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Identification of transmembrane domain 6 & 7 residues that contribute to the binding pocket of the urotensin II receptor

Brian J. Holleran, Ivana Domazet, Marie-Eve Beaulieu, Li Ping Yan, Gaétan Guillemette, Pierre Lavigne, Emanuel Escher, Richard Leduc *

Department of Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

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ABSTRACT

Urotensin II (U-II), a cyclic undecapeptide, is the natural ligand of the urotensin II (UT) receptor, a G protein-coupled receptor. In the present study, we used the substituted-cysteine accessibility method to identify specific residues in transmembrane domains (TMDs) six and seven of the rat urotensin II receptor (rUT) that contribute to the formation of the binding pocket of the receptor. Each residue in the R256$^{(6.32)}$-Q283$^{(6.59)}$ fragment of TMD6 and the A295$^{(7.31)}$-T321$^{(7.57)}$ fragment of TMD7 was mutated, individually, to a cysteine. The resulting mutants were expressed in COS-7 cells, which were subsequently treated with the positively charged methanethiosulfonate-ethylammonium (MTSEA) or the negatively charged methanethiosulfonate-ethylsulfonate (MTSES) sulfhydryl-specific alkylating agents. MTSEA treatment resulted in a significant reduction in the binding of TMD6 mutants F268C$^{(6.44)}$ and W278C$^{(6.54)}$ and TMD7 mutants L298C$^{(7.34)}$, T302C$^{(7.38)}$, and T303C$^{(7.39)}$ to ^{125}I-U-II. MTSES treatment resulted in a significant reduction in the binding of two additional mutants, namely L282C$^{(6.58)}$ in TMD6 and Y300C$^{(7.36)}$ in TMD7. These results suggest that specific residues orient themselves within the water-accessible binding pocket of the rUT receptor. This approach, which allowed us to identify key determinants in TMD6 and TMD7 that contribute to the UT receptor binding pocket, enabled us to further refine our homology-based model of how U-II interacts with its cognate receptor.

1. Introduction

Urotensin II (ETPDCFWKYCV, U-II) is a cyclic undecapeptide with vasoactive, proliferative, neuronal, and chemotactic properties. U-II is the endogenous ligand of the urotensin II (UT) receptor, which is considered a pharmacological target for treating cardiovascular diseases (hypertension, heart failure, cardiac fibrosis, and hypertrophy), atherosclerosis, liver diseases, and diabetes [14,15,26,28]. U-II was originally isolated from the urophysis of teleost fish [30], and the cDNA encoding its precursor has been identified in many species [9,10]. The UT receptor is a member of family 'A' of the larger G protein-coupled receptor (GPCR) superfamily [2]. Many features associated with this family such as a short N-terminus, a highly conserved residue in each transmembrane domain (TMD), a D/ERY motif in the second intracellular loop, a CW/FxP 'toggle switch' motif [32] in TMD6, a NPxxY motif in TMD7, and potential serine/threonine phosphorylation sites in the cytoplasmic tail [31] are found in the UT receptor.

The molecular mechanisms by which agonists bind to and activate GPCRs through conformational changes remain obscure. Although for many years, the only available structural model was rhodopsin [27], the structures of other GPCRs such as the β2 adrenergic [7], β1 adrenergic [34], opsin [29], and A_{2A} adenosine receptors [19] have recently been determined. These studies have enabled us to better understand how diffusible ligands can recognize and bind to GPCRs, and how TMDs are involved in this process. For instance, the A_{2A} adenosine structure clearly shows how both TMD6 and TMD7 play a role in the formation of the receptor's binding pocket [19].

Despite these major advances, many questions remain regarding the dynamics by which conformers shift from the ground state to an active state. A variety of biophysical and biochemical approaches are needed to help address these issues. The substituted-cysteine accessibility method (SCAM) [1,21,22] is an ingenious approach for systematically identifying TMD residues that contribute to the binding-site pocket of GPCRs. Consecutive residues within TMDs are mutated to cysteine, one at a time, and the mutant receptors are expressed in heterologous cells. If ligand

* Corresponding author. Tel.: +1 819 564 5413; fax: +1 819 564 5400.
E-mail address: Richard.Leduc@USherbrooke.ca (R. Leduc).

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binding to a cysteine-substituted mutant is unchanged compared to wild-type receptor, it is assumed that the structure of the mutant receptor, especially around the binding site, is similar to that of the wild-type receptor and that the substituted cysteine lies in a orientation similar to that of the residue of the wild-type receptor. In TMDs, the sulfhydryls of cysteines oriented toward the aqueous binding-site pocket should react more quickly with charged sulfhydryl reagents like methanethiosulfonate-ethylammonium (MTSEA) and methanethiosulfonate-ethylsulfonate (MTSES) than the sulfhydryls of cysteines that face the interior of the protein or the lipid bilayer. Two criteria are used to determine whether engineered cysteines are positioned at the surface of the binding-site pocket: (i) the reaction with the MTS reagent alters binding irreversibly and (ii) the reaction is retarded by the presence of the ligand. This approach has been used by us and others to identify residues that line the surface of GPCR binding-site pockets [3,17,20,24,25,36]. Here, we report the application of SCAM to probe TMD6 and TMD7 of the rat UT receptor.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) and bacitracin were from Sigma–Aldrich (Oakville, ON, Canada). FUGENE®-6 was from Roche Molecular Biochemicals (Mannheim, Germany). The sulfhydryl-specific alkylating reagents MTSEA (CH_3SO_2-$SCH_2CH_2NH_3^+$) and MTSES (CH_3SO_2-$SCH_2CH_2SO_3^-$) were from Toronto Research Chemicals (Toronto, ON, Canada). The cDNA clone of the rUT receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Brian O'Dowd (Department of Pharmacology, University of Toronto, Toronto, ON, Canada). DMEM (Dulbecco's modified Eagle's media), FBS (fetal bovine serum), and penicillin/streptomycin were from Gibco Life Technologies (Gaithersburg, MD, USA). Oligonucleotide primers were from IDT (Coralville, IA, USA). Human U-II was from Phoenix Pharmaceuticals (Belmont, CA, USA). ^{125}I-U-II (specific activity 1000 Ci/mmol) was prepared using IODO-GEN® (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril; Pierce Chemical Co.,) as described by Fraker and Speck [16]. Briefly, 10 μl of a 1 mM peptide solution was incubated with 20 μg of IODO-GEN®, 80 μl of 100 mM borate buffer (pH 8.5), and 1 mCi of $Na^{125}I$ for 30 min at room temperature, and was then purified by HPLC on a C-18 column. The specific radioactivity of the labeled peptide was determined by self-displacement and saturation-binding analysis.

2.2. PCR mutagenesis

Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Expand High Fidelity PCR System; Roche Diagnostics) using rUT inserted into pcDNA3 (Invitrogen, Burlington, ON, Canada) as a template. A set of forward and reverse oligonucleotides were constructed to introduce cysteine mutations between R256$^{(6.32)}$ and Q283$^{(6.59)}$ for TMD6 and between A295$^{(7.31)}$ and Q283$^{(7.57)}$ for TMD7. PCR products were subcloned using KpnI and XbaI sites of pcDNA3 after digestion by the same restriction enzymes and the mutations were confirmed by nucleotide sequencing.

2.3. Cell culture and transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C. Semi-confluent cells (70%) in 100-mm-diameter Petri dishes were transfected using FUGENE®-6 as described by the manufacturer. Transfected cells were grown for 48 h before using them for the binding and SCAM assays.

2.4. Binding experiments

COS-7 cells were washed once with PBS and subjected to one freeze-thaw cycle. Broken cells were gently scraped into washing buffer (20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$), centrifuged at 2500 × *g* for 15 min at 4 °C, and resuspended in binding buffer (20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.1% BSA, 0.01% bacitracin). Saturation binding experiments were performed by incubating broken cells (20–40 μg of protein) for 1 h at room temperature with increasing concentrations of ^{125}I-U-II (0.15–20 nM) in a final volume of 500 μl. Non-specific binding was determined in the presence of 1 μM unlabeled U-II. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for at least 1 h in binding buffer. Receptor-bound radioactivity was evaluated by γ-radiation counting. Results are presented as means ± SD. Binding data (B_{max} and K_d) were analyzed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) using a one-site binding hyperbola nonlinear regression analysis.

2.5. Treatment with MTS reagents

MTS treatments were performed according to the procedure of Javitch et al. [21], with minor modifications. Two days after transfection, the cells, which were grown in 12-well plates, were washed with PBS and incubated for 3 min at room temperature with freshly prepared MTSEA or MTSES at the desired concentrations (typically 0.5–6 mM) in a final volume of 200 μl. The reaction was stopped by washing the cells with ice-cold PBS. Intact cells were then incubated in binding medium (DMEM, 25 mM HEPES, pH 7.4, 0.1% BSA) containing 0.05 nM ^{125}I-U-II for 120 min at room temperature. After washing with ice-cold PBS, cells were lysed with 0.1N NaOH and the radioactivity was evaluated by γ counting. The percentage of fractional binding inhibition was calculated as [1 − (specific binding after MTS-X treatment/specific binding without treatment)] × 100.

2.6. Protection against MTS reagents by U-II

Transfected cells grown in 12-well plates were washed once with PBS and incubated in the presence or absence of 100 nM U-II for 1 h at 16 °C (to avoid internalization of receptors). Cells were washed to remove excess ligand and were treated with the MTS reagent. Cells were washed three times with ice-cold PBS and once with an acidic buffer (150 mM NaCl, 50 mM acetic acid, pH 3.0) to dissociate bound ligand. They were then incubated for 3 h at 16 °C in binding medium (DMEM, 25 mM HEPES, pH 7.4, 0.1% BSA) containing 0.05 nM ^{125}I-U-II. The percentage of protection was calculated as [(inhibition in the absence of U-II) − (inhibition in the presence of U-II)/(inhibition in the absence of U-II)] × 100.

2.7. Molecular modeling

All calculations were performed on a Silicon Graphics Octane2 workstation (Silicon Graphics Ins. Mountain View, CA, USA). The U-II and rUT receptor models were built using Insight II modules (Biopolymer, Homology, Discover; Accelrys, San Diego, CA, USA). The molecular model of U-II was constructed in a linear form using the Biopolymer module from Insight II. Subsequently, a disulfide bond was added between the residues in positions 5 and 10 of the ligand. The potential energy of the peptide was minimized for 500 steps with the steepest descents and a consistent valence force