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Identification of transmembrane domain 3, 4 & 5 residues that contribute to the formation of the ligand-binding pocket of the urotensin-II receptor

Xavier Sainsily, Jérôme Cabana, Philip E. Boulais, Brian J. Holleran, Emanuel Escher, Pierre Lavigne, Richard Leduc *

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

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ABSTRACT

Urotensin-II (UII), a cyclic undecapeptide, selectively binds the urotensin-II receptor (UT receptor), a G protein-coupled receptor (GPCR) involved in cardiovascular effects and associated with numerous pathophysiological conditions including hypertension, atherosclerosis, heart failure, pulmonary hypertension and others. In order to identify specific residues in transmembrane domains (TM) three (TM3), four (TM4) and five (TM5) that are involved in the formation of the UT receptor binding pocket, we used the substituted-cysteine accessibility method (SCAM). Each residue in the F118$^{(3.20)}$ to S146$^{(3.48)}$ fragment of TM3, the L168$^{(4.44)}$ to G194$^{(4.70)}$ fragment of TM4 and the W203$^{(5.30)}$ to V232$^{(5.59)}$ fragment of TM5, was mutated, individually, to a cysteine. The resulting mutants were then expressed in COS-7 cells and subsequently treated with the positively charged sulfhydryl-specific alkylating agent methanethiosulfonate-ethylammonium (MTSEA). MTSEA treatment resulted in a significant reduction in the binding of ^{125}I-UII to TM3 mutants L126C$^{(3.28)}$, F127C$^{(3.29)}$, F131C$^{(3.33)}$ and M134C$^{(3.36)}$ and TM4 mutants M184C$^{(4.60)}$ and I188C$^{(4.64)}$. No loss of binding was detected following treatment by MTSEA for all TM5 mutants tested. In absence of a crystal structure of UT receptor, these results identify key determinants in TM3, TM4 and TM5 that participate in the formation of the UT receptor binding pocket and has led us to propose a homology model of the UT receptor.

1. Introduction

Urotensin-II (Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val, UII) is a cyclic undecapeptide described as the most potent vasoconstrictor identified in mammals, firstly isolated from the caudal neurosecretery system of the Teleost fish [1]. UII signals through the urotensin-II receptor (UT receptor) detected in the central nervous system and widely expressed in human tissues, including the left atrium and ventricle of the heart, smooth muscle cells of the coronary artery and aorta, as well as endothelial cells from several vascular beds [2]. The UII/UT receptor system is considered as a pharmacological target in the pathophysiology of hypertension, heart failure, and cardiac fibrosis and hypertrophy [3,4]. 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 (TM), a D/ERY motif in the second intracellular loop, a CW/FxP 'toggle switch' motif [5] in TM6, a NPxxY motif in TM7, and potential serine/threonine phosphorylation sites in the cytoplasmic tail are found in the UT receptor [6].

The molecular mechanisms by which agonists bind to and activate GPCRs through conformational changes are not completely understood. Although for many years, the only available structural model of a GPCR was rhodopsin [7], recently the three-dimensional structures of other GPCRs such as the β adrenergic receptors [8,9], adenosine A2A receptor [10], chemokine CXCR4 receptor [11], and opioid receptors [12–14] have been determined. These studies have enabled a better understanding of how diffusible ligands can recognize and interact with residues of the binding pocket of GPCRs.

Despite these major advances, many questions remain regarding the subtle variations found in different GPCR binding pockets. Hence, a variety of biophysical and biochemical approaches are needed to identify those key determinants that make-up the binding cavity. The substituted-cysteine accessibility method

* Corresponding author.
E-mail address: richard.leduc@usherbrooke.ca (R. Leduc).

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(SCAM) [15–17] is an ingenious approach for systematically identifying TM residues that contribute to the formation of the binding-site pocket of GPCRs. In this approach, consecutive residues within TMs are mutated to cysteine, one at a time and the mutant receptors are expressed in heterologous cells. If ligand 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 an orientation similar to that of the residue of the wild-type receptor. In TMs, the sulfhydryls of cysteines oriented towards the aqueous binding-site pocket should react more quickly with charged sulfhydryl reagents like methanethiosulfonate-ethylammonium (MTSEA) 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 MTSEA 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 [18–24]. Indeed, using SCAM analysis, we have previously identified five MTSEA-sensitive residues in TM6 and TM7 of the rat UT receptor (rUT receptor) [23]. In this study, we report the application of SCAM to probe TM3, TM4 and TM5 of the rUT receptor.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) and bacitracin were from Sigma–Aldrich (Oakville, ON, Canada). X-tremeGENE HP transfection reagent was from Roche Applied Science (Indianapolis, IN, USA). The sulfhydryl-specific alkylating reagent MTSEA (CH_3SO_2–$SCH_2CH_2NH_3^+$) was from Toronto Research Chemicals (Toronto, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), phosphate-buffered saline (PBS), and penicillin/streptomycin were from Wisent Bioproduct (St-Bruno, QC, Canada). Oligonucleotide primers were from IDT (Coralville, IA, USA). Human UII was from Phoenix Pharmaceuticals (Belmont, CA, USA). ^{125}I-UII (specific activity 1000 Ci/mmol) was prepared using IODO-GEN (1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril; Thermo Scientific Pierce, Nepean, ON, Canada) as described by Fraker and Speck [25]. 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 reversed-phase HPLC on a C-18 column. The specific radioactivity of the labeled peptide was determined by self-displacement and saturation-binding analysis.

2.2. Numbering of residues

Residues in TM3, TM4 and TM5 of the rUT receptor were given two numbering schemes. First, residues were numbered according to their positions in the rUT receptor sequence. Second, residues were also indexed according to their position relative to the most conserved residue in each TM in which they are located [26]. By definition, the most conserved residue was assigned the position index "50" e.g. in TM5, P223 is the most conserved residue and was designated P223$^{(5.50)}$, whereas the upstream residue was designated G222$^{(4.49)}$ and the downstream residue G224$^{(4.51)}$.

2.3. PCR mutagenesis

Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuickChange Lightning Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA) using rUT receptor subcloned in the mammalian expression vector pcDNA3.1 as a template. A set of forward and reverse oligonucleotides were synthesized to introduce cysteine mutations between F118$^{(3.20)}$ and S146$^{(3.48)}$ for TM3, between L168$^{(4.44)}$ and G194$^{(4.70)}$ for TM4 and between W203$^{(5.30)}$ and V232$^{(5.59)}$ for TM5, and the mutations were confirmed by nucleotide sequencing.

2.4. Cell culture

COS-7 cells were grown in 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 X-tremeGENE HP as described by the manufacturer. Transfected cells were used 24 h post-transfection for binding and 48 h post-transfection for SCAM assays.

2.5. 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 (4-10 μg of protein) for 1 h at room temperature with increasing concentrations of ^{125}I-UII (0.15–20 nM) in a final volume of 200 μl. Non-specific binding was determined in the presence of 1 μM unlabeled UII. Bound radioactivity separated from free ligand by filtration through glass fiber filter plates (Millipore, MA, Billerica,) and washed 3 times with 200 μL of ice-cold washing 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 6 for Windows (GraphPad Software, San Diego, CA, USA) using a one-site binding hyperbola nonlinear regression analysis.

2.6. Enzyme-linked immunosorbent assay

COS-7 cells were seeded in 24-well plates at 30,000 cells/well. 48 h post-transfection, cells were fixed in 3.7% (v/v) formaldehyde/Tris-buffered saline (TBS) (20 mM Tris–HCl pH 7.5 and 150 mM NaCl) for 5 min at room temperature. Cells were then washed twice with TBS and incubated 30 min with TBS containing 1% BSA at room temperature to block non-specific binding. A mouse monoclonal anti-c-Myc (clone 9E10, Roche Applied Science, Indianapolis, IN, USA) was added at a dilution of 1:5000 in TBS-BSA 1% for 60 min. Following the incubation with the primary antibody, cells were washed twice with TBS and anti-mouse IgG-peroxydase antibody (Sigma–Aldrich, Oakville, ON, Canada) was then added at a dilution of 1:10000 in TBS-BSA 1% for 60 min. Following the incubation with the secondary antibody, cells were washed twice with TBS and 250 μL of 3,3′,5,5′-tetramethylbenzidine (T0440, Sigma–Aldrich, Oakville, ON, Canada) was added. The plates were incubated at room temperature and the reaction was stopped using 250 μL of HCl 2 N. 200 μL of the colorimetric reaction was transferred to a 96-well plate and the absorbance was measured at 450 nm. Cells transfected with empty vector (pcDNA3.1) were used to determine background.

2.7. Treatment with MTSEA reagent

MTS treatments were performed according to the procedure of Javitch et al. [16], with minor modifications. Two days after