTRACT

Defining key differences between agonist and antagonist binding to hormone receptors is important and will aid rational drug design. $Glu^{1.35}$ in transmembrane helix 1 (TM1) of the human oxytocin receptor (OTR) is absolutely conserved in all OTRs cloned to date. We establish that $Glu^{1.35}$ is critical for high affinity binding of agonists (full and partial) but is not required for antagonist binding (peptide or non-peptide). Consequently, the mutant receptor [E1.35A]OTR exhibited markedly decreased OT affinity (>1200-fold) and disrupted second messenger generation. Substitutions of $Glu^{1.35}$ by Asp, Gln or Arg were incapable of supporting wild-type OTR agonist binding or signaling. Molecular modeling revealed that $Glu^{1.35}$ projects into the receptor's central binding crevice and provides agonist-specific contacts not utilized by antagonists. This study explains why Glu is absolutely conserved at residue-1.35 in all receptors binding OT and related peptides, and provides molecular insight into key differences between agonist–receptor and antagonist–receptor binding modes.

1. Introduction

The neurohypophysial peptide hormone oxytocin (OT) and oxytocin-like peptides, such as mesotocin and isotocin, facilitate reproduction in all vertebrates (Acher et al.,1995; Parry and Bathgate, 2000; Gimpl and Fahrenholz, 2001). Even in the relatively simple earthworm *Eisenia foetida*, the OT-related peptide annetocin induces egg-laying behaviour (Oumi et al., 1996). In humans, OT mediates a wide range of central and peripheral effects (Gimpl and Fahrenholz, 2001; Opar, 2008), including increasing the frequency and intensity of uterine contraction at parturition and contraction of the mammary gland myoepithelium during lactation (Gimpl and Fahrenholz, 2001; Soloff et al., 1979). The potent uterotonic role played by OT in birth has resulted in extensive use of this peptide clinically to induce and augment labor (Owen and Hauth, 1992). The physiological effects of OT are mediated by a specific oxytocin receptor (OTR) expressed by target tissues. As pregnancy approaches term there is an increase in the abundance of OTRs expressed by the myometrium which results in a specifically timed increased responsiveness of the uterus to OT (Fuchs et al., 1995; Kimura and Saji, 1995; Parry and Bathgate, 2000). Binding antagonists to the OTRs can effectively blockade the receptors, thereby reducing receptor availability to OT resulting in increased uterine quiescence. Both peptide antagonists such as Atosiban (d[DTyr(Et)^2,Thr^4,Orn^8]OT) (Valenzuela et al., 2000) and non-peptide antagonists (Pettibone and Freidinger, 1997; Hawtin et al., 2005a) have been developed for this tocolytic purpose. The OTR is a Family A (rhodopsin-like) G-protein-coupled receptor (GPCR) and exhibits structural features typical of this family, including seven transmembrane (TM) helices (Kimura et al., 1992). Only one OTR subtype has been cloned from humans, implying that the wide range of physiological effects of OT is mediated by a single receptor which signals primarily by coupling to phospholipase C to generate inositol trisphosphate ($InsP_3$) as second messenger (Gimpl and Fahrenholz, 2001).

As agonists induce OTR signaling and antagonists do not, defining differences between the agonist–OTR interaction and the antagonist–OTR interaction at the molecular level, will provide insight into OT action and may aid rational drug design. In this study we establish that a Glu in TM1 of the human OTR is critical for agonist binding and signaling but is not required for antagonist binding (peptide antagonist or non-peptide antagonist). Furthermore, we demonstrate that there is a specific requirement for Glu

Abbreviations: AVP, [arginine^8]vasopressin; ELISA, enzyme-linked immunosorbent assay; GPCR, G-protein-coupled receptor; InsP, inositol phosphate; $InsP_3$, inositol trisphosphate; OT, oxytocin; OTA, d$(CH_2)_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr$(NH_2)^9$ vasotocin; OTR, oxytocin receptor; TM, transmembrane helix.

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at this locus which explains why this Glu is absolutely conserved in all receptors for OT and OT-related peptides cloned to date.

2. Materials and methods

2.1. Materials

The radioligand [Tyr^9-^{125}I]OTA (specific activity of 2200 Ci/mmol) was from Perkin Elmer (Beaconsfield, UK). OT and AVP were purchased from Sigma (Poole, UK). The cyclic peptide antagonist [d(CH$_2$)$_5$Tyr(Me)^{2}Thr^{4}Orn^{8}TyrNH$_2$^{9}]vasotocin (OTA) was from Bachem (St. Helens, UK) and L-368,899 was a kind gift from Dr. Douglas J. Pettibone (Merck Research Laboratories, West Point, PA). Cell culture media, buffers and supplements were purchased from Gibco (Uxbridge, UK). *Dpn*I and *Pfu* polymerase were from New England Biolabs (Hitchin, UK).

2.2. Mutant receptor constructs

Human OTR cDNA with an N-terminal haemagglutinin (HA) tag was subcloned into pcDNA3.1(+) (Invitrogen) prior to mutagenesis. Introduction of the epitope tag did not affect the pharmacology of the receptor. Mutants were engineered using the QuikChange™ site-directed mutagenesis kit (Stratagene, Cambridge, UK) according to the manufacturer's instructions using forward and reverse oligonucleotide primers synthesized by Invitrogen (UK). The forward primers for [E1.35A]OTR, [E1.35D]OTR, [E1.35Q]OTR and [E1.35R]OTR were 5′-C-CTG-GCG-CGC-GTG-G**CA**-GTG-GCG-GTG-CTG-3′, 5′-CTG-GCC-AAA-CTG-GA**C**-ATC-GCC-GTG-CTG-3′, 5′-CTG-GCC-AAA-CTG-**C**AG-ATC-GCC-GTG-CTG-3′ and 5′-CTG-GCC-AAA-CTG-**CGC**-ATC-GCC-GTG-CTG-3′, respectively, with appropriate base changes shown in bold. All receptor constructs were confirmed by automated fluorescent sequencing in their entirety in both sense and antisense directions (Functional Genomics Laboratory, University of Birmingham, Birmingham, UK) and subcloned using unique *Hind*III and *Kpn*I restriction sites.

2.3. Cell culture and transfection

HEK 293T cells were routinely cultured in Dulbecco's modified Eagles 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) in humidified 5% (v/v) CO_2 in air at 37 °C. For radioligand binding assays, cells were seeded at a density of ~5×10^5 cells/100 mm dish and transfected after 48 h. For measurements of cell-surface expression, cells were seeded at a density of 1.5×10^5 cells per poly D-lysine-coated well (24-well plate) and transfected after 30 h. For measurement of agonist-induced inositol phosphates production, HEK 293T cells were seeded onto poly D-lysine-coated 12-well plates at a density of 2.5×10^5 cells per well and transfected after 30 h. Cells were transfected using a mixture (per 1 μg DNA) of 6 μl 10 mM polyethyleneimine and 45 μl 5% glucose solution, incubated for 30 min at room temperature and added to an appropriate final volume of full media. 12- and 24-well plates were treated with 0.5 μg and 1 μg DNA per well respectively and 100 mm dishes were treated with 5 μg DNA/dish. Characterization of expressed receptors was performed 48–72 h after transfection.

2.4. Radioligand binding assays

A washed membrane fraction was prepared from transfected HEK 293T cells and the protein concentration determined using the BCA protein assay kit (Pierce Chemical Co., UK) with BSA as standard. Radioligand binding assays were performed as previously described (Hawtin et al., 2000) using the OTR-selective peptide antagonist [^{125}I]OTA (2200 Ci/mmol) as tracer ligand. Competition binding assays (final volume of 500 μl) containing radioligand (0.5–10 pM), cell membranes (100–500 μg) and competing ligand (at the concentration indicated) were incubated at 30 °C for 90 min to establish equilibrium. Bound and free ligand were separated by centrifugation (13,000 × *g*, 10 min), membranes washed, dissolved in Soluene-350 (Packard) and the radioactivity quantified by liquid scintillation spectroscopy using HiSafe3 (Wallac, UK) as cocktail. Non-specific binding was determined in parallel incubations using OTA (1 μM). Binding data were analyzed by non-linear regression to fit theoretical Langmuir binding isotherms to the experimental data using GraphPad PRISM (Graphpad Software Inc., San Diego, CA). IC_{50} values for competing ligands were corrected for radioligand occupancy (Cheng and Prusoff, 1973) using the radioligand affinity (K_d) experimentally determined for each individual construct.

2.5. Determination of cell-surface expression using enzyme-linked immunosorbent assay (ELISA)

All receptor constructs incorporated an HA epitope tag in the N-terminus which enabled cell-surface expression to be determined 48 h after transfection, in fixed cells, using an ELISA as described previously (Hawtin et al., 2006). Results were normalized against a wild-type OTR control processed in parallel. Non-transfected cells were used to determine background. All experiments were performed in quadruplicate.

2.6. Inositol phosphates production

The assay for accumulation of inositol phosphates induced by OT or AVP was based on that previously described (Howl et al., 1995). Briefly, following pre-labeling of transfected cells with 1 μCi/ml myo-[2-^{3}H]inositol (Perkin Elmer) in inositol free DMEM containing 1% (v/v) FCS, a mixed fraction containing mono-, bis-, and tris-, phosphates (InsP–InsP$_3$) was collected following stimulation by agonist for 30 min, at the concentrations indicated, in the presence of 10 mM LiCl. EC_{50} values were determined by nonlinear regression after fitting of sigmoidal curves to experimental data.

2.7. Secondary structure prediction

The sequence of the OTR was submitted to the hidden Markov model-based protein structure prediction, SAM-T02 (Karplus et al., 2003), which utilizes a hidden Markov model engine to search for homologous proteins from which a sequence alignment is produced and a structure prediction obtained.

2.8. Receptor modeling

The OTR sequence was aligned against the crystal structure coordinates of bRho using CLUSTALW (Thompson et al., 1994). The alignment was then used to generate homology models using MODELLER version 6.2 (Sali and Blundell, 1993). A collection of 200 model structures was generated and ranked based on an objective function provided by MODELLER version 6.2. From this ensemble, a single structure was selected for further analysis. Further refinement of the homology model was achieved through molecular dynamics simulations of the receptor embedded in a hydrated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine bilayer. Molecular dynamics simulations were carried out using the GROMOS96 force-field parameters, with minor modifications, as implemented in GROMACS (Lindahl et al., 2001).

3. Results

3.1. Glu$^{1.35}$ provides critical agonist-specific binding contacts

Elucidation of peptide hormone binding sites within the GPCR architecture is of fundamental importance for understanding the molecular basis of agonist-induced receptor activation. Furthermore, defining the specific ligand-receptor interactions which result in a ligand functioning as an agonist, versus an antagonist, will aid rational drug design. For the OTR, the ligand binding site is composed of residues located within both the TM helical bundle plus residues in extracellular domains (Gimpl and Fahrenholz, 2001). It has been shown previously that the N-terminus of the OTR is required for high affinity binding of OT but not for binding antagonists (Hawtin et al., 2001; Postina et al., 1996). Furthermore, this agonist-specific effect was attributed to Arg^{34} (residue 1.27 using standard GPCR residue nomenclature (Ballesteros and Weinstein, 1995)) located within the N-terminal domain proximal to the membrane and close to the top of TM1 (Wesley et al., 2002). Examination of all the known sequences of receptors for OT and OT-like peptides in the GPCR database (www.gpcr.org) revealed that a glutamic acid residue is absolutely conserved within the TM1 helix, at residue-1.35. Furthermore, molecular modeling indicates that the side-chain of this Glu$^{1.35}$ orientates into the binding crevice within the TM helical bundle (Fig. 1) where it would be well placed to provide binding contacts for ligand interaction.

To investigate the role of Glu$^{1.35}$ in ligand recognition, a mutant human OTR was engineered in which Glu$^{1.35}$ was replaced by Ala ([E1.35A]OTR). [E1.35A]OTR was expressed in HEK 293T cells, pharmacologically characterized and compared to wild-type OTR. Three different classes of ligand were employed to probe the contribution of Glu$^{1.35}$ to the binding site: (i) the natural agonist OT; (ii) the cyclic peptide antagonist [d(CH$_2$)$_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr(NH$_2$)^9]vasotocin (OTA), which is structurally related to OT (Elands et al., 1988); and (iii) a non-peptide antagonist L-368,899 which is camphor-based and has no structural similarity to OT (Williams et al., 1994). The wild-type receptor and [E1.35A]OTR exhibited the same cell surface expression level of 1–2 pmol/mg protein (Table 1). Competition radioligand binding curves were determined for the different