Photolabelling the urotensin II receptor reveals distinct agonist- and partial-agonist-binding sites

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The mechanism by which GPCRs (G-protein-coupled receptors) undergo activation is believed to involve conformational changes following agonist binding. We have used photoaffinity labelling to identify domains within GPCRs that make contact with various photoreactive ligands in order to better understand the activation mechanism. Here, a series of four agonist {[Bpa¹]U-II (Bpa is *p*-benzoyl-L-phenylalanine), [Bpa²]U-II, [Bpa³]U-II and [Bpa⁴]U-II} and three partial agonist {[Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II (Pen is penicillamine), [Bpa²Pen⁵D-Trp⁷Orn⁸]U-II and [Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II} photoreactive urotensin II (U-II) analogues were used to identify ligand-binding sites on the UT receptor (U-II receptor). All peptides bound the UT receptor expressed in COS-7 cells with high affinity (K_d of 0.3–17.7 nM). Proteolytic mapping and mutational analysis led to the identification of Met²⁸⁸ of the third extracellular loop of the UT receptor as a binding site for all four agonist peptides. Both partial agonists containing

the photoreactive group in positions 1 and 2 also cross-linked to Met²⁸⁸. We found that photolabelling with the partial agonist containing the photoreactive group in position 6 led to the detection of transmembrane domain 5 as a binding site for that ligand. Interestingly, this differs from Met¹⁸⁴/Met¹⁸⁵ of the fourth transmembrane domain that had been identified previously as a contact site for the full agonist [Bpa⁶]U-II. These results enable us to better map the binding pocket of the UT receptor. Moreover, the data also suggest that, although structurally related agonists or partial agonists may dock in the same general binding pocket, conformational changes induced by various states of activation may result in slight differences in spatial proximity within the cyclic portion of U-II analogues.

Key words: G-protein-coupled receptor (GPCR), ligand binding, photolabelling, urantide, urotensin II.

INTRODUCTION

U-II (urotensin II, Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val) is a cyclic undecapeptide (the underlined residues represent the cyclic portion of the peptide) with activity on the cardiovascular and nervous system [1]. U-II was originally isolated from the urophysis of teleost fish [2], but the cDNA encoding its precursor has now been identified in many species [3,4]. *In situ* hybridization and immunohistochemistry have revealed that the peptide is localized to the medulla oblongata of the brain and the spinal cord in the central nervous system, but it is also present in tissues such as adrenal glands, kidney and spleen, suggesting neurohormonal or hormonal properties [3,5]. Although it is clear that U-II influences the physiological regulation of mammalian cardiovascular function, early reports showed conflicting results with regard to the peptide's vasoactive responses, which depend on the species or tissues used [1], whereas other studies described that administration of human U-II leads to potent vasodilator and cardiostimulant responses in human tissues [6,7]. More recently, a second peptide possessing structural similarity to U-II, while having a markedly different expression pattern, has been identified and termed U-II-related peptide (URP) [8].

U-II is the endogenous ligand of the UT receptor, a member of family A or rhodopsin-like family of GPCRs (G-protein-coupled receptors) [9]. Many features associated with this family, such as a short N-terminus, a highly conserved residue in each transmembrane domain, the presence of the D/ERY motif in the second intracellular loop, the NPXXY motif within TM7 (transmembrane domain 7) as well as potential serine/threonine phosphorylation

sites in the cytoplasmic tail, are found in the UT receptor [10]. The UT receptor is detected in the central nervous system and is 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 [9]. Compelling evidence has implicated the U-II–UT receptor system in the pathophysiology of hypertension, heart failure and cardiac fibrosis and hypertrophy [1,11].

Elucidation of the molecular mechanisms by which an agonist binds to and activates a GPCR needs to be addressed using a variety of biophysical and biochemical approaches. We have sought to probe into the interactions of U-II with the UT receptor by covalently linking U-II to its receptor with a series of photoreactive analogues containing the photoactivatable amino acid Bpa (*p*-benzoyl-L-phenylalanine). Bpa-based photoaffinity labelling [12] has been employed successfully to identify binding sites in several peptide receptors [13–15]. In the present study, this approach was employed in order to identify contact points using two series of analogous probes that either fully or weakly activate the UT receptor. We hypothesized that photoaffinity labelling with photoprobes having different intrinsic activities may yield different contact points. Such differences may reflect the distinct ensembles of conformations that can be stabilized by both ligands [16]. We report the specific interaction of the first four positions of the full agonist, U-II, within ECL (extracellular loop) 3 of the receptor, more specifically at Met²⁸⁸. The first two positions of a partial U-II agonist also interact with this amino acid. However, position 6 of the partial agonist photoprobe does not appear to interact with the same binding site compared with its analogous full agonist probe

Abbreviations used: Bpa, *p*-benzoyl-L-phenylalanine; DMEM, Dulbecco's modified Eagle's medium; ECL, extracellular loop; Endo, endoproteinase; FBS, foetal bovine serum; GPCR, G-protein-coupled receptor; Pen, penicillamine; RMSD, root mean square deviation; TM, transmembrane domain; U-II, urotensin II; UT receptor, U-II receptor; hUT, human U-II receptor; rUT receptor, rat U-II receptor.

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[17]. The present study, coupled with our previous work [17], has allowed us to propose a model which defines for the first time the orientation of U-II within the rUT (rat UT) receptor binding pocket.

EXPERIMENTAL

Materials

BSA, bacitracin, soya-bean trypsin inhibitor and CNBr were from Sigma. Staphylococcal V8 protease (Endo Glu-C; EC 3.4.21.19), Endo Arg-C (endoproteinase Arg-C; EC 3.4.22.8), Endo Lys-C (endoproteinase Lys-C; EC 3.4.21.50), FuGENE[®]-6 and Complete[™] protease cocktail inhibitor were from Roche. The cDNA clone of the rUT receptor subcloned in the mammalian expression vector pcDNA3.0 was kindly provided by Dr Brian O'Dowd (Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada). DMEM (Dulbecco's modified Eagle's medium), FBS (foetal bovine serum), penicillin/streptomycin and oligonucleotide primers were obtained from Gibco Life Technologies. Human U-II was from Phoenix Pharmaceuticals. Bpa-containing U-II peptide analogues were synthesized in our laboratories using Fmoc (fluoren-9-ylmethoxycarbonyl)-protected amino acids and Wang resin (a *p*-benzyloxybenzyl alcohol resin from Novabiochem). Cysteine residues were protected by acetamidomethyl groups, which were removed by using mercury acetate. Disulfide bond formation was achieved by oxidation with DMSO. The synthetic peptides were purified to homogeneity by reverse-phase HPLC. ¹²⁵I-U-II, ¹²⁵I-[Bpa¹]U-II, ¹²⁵I-[Bpa²]U-II, ¹²⁵I-[Bpa³]U-II, ¹²⁵I-[Bpa⁴]U-II, ¹²⁵I-[Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II (Pen is penicillamine), ¹²⁵I-[Bpa²Pen⁵D-Trp⁷Orn⁸]U-II and ¹²⁵I-[Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II (specific activity 400 Ci/mmol) were prepared with Iodo-Gen[®] (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril; Pierce Chemical Co.) as described by Fraker and Speck [18]. Briefly, 10 μ l of a 1 mM peptide solution was incubated with 20 μ g of Iodo-Gen[®], 80 μ l of 100 mM borate buffer, pH 8.5, and 1 mCi of Na¹²⁵I for 30 min at room temperature (25 °C) before being purified by HPLC on a C₁₈ column. The specific radioactivity of the labelled peptides was determined by self-displacement and saturation-binding analysis.

PCR mutagenesis

Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Expand High Fidelity PCR System; Roche Diagnostics) using the rUT receptor inserted into pcDNA3.0 (Invitrogen) as a template. A set of forward and reverse oligonucleotides were constructed in order to introduce a Met $\rightarrow$ Leu mutation at position 288 of the rUT receptor to generate M288L-rUT. PCR products were re-inserted in pcDNA3.0, and mutagenesis was confirmed by nucleotide sequencing.

Cell culture and transfection

COS-7 cells were grown in DMEM containing 10% (v/v) FBS, 100 i.u./ml penicillin and 100 μ g/ml 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 photoaffinity labelling and binding assays.

Binding experiments

COS-7 cells were washed once with PBS and subjected to one freeze-thaw cycle. Broken cells were then gently scraped into

washing buffer (20 mM Tris/HCl, pH 7.4, and 5 mM MgCl₂), centrifuged at 2500 *g* for 15 min at 4 °C and resuspended in binding buffer (20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 0.1% BSA and 0.01% bacitracin). For competition binding assays, broken cells (50–75 μ g of protein) were incubated for 1 h at room temperature in binding buffer with 0.1 nM ¹²⁵I-U-II (400 Ci/mmol) as tracer and increasing concentrations of U-II peptide analogues in a final volume of 0.5 ml. Bound radioactivity was separated from free ligand by filtration through GF/C filters pre-soaked for at least 1 h in binding buffer. Receptor-bound radioactivity was evaluated by γ -radiation counting.

Measurement of agonist-induced production of inositol phosphates

Accumulation of inositol phosphates was determined essentially as described in [19], with the following modification. Production of inositol phosphates was induced with 100 nM U-II or each analogue for 10 min at 37 °C in buffer containing 10 mM LiCl. Inositol phosphates were sequentially eluted from the anion-exchange column by the addition of an ammonium formate/formic acid solution of increasing ionic strength. Fractions containing inositol phosphates were collected and counted for radioactivity in a liquid-scintillation counter.

Photoaffinity labelling

Photoaffinity labelling was performed as described previously [13]. Transfected COS-7 cells were incubated for 1 h at room temperature in 0.5 ml of binding buffer containing the photoreactive radioligand (5 nM), in the presence (10 μ M) or absence of U-II. Cells were then washed, irradiated for 1 h at 0 °C under filtered UV light in washing buffer and centrifuged at 6000 *g* for 15 min at 4 °C. The pellet was solubilized on ice for 30 min in modified radioimmunoprecipitation buffer [m-RIPA; 50 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5 mM sodium azide, 1% (v/v) Nonidet P40, 0.25% (v/v) deoxycholic acid and 0.1% (w/v) SDS] containing Complete[™] protease inhibitor cocktail. After centrifugation at 15000 *g* for 30 min at 4 °C, the supernatants were kept at –80 °C until further analysis.

Partial purification of the labelled complex

Photolabelled receptors were separated by SDS/PAGE using 10% (w/v) acrylamide/Tris/glycine gels, before being exposed to BioMax[®] MS films (Kodak). Bands corresponding to receptor–ligand complexes were excised from the gels and submitted to electroelution. Eluted proteins were then concentrated and kept at –20 °C.

Proteolytic and chemical digestion

For V8 protease digestions, partially purified photolabelled receptors (5000 c.p.m.) were resuspended in 50 μ l of digestion buffer (100 mM ammonium bicarbonate buffer, pH 8.0) at room temperature for the indicated times. Under these conditions, V8 protease cleaves at the C-terminal side of glutamate residues. When subsequent digestions were needed, products of the first digestion were run on a 16.5% (w/v) acrylamide/Tris/Tricine gel, and the corresponding band was electroeluted. Eluted proteins were concentrated and submitted to subsequent digestion. For Endo Arg-C digestion, partially purified photolabelled receptors (5000 c.p.m.) were resuspended in 100 μ l of digestion buffer (100 mM Tris/HCl, pH 7.6, 100 mM CaCl₂, 0.5 mM EDTA and 0.01% SDS), and samples were incubated with Arg-C (0.5 μ g) for 16 h at 37 °C. For Endo Lys-C digestion, partially purified

photolabelled receptors (5000 c.p.m.) were resuspended in 50 μ l of digestion buffer (25 mM Tris/HCl, pH 8.5, 1 mM EDTA and 0.1 % SDS), and samples were incubated with Lys-C (0.5 μ g) for 16 h at 25 °C. For CNBr hydrolysis, partially purified photolabelled receptors (5000 c.p.m.) were resuspended in a 1:1 mixture of 10 % trifluoroacetic acid and CNBr (100 mg/ml) and incubated at room temperature in the dark for 18 h. Reactions were terminated by adding 0.6 ml of water and the material was freeze-dried.

Analysis of the products of proteolysis and chemical cleavage

The products of proteolysis and chemical cleavage were analysed by SDS/PAGE using 16.5 % (w/v) acrylamide/Tris/Tricine gels, followed by autoradiography on X-ray films (BioMax[®] MS) with intensifying screens. ¹⁴C-Labelled low-molecular-mass protein standards (Amersham Biosciences) were used to determine apparent molecular masses.

Molecular modelling

All calculations were performed on a Silicon Graphics Octane2 workstation. The U-II-derived ligands and the rUT receptor model were built using Insight II modules (Biopolymer, Homology, Discover; Accelrys). The molecular models of the agonist (U-II, [Bpa¹]U-II, [Bpa²]U-II, [Bpa³]U-II, [Bpa⁴]U-II and [Bpa⁶]U-II) and the partial-agonist peptides ([Pen⁵D-Trp⁷Orn⁸]U-II, [Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II, [Bpa²Pen⁵D-Trp⁷Orn⁸]U-II and [Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II) were constructed in a linear form using the Biopolymer module from InsightII. Subsequently, a disulfide bond was added between residues in position 5 and 10 of each ligand. The potential energy of each peptide was minimized for 500 steps with steepest descents with a consistent valence force field [20] using the Discover module. All the minimized structures adopted the overall conformation reported previously for U-II [21]. A distance-dependent dielectric constant of 4 was used.

The theoretical structure of the rUT receptor (GenBank[®] accession number P49684) was generated by homology modelling based on the crystal structure of bovine rhodopsin (PDB code 1L9H, chain A) [22], as described previously [23]. The resulting structure represents an inactive form of the rUT receptor with an overall configuration very similar to that of bovine rhodopsin [2.35 Å (1 Å = 0.1 nm) RMSD (root mean square deviation) between the backbone atoms of the transmembrane domains].

In order to allow the incorporation of the ligand within the receptor's orthosteric cleft, a cavity had to be generated. This was accomplished by rotation of the Φ angle of Thr⁴⁰ from -95.69° to -23.89° (displaces the N-terminal segment outwards from the helix bundle) followed by rotation of the Φ angle of Lys²⁴⁷ from -133.86° to -126.66° in order to displace TM6 and TM7. Rearrangement of TM6 and TM7 following receptor activation is supported by experimental evidence [24–26]. The bonds between S–S from Cys¹²³–Cys¹⁹⁹, C–N from Leu¹⁸⁶–Ala¹⁸⁷ and C–N from Ala²⁰²–Trp²⁰³ were then broken, and the Φ angle of Cys¹⁹⁹ was rotated from -86.83° to 136.93°, the Φ angle of Ser¹⁹⁷ from 49.67° to 92.07°, the Ψ angle of Lys¹⁹⁶ from -125.35° to -146.06°, and the Ψ angle of Arg¹⁹³ from -25.76° to -49.76°. The broken bonds were then reformed, and a potential energy minimization was performed with transmembrane domains fixed until the maximum derivative was less than 0.1 kcal/mol (1 kcal $\approx$ 4.184 kJ). The resulting structure gives an RMSD value of 0.36 Å for TM1–TM5 backbone atoms compared with the initial model and a RMSD value of 1.96 Å for all transmembrane backbone atoms.

The modelled U-II structure was placed in between the transmembrane domains of the rUT receptor, as suggested by photo-

labelling results obtained with the agonist ligands. Information about ligand–receptor interactions obtained in previous photoaffinity labelling and mutagenesis experiments [17,27] was considered along with the contact sites reported here, and was used as distance restraints for the energy minimization of the U-II–rUT receptor complex. A first minimization of the complex between U-II and the rUT receptor was performed using distance restraints ($2 \text{ Å} < d < 7 \text{ Å}$) between the S δ atoms of photolabelled methionine residues and the C γ atom of the corresponding side chains on U-II. Explicitly, Glu¹ was kept close to Met²⁸⁸, Phe⁶ was restrained to Met¹⁸⁴ and Met¹⁸⁵ [17], whereas Lys⁸ was restrained to Asp¹³⁰ [27]. The backbone atoms of the rUT receptor transmembrane domains were held in their position during this step; the ligand, extracellular and intracellular loops were free to move and interact. The U-II–rUT receptor complex was refined by several energy-minimization steps, until the maximum derivative was less than 0.1 kcal/mol. Molecular Graphics Figures were created using PyMol (Delano Scientific).

RESULTS

Binding specificity of photoreactive analogues

Figure 1(A) shows the primary structure of U-II and the photoreactive U-II analogues used in the present study. For the agonist peptides, Glu¹, Thr², Pro³ and Asp⁴ were successively replaced by Bpa to give [Bpa¹]U-II (1), [Bpa²]U-II (2), [Bpa³]U-II (3) and [Bpa⁴]U-II (4) respectively. We also synthesized peptides based on the structure of urantide. Although urantide was originally described as an antagonist [29], recent reports have demonstrated that this peptide acts as a weak partial agonist in various cells heterologously expressing the UT receptor [30]. Residues 1, 2 and 6 were successively replaced with Bpa in a 'humanized' version of the urantide peptide [Pen⁵D-Trp⁷Orn⁸]U-II (5) to give [Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II (6), [Bpa²Pen⁵D-Trp⁷Orn⁸]U-II (7) and [Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II (8). All peptides also contained a tyrosine residue, present in the native peptide at position 9, for radioiodination.

In competitive binding assays, all analogues exhibited high affinities for the UT receptor expressed in COS-7 cells (Table 1) with the exception of 8, which showed a slightly reduced affinity (17.7 nM). The functional properties of these analogues were then assessed by measuring the ligand-induced Ins(1,4,5) P_3 production in COS-7 cells expressing the rUT receptor (Table 1). Incubation of the cells with 1, 2, 3 or 4 caused an increase in Ins(1,4,5) P_3 comparable with the level produced by native U-II, suggesting the full agonist nature of these analogues. All four peptides based on urantide (5, 6, 7 and 8) elicited very weak Ins(1,4,5) P_3 release in this assay, indicating their weak partial agonistic nature. It was therefore assumed that the structure probed by these peptides could differ from the one derived from the full agonist-probed receptor structure.

Photoaffinity labelling of the rUT receptor

In the photoaffinity labelling experiments, all peptides labelled a protein migrating as a 60 kDa band on SDS/PAGE (Figure 2). This adduct has been observed previously when photolabelling with ¹²⁵I-[Bpa⁶]U-II [17] and corresponds to the intact glycosylated receptor. Incubation with 10 μ M U-II abolished the labelling, indicating the specificity of the interaction. In order to identify the covalently modified receptor region, the labelled species were partially purified and treated with the chemical agent CNBr or specific endoproteases, i.e. V8 protease, Endo Arg-C or Endo Lys-C.

Figure 1 (A) Amino acid sequence of human U-II and urantide-based photoreactive analogues, and (B) two-dimensional representation of the primary structure of the rUT receptor

(A) Sequences of U-II and synthetic photoprobes. The photoreactive Bpa residue is indicated in bold on the right, and Tyr⁹ is the radioiodine incorporation site. (B) Black closed circles indicate sites of hydrolysis for CNBr. Bold circles indicate recognition sites for V8 protease. Light-grey closed circles indicate recognition sites for Endo Arg-C. Dark-grey closed circles indicate recognition sites for Endo Lys-C. Putative sites of N-glycosylation on Asn²⁹ and Asn³³ are also indicated.

V8 protease digestion of the ¹²⁵I-[Bpa¹]U-II-rUT, ¹²⁵I-[Bpa²]U-II-rUT, ¹²⁵I-[Bpa³]U-II-rUT and ¹²⁵I-[Bpa⁴]U-II-rUT receptor complexes

In an initial attempt to map the ligand-receptor contact site for agonist peptide ligands, the 60 kDa forms of ¹²⁵I-1, ¹²⁵I-2, ¹²⁵I-3 and ¹²⁵I-4-photolabelled rUT receptor complexes were partially purified (Figure 3A, lane 1) and submitted to V8 protease digestion.

This enzyme cleaves on the C-terminal side of glutamate residues (Figure 1B) and the cleavage was carried out in a time-course experiment over a period of 162 h. This digestion produced a band of apparent molecular mass 17 kDa after 2 h for each photolabelled receptor complex (Figure 3A, lane 2). One receptor domain appearing as a potential candidate is fragment Leu²⁴-Glu¹⁴⁷ with a calculated molecular mass of 14.8 kDa glycosylated on Asn²⁹ and/or Asn³³ (Figure 3D). Other fragments corresponding to

Table 1 Pharmacological characterization of photolabile urotensin II derivatives

Affinities of compounds **1**, **2**, **3**, **4**, **5**, **6**, **7** and **8** were obtained by competition binding curves of ^{125}I -U-II (0.1 nM) displaced by U-II in cell membrane preparations of COS-7 cells transiently transfected with rUT receptor cDNA. Inositol phosphate production was measured following stimulation with 100 nM of either U-II or analogues for 15 min at 37 °C and as described in the Experimental section. Experiments were repeated three times, with similar results. The relative response is compared with that found using 100 nM U-II.

	K_i (nM)	Relative response (%)
U-II	2.8	—
[Bpa ¹]U-II	2.3	132
[Bpa ²]U-II	2.5	101
[Bpa ³]U-II	2.6	94
[Bpa ⁴]U-II	3.6	114
[Pen ⁵ D-Trp ⁷ Orn ⁸]U-II	4.7	10
[Bpa ¹ Pen ⁵ D-Trp ⁷ Orn ⁸]U-II	0.8	8
[Bpa ² Pen ⁵ D-Trp ⁷ Orn ⁸]U-II	0.3	7
[Pen ⁵ Bpa ⁶ D-Trp ⁷ Orn ⁸]U-II	17.7	12

Figure 2 Specific photoaffinity labelling of rUT receptor from transiently transfected COS-7 cells

(A) COS-7 cells expressing the rUT receptor were photolabelled with ^{125}I -**1**, ^{125}I -**2**, ^{125}I -**3** or ^{125}I -**4**. (B) COS-7 cells expressing the rUT receptor were photolabelled with ^{125}I -**6**, ^{125}I -**7** or ^{125}I -**8**. All complexes were solubilized, denatured and separated by SDS/PAGE on a 10% gel (40 µg of protein per lane) and terminated before exit of the migration front into the buffer. Gels were dried and subjected to autoradiography. Total labelling and labelling in the presence of 10 µM U-II are shown. Protein standards of the indicated molecular masses (in kDa) were run in parallel. These results are representative of at least three separate experiments.

Arg¹⁴⁸–Glu²⁸⁶ or the incompletely digested Arg¹⁴⁸–Glu²⁹³ with a calculated molecular mass of 17.5 kDa (non-glycosylated) are additional candidates. Further digestion of all four photolabelled rUT receptor complexes progressively yielded a final fragment of 3 kDa (Figure 3A, lane 6). Taking into account the molecular mass of the ligand and cleavage specificity, this small fragment can only correspond to receptor fragment Ala²⁸⁷–Glu²⁹³ of ECL3 for all four photoprobes (Figure 3D).

CNBr chemical cleavage of the ^{125}I -[Bpa¹]U-II-rUT, ^{125}I -[Bpa²]U-II-rUT, ^{125}I -[Bpa³]U-II-rUT and ^{125}I -[Bpa⁴]U-II-rUT receptor complexes

To further characterize the agonist–receptor contact site, the 60 kDa forms of ^{125}I -**1**-, ^{125}I -**2**-, ^{125}I -**3**- and ^{125}I -**4**-photolabelled rUT receptor complexes were partially purified (Figure 3B, lanes 1, 3, 5 and 7) and submitted to CNBr cleavage, which specifically cleaves at the C-terminal end of methionine residues (Figure 1B). Each agonist–receptor complex (Figure 3B, lanes 2, 4, 6 and 8) when submitted to CNBr digestion produced two bands migrating at apparent molecular masses of 22 and 12.5 kDa. Owing to the large size of the fragments obtained, the best candidates for attribution of these fragments are Leu¹⁸⁶–Met²⁸⁸ for the 12.5 kDa band and Leu¹⁸⁶–Met³⁷⁶ for the 22 kDa band, which corresponds to an incomplete digestion at Met²⁸⁸. In the case of ^{125}I -**1**- and ^{125}I -**4**-photolabelled rUT receptor complexes, an additional band of 2 kDa was also produced. The presence of this 2 kDa band is consistent with ϵ -labelling of Met²⁸⁸ of the rUT receptor and corresponds to the cleaved methylthiocyanate adduct of the photoligands. Moreover, the absence of ligand release in the CNBr-digestion pattern of the ^{125}I -**2**- and ^{125}I -**3**-photolabelled rUT receptor complexes, together with the results obtained by the V8 protease digestion, suggests that both of these photoprobes label on the γ -carbon of Met²⁸⁸.

To confirm further that the first four positions of the agonist U-II are in close proximity to Met²⁸⁸ of ECL3 of the rUT receptor, we examined the effect of replacing this residue with leucine by expressing the M288L-rUT receptor mutant. Photolabelling of M288L-rUT with ^{125}I -**1**-, ^{125}I -**2**-, ^{125}I -**3**- and ^{125}I -**4**- was performed and each complex was partially purified (Figure 3C, lanes 1, 3, 5 and 7) and submitted to CNBr chemical cleavage. For all four probes, CNBr cleavage of the photolabelled mutant complex yielded a 22 kDa band corresponding to the receptor fragment Leu¹⁸⁶–Met³⁷⁶ (Figure 3C, lanes 2, 4, 6 and 8). The ^{125}I -**1**- and ^{125}I -**4**-photolabelled M288L-rUT mutant receptor complexes failed to show any ligand release, in contrast with the wild-type receptor. Furthermore, V8 protease cleavage of the ligand–mutant complexes yielded a fragment of 3 kDa similar to that of wild-type receptor (results not shown), indicating that labelling on the mutant receptor still occurred on the Arg²⁸⁷–Glu²⁹³ domain. Taken together, our results strongly suggest that Met²⁸⁸ of ECL3 of the rUT receptor is a common contact point for all four N-terminal positions of U-II (Figure 3E).

CNBr chemical cleavage of the ^{125}I -[Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II-rUT and ^{125}I -[Bpa²Pen⁵D-Trp⁷Orn⁸]U-II-rUT complexes

In order to compare the binding mode of the full U-II agonists with that of the weak partial agonist [Pen⁵D-Trp⁷Orn⁸]U-II, photoprobes incorporating Bpa in positions 1, 2 and 6 were used to determine peptide–receptor contact sites. Initially, the peptides ^{125}I -**6** and ^{125}I -**7** containing Bpa in the first two positions were used. The 60 kDa forms of ^{125}I -**6**- and ^{125}I -**7**-photolabelled rUT receptor complexes were partially purified (Figure 4, lanes 1 and 5) and submitted to CNBr cleavage. Each ligand–receptor complex (Figure 4, lanes 2 and 6) following CNBr digestion produced two bands migrating at apparent molecular masses of 22 and 12.5 kDa, with the ^{125}I -**6**-photolabelled receptor complex additionally producing ligand release. This CNBr-digestion pattern is identical with that obtained with their full agonist counterparts ^{125}I -**1** and ^{125}I -**2**, indicating that labelling for both partial agonist photoprobes also occurred on Met²⁸⁸ of ECL3 of the rUT receptor (Figure 3E). To corroborate this result, photolabelling of M288L-rUT with ^{125}I -**6** and ^{125}I -**7**, was performed, both photolabelled receptor mutant

Figure 3 Proteolytic and chemical cleavage of ^{125}I -[Bpa¹]U-II, ^{125}I -[Bpa²]U-II, ^{125}I -[Bpa³]U-II and ^{125}I -[Bpa⁴]U-II photolabelled rUT receptor

(A) Partially purified 60 kDa form of ^{125}I -2-photolabelled rUT receptor (5000 c.p.m.) was incubated in the absence (lane 1) or the presence of V8 protease (5 μg). Digestions were carried out for the indicated times at room temperature. Samples were run on a 16.5% acrylamide Tris/Tricine separating gel followed by autoradiography. ^{14}C -Labelled protein standards of the indicated molecular masses (in kDa) were run in parallel. The same results were obtained for ^{125}I -1-, ^{125}I -3- and ^{125}I -4-photolabelled rUT receptor complexes. These results are representative of at least three separate experiments. (B and C) Partially purified 60 kDa forms of ^{125}I -1- (lanes 1 and 2), ^{125}I -2- (lanes 3 and 4), ^{125}I -3- (lanes 5 and 6) and ^{125}I -4- (lanes 7 and 8) photolabelled rUT receptor (B) or M288L-rUT (C) (5000 c.p.m.) were respectively incubated in the absence (lanes 1, 3, 5 and 7) or in the presence (lanes 2, 4, 6 and 8) of CNBr (50 mg/ml). All digestions were carried out for 18 h at room temperature in the dark. Samples were run on a 16.5% acrylamide Tris/Tricine separating gel followed by autoradiography. ^{14}C -Labelled protein standards of the indicated molecular masses (in kDa) were run in parallel. These results are representative of at least three separate experiments. (D and E) Serpentine representation of the theoretical V8 protease (D) and CNBr (E) fragmentation footprints for the rUT receptor with cleavage at all potential sites. The numbers in square brackets indicate the molecular mass of the corresponding photoaffinity cross-linked fragment plus ligand. The putative contact domains, corresponding to the non-glycosylated receptor fragments 287–293 for V8 protease and 186–288 for CNBr, are highlighted as dark-grey shading. The photolabelled Met²⁸⁸ is indicated in (E) as a black closed circle.

complexes were partially purified (Figure 4, lanes 3 and 7) and submitted to CNBr chemical cleavage. Following this cleavage (Figure 4, lanes 4 and 8), ligand release for ^{125}I -6 was completely abolished. These results indicate that labelling of the N-terminal portion of both agonist and partial agonist U-II ligands occurs on Met²⁸⁸.

CNBr chemical cleavage of the ^{125}I -[Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II-rUT receptor complex

We had shown previously that the full agonist [Bpa⁶]U-II labelled Met¹⁸⁴/Met¹⁸⁵ of the fourth transmembrane domain of the rUT receptor [17]. The photoprobe [Pen⁵D-Trp⁷Orn⁸]U-II

Figure 4 CNBr cleavage of ^{125}I -[Pen⁵Bpa¹D-Trp⁷Orn⁸]U-II and ^{125}I -[Bpa²Pen⁵D-Trp⁷Orn⁸]U-II photolabelled rUT receptor

Partially purified 60 kDa forms of ^{125}I -6- and ^{125}I -7- photolabelled rUT receptor and M288L-rUT (5000 c.p.m.) were incubated in the absence (lanes 1, 3, 5 and 7) or the presence (lanes 2, 4, 6 and 8) of CNBr (50 mg/ml) for 18 h at room temperature in the dark. Samples were run on a 16.5% acrylamide Tris/Tricine separating gel followed by autoradiography. ^{14}C -Labelled protein standards of the indicated molecular masses (in kDa) were run in parallel. These results are representative of at least three separate experiments.

incorporating Bpa in the sixth position, a weak partial agonist, was used to assess whether labelling would also occur at Met¹⁸⁴/Met¹⁸⁵. First, the 60 kDa form of the ^{125}I -8-photolabelled receptor complex was partially purified and digested with V8 protease. The resulting 17 kDa fragment (Figure 5A, lane 1), which corresponds to Arg¹⁴⁸–Glu²⁸⁶ [17], was partially purified. Cleavage of this fragment with CNBr (Figure 5A, lane 2) led to the production of a 12.5 kDa fragment, which could be attributed to the fragment Met¹⁸⁵–Glu²⁸⁶ (Figure 5C). More importantly, the lack of any detected ligand release (Figure 5A, lane 2) indicates that ^{125}I -8 did not cross-link to Met¹⁸⁴/Met¹⁸⁵ as was observed for [Bpa⁶]U-II. To identify further the binding domain of ^{125}I -8, the 17 kDa V8 protease fragment (Figure 5B, lane 1) was submitted to Endo Lys-C digestion, which cleaves specifically at the C-terminal end of lysine residues (Figure 1B). Endo Lys-C digestion produced two bands migrating at apparent molecular masses of 10 and 7.5 kDa (Figure 5B, lane 2). Since the 10 kDa band is progressively cleaved to give the 7.5 kDa band after prolonged digestion times (results not shown), the former is believed to originate from partial enzyme cleavage. Taking into account the region delimited by the V8 protease digestion, the only candidate for the 7.5 kDa band is the fragment Ser¹⁹⁷–Lys²⁴⁷. This fragment encompasses part of ECL2, TM5 and part of intracellular loop 3 (Figure 5D). To narrow down the domain of interaction of ^{125}I -8 further, the 17 kDa V8 protease fragment was submitted to Endo Arg-C digestion, which specifically cleaves at the C-terminal end of arginine residues (Figure 1B). This digestion produced a single band migrating at a molecular mass of 4 kDa (Figure 5B). Taking into account the results obtained from the Lys-C digestion, the only possible candidate for this band is the fragment Thr²¹⁰–Arg²³³, therefore localizing the labelling of ^{125}I -8 to TM5 of the rUT receptor (Figure 5E).

DISCUSSION

Identifying the molecular determinants involved in the bimolecular recognition of ligand and receptor is of great importance in

order to understand the initial steps leading to receptor activation. In the present study, photoaffinity labelling was used as a strategy to investigate the interactions of U-II peptides with the rUT receptor. This approach depends on the spatial proximity between radioactive photoligands and receptor domains to form a covalent conjugate that is subsequently mapped to identify the interaction sites. We hypothesized that the receptor conformation stabilized by a U-II agonist might differ from a conformation stabilized by a weak partial agonist and that these changes may be detected by photoaffinity labelling. Such an approach has recently been exploited for the cholecystokinin and secretin receptors [31,32].

To allow for the most optimal comparison between both N-terminal and C-terminal positions of U-II, a humanized version of urantide containing the first four residues of human U-II was designed. Four analogues of U-II containing Bpa in the variable N-terminal part of U-II at positions 1, 2, 3 and 4, as well as three humanized urantide analogues containing Bpa in both the variable (positions 1 and 2) and cyclic (position 6) were prepared. These peptides were characterized for their capacity to recognize and activate the UT receptor and the photolabelling patterns of agonist peptides were compared with those of the partial agonists. A recent report showed that N-terminal deletions or substitutions of U-II had little or no effect on agonistic properties or binding affinities of the peptides [33], therefore we expected that Bpa substitution within this region should be permissible. Indeed, all U-II photoligands with Bpa in positions 1, 2, 3 and 4 demonstrated full agonist properties with high affinity for the UT receptor. Replacing positions 1, 2 and 6 in humanized urantide ([Pen⁵D-Trp⁷Orn⁸]U-II) with Bpa led to the production of partial agonists, as demonstrated by their capacity at producing low levels of Ins(1,4,5) P_3 , which is in line with the reported partial agonist nature of urantide [30]. It is noteworthy to mention, however, that studies have demonstrated that urantide is a partial agonist towards heterologously expressed hUT receptor (human UT receptor), but an antagonist towards rat aorta preparations [35], thereby suggesting that differences probably arise because of the cellular or tissue preparation used. Also, previous studies from our group indicated that substituting Bpa at position 6 in the cyclic portion of U-II analogues did not greatly affect its affinity [17]. Introduction of Bpa at position 6 in the humanized urantide peptide lowered its affinity for the UT receptor somewhat when compared with humanized urantide itself (5). Nevertheless, on the basis of their pharmacological properties (Table 1) and their complete displacement by the parent U-II peptide in the photolabelling experiments, it is reasonable to assume that each analogue, agonist or partial agonist, would adopt a orthosteric binding mode similar to that taken by its parent peptide.

Previously, it has been shown that position 6 of U-II photolabelled Met¹⁸⁴/Met¹⁸⁵ of the rUT receptor and that a U-II analogue containing Bpa in position 7 failed to bind the receptor [17]. Analogues containing Bpa in position 8 and 9 of U-II also failed to bind the rUT receptor (results not shown). This is not surprising in the light of structure–activity studies on U-II [33]. Our present results from enzymatic and chemical degradation of the photoligand–receptor complexes incorporating Bpa in an N-terminal position for both full and partial agonists have identified Met²⁸⁸ of ECL3 as the sole contact point. It could be reasoned that the N-terminal part of U-II analogues can interact weakly with the extracellular loops of the UT receptor; this would explain why these residues are less important for affinity and receptor activation. It is noteworthy to mention that the rUT and hUT receptors share 90% sequence similarity. Interestingly, whereas most residues of ECL3 are conserved between the rUT and hUT receptor, the hUT receptor lacks Met²⁸⁸ in ECL3. Because it has been shown using surface plasmon resonance that ECL3 from the

Figure 5 Proteolytic and chemical cleavage of the partially purified V8-protease-generated fragment of ^{125}I -[Pen⁵Bpa⁶D-Trp⁷Orn⁸]U-II photolabelled rUT receptor

The partially purified 60 kDa form of ^{125}I -8-photolabelled rUT receptor and M184/185L-rUT (30 000 c.p.m.) were incubated in the presence of V8 protease (1 μg) for at least 7 days at 37°C. The resulting 17 kDa labelled receptor fragments was then excised from the gel, electroeluted and concentrated. (A) The partially purified 17 kDa fragment was incubated in the absence or presence of 50 mg/ml CNBr for 18 h at room temperature in the dark. (B) The partially purified 17 kDa fragments were incubated in the absence (lanes 1) or presence of Endo Lys-C (5 μg) (lane 2) or Endo Arg-C (5 μg) (lane 3) for 18 h at 37°C. Samples were run on a 16.5% acrylamide Tris/Tricine separating gel followed by autoradiography. ^{14}C -labelled protein standards of the indicated molecular masses (in kDa) were run in parallel. These results are representative of at least three separate experiments. (C, D and E) Serpentine representation of the idealized CNBr (C), Endo Lys-C (D) and Endo Arg-C (E) fragmentation footprints of the 17 kDa V8 protease fragment with cleavage at all potential sites. The numbers in square brackets indicate the molecular mass of the corresponding photoaffinity cross-linked fragment plus ligand. The putative contact domains, corresponding to the receptor fragments 197–247 for Endo Lys-C and 210–233 for Endo Arg-C, are highlighted as dark-grey shading.

human receptor specifically binds U-II [36], it is reasonable to hypothesize that the interaction mode of the N-terminus of U-II with either the rat or the human receptor will be similar.

Interaction of benzophenone residues with methionine residues of receptors has been widely reported [15,37]. This type of interaction appears to be favoured, since the free radical is stabilized as a charge-transfer complex by the presence of the thioether moiety found in methionine. Such a complex would drive the equilibrium towards the formation of covalent products with the adjacent methyl or methylene groups. A benzophenone-containing ligand could thus be attracted to methionine residues located in close

proximity to the binding site, but beyond the van der Waals radius, although, if no methionine is present, Bpa is still able to covalently link side chains of other residues [38–40]. Photolabelling of methionine residues can lead to two different labelling patterns: ϵ -labelling, which leads to ligand release following CNBr cleavage, and γ -labelling, which does not lead to ligand release [23]. This property can be exploited to refine the mapping studies. Our results show that CNBr cleavage gives rise to ligand release for the ^{125}I -1-, ^{125}I -4- and ^{125}I -6-photolabelled rUT receptor complexes, whereas ligand release is not observed in the case of photolabelling with ^{125}I -2-, ^{125}I -3 and ^{125}I -7. CNBr cleavage of these latter

Figure 6 Molecular models of U-II analogues and U-II-rUT receptor complexes

(A) The U-II ligand structures [Bpa¹Pen⁵D-Trp⁷Orn⁸]U-II (dark grey), [Bpa²Pen⁵D-Trp⁷Orn⁸]U-II (pink), [Bpa³]U-II (blue), [Bpa⁴]U-II (orange), [Bpa⁵]U-II (yellow) and [Bpa⁶]U-II (green) were superimposed. (B) Orientation of U-II in the rUT receptor binding pocket. The receptor backbone is shown as a light-grey ribbon. The ligand U-II backbone is shown as a pink ribbon, except for Glu¹-Asp⁴ (yellow), Phe⁶ (green) and Lys⁸ (blue), which interact with Met²⁸⁸, Met¹⁸⁴/Met¹⁸⁵ (all methionine residues are light orange) and Asp¹³⁰ (red) of the rUT receptor respectively. The inset highlights the interaction of Lys⁸ of U-II with Asp¹³⁰ of the receptor from another angle with TM1, TM6 and TM7 removed for clarity. Oxygen atoms are red, nitrogen atoms are blue, and sulfur atoms are orange for all molecules. All structures were modelled as described in the Experimental section. See also the animated Supplementary Figure 1 and Supplementary pdb file at <http://www.BiochemJ.org/bj/402/bj4020051add.htm>.

complexes generates only traces of the 12.5 kDa fragment and mostly the complete 186–375 fragment of 22 kDa; this is indicative of γ -labelling. Our results show that the CNBr reaction is particularly difficult to drive to completion within the Met²⁸⁸ region. This can be explained in two ways: first, it has been observed that peptide bonds containing proline residues are difficult to cleave (residue 289 is proline), and, secondly, as reported above, the close proximity of ligand attachment to Met²⁸⁸ could restrict the formation of CNBr reaction intermediates, thus preventing the reaction from proceeding optimally [41]. Such steric hindrance can be induced by label incorporation into the γ -methylene group of Met²⁸⁸

and would thus demonstrate that ¹²⁵I-2, ¹²⁵I-3 and ¹²⁵I-7 also interact with Met²⁸⁸. Exclusive γ -labelling of ¹²⁵I-2, ¹²⁵I-3 and ¹²⁵I-7 indicates that the Bpa moiety of these peptides is in closer proximity to the Met²⁸⁸ backbone than that of ¹²⁵I-1, ¹²⁵I-4 and ¹²⁵I-6 which shows ϵ -labelling. Molecular modelling, based on the reported U-II structure which conserved the type II' β -turn [21] (Figure 6A), suggests further that Bpa would indeed occupy distinct conformational spaces in the receptor's orthosteric binding pocket depending on the residue's position within either an agonist or partial agonist peptide. Although these models cannot be seen as experimental structures, they nonetheless offer a reasonable

structural framework to explain the position-dependence of the photolabelling of Met²⁸⁸.

It is generally accepted that binding of ligands with different potencies to a given receptor should lead to the stabilization of distinct receptor conformations [42]. We had found previously that photolabelling with a full agonist U-II peptide containing Bpa in position 6 led to the identification of Met¹⁸⁴/Met¹⁸⁵ of TM4 of the rUT receptor as a contact site. We now show that photolabelling with the weak partial agonist ¹²⁵I-8, which also incorporates Bpa in position 6, identified a different labelling site which was subsequently mapped to TM5. Thus these differences may reflect changes in the conformations adopted by the receptor during the activation process and more particularly the different relative orientations adopted by TM4 and TM5 within the receptor core. We propose that, during activation, TM4 may move closer to the receptor's orthosteric binding pocket and/or that TM5 may move away from the receptor's binding pocket, allowing labelling of TM4 by the agonist. At this point, it is not possible to determine the exact nature or extent of these movements. Our results support the notion that full agonist and partial agonist U-II peptides bind to the UT receptor differently [36] and hence may stabilize different rUT receptor conformations.

In order to assess the relative position of the full agonist peptide in the binding pocket of the rUT receptor, we sought to model the receptor in complex with the U-II peptide. To correctly position U-II within the receptor binding pocket, distance restraints needed to be defined. A first point used was the putative ionic interaction between Lys⁸ of U-II and Asp¹³⁰ of the rUT receptor (Figure 6B, inset). In support of this interaction, the corresponding Asp¹²² of the somatostatin receptor subtype 2, a member of the same GPCR subfamily as the UT receptor, was found to interact with Lys⁹ of somatostatin, a peptide analogous to U-II [43]. Furthermore, substitution of alanine for Asp¹³⁰ in the rUT receptor totally abolished receptor binding (results not shown), and structure-activity studies of U-II consistently show that Lys⁸ is critical to U-II binding and function [33]. Our photolabelling results were used to define a second interaction point: an area defined as a mean position between the C α of Glu¹-Asp⁴ of U-II with Met²⁸⁸ of the rUT receptor. A third anchoring point is position Bpa⁶ which is in close proximity to Met¹⁸⁴/Met¹⁸⁵ of TM4, which we had identified previously. Using these anchoring points, a U-II-rUT receptor complex was modelled (Figure 6B, see also animated Supplementary Figure 1 and Supplementary pdb file at <http://www.BiochemJ.org/bj/402/bj4020051add.htm>). Following minimization of the complex with distance restraints defined, the structure of docked U-II has a RMSD value of 1.4 Å compared with the initial U-II structure and the rUT receptor transmembrane domains have a RMSD value of 1.5 Å compared with the initial model. These values indicate that the structure of both ligand and receptor varied little during the docking process. In our model, the critical Trp⁷, Lys⁸ and Tyr⁹ residues which compose the pharmacophore of U-II [34] are well oriented within a pocket formed by the cytoplasmic side of the transmembrane domains of the rUT receptor, highlighting their importance for receptor binding and activation.

In conclusion, our results support the hypothesis that agonist or partial agonist ligands may stabilize different receptor conformations upon binding. For the N-terminal portion of U-II peptides, we show that binding determinants are present in ECL3 of the rUT receptor. We also show a possible contribution of TM4 and TM5 during receptor activation. Although most studies to date implicate either TM6 or TM3 movements during receptor activation [25,26], it is increasingly becoming recognized that TM5 may play a more important role during the GPCR activation process than appreciated previously [44]. Further studies combining muta-

genesis, SCAM (substituted cysteine accessibility method) and molecular modelling should provide further information crucial to the development of models defining the binding pocket, and perhaps the activation mechanism of this receptor. Because of the putative role of U-II in several pathophysiologicals, including cardiovascular disease, the design of novel ligands could be facilitated by the knowledge brought on by these models.

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REFERENCES

- Douglas, S. A. (2003) Human urotensin-II as a novel cardiovascular target: 'heart' of the matter or simply a fishy 'tail'? *Curr. Opin. Pharmacol.* **3**, 159–167.
- Pearson, D., Shively, J. E., Clark, B. R., Geschwind, I. I., Barkley, M., Nishioka, R. S. and Bern, H. A. (1980) Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5021–5024.
- Coulouarn, Y., Lihrmann, I., Jegou, S., Anouar, Y., Tostivint, H., Beauvillain, J. C., Conlon, J. M., Bern, H. A. and Vaudry, H. (1998) Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15803–15808.
- Coulouarn, Y., Jegou, S., Tostivint, H., Vaudry, H. and Lihrmann, I. (1999) Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett.* **457**, 28–32.
- Nothacker, H. P., Wang, Z., McNeill, A. M., Saito, Y., Merten, S., O'Dowd, B., Duckles, S. P. and Civelli, O. (1999) Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat. Cell Biol.* **1**, 383–385.
- Katano, Y., Ishihata, A., Aita, T., Ogaki, T. and Horie, T. (2000) Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors, on rat coronary arteries. *Eur. J. Pharmacol.* **402**, R5–R7.
- MacLean, M. R., Alexander, D., Stirrat, A., Gallagher, M., Douglas, S. A., Ohlstein, E. H., Morecroft, I. and Pollard, K. (2000) Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br. J. Pharmacol.* **130**, 201–204.
- Sugo, T., Murakami, Y., Shimomura, Y., Harada, M., Abe, M., Ishibashi, Y., Kitada, C., Miyajima, N., Suzuki, N., Mori, M. and Fujino, M. (2003) Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem. Biophys. Res. Commun.* **310**, 860–868.
- Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., Romanic, A. M., Loudon, C. S., Foley, J. J., Sauermeier, C. F., Coatney, R. W. et al. (1999) Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* **401**, 282–286.
- Proulx, C. D., Simaan, M., Escher, E., Laporte, S. A., Guillemette, G. and Leduc, R. (2005) Involvement of a cytoplasmic-tail serine cluster in urotensin II receptor internalization. *Biochem. J.* **385**, 115–123.
- Ong, K. L., Lam, K. S. and Cheung, B. M. (2005) Urotensin II: its function in health and its role in disease. *Cardiovasc. Drugs Ther.* **19**, 65–75.
- Dorman, G. and Prestwich, G. D. (2000) Using photolabile ligands in drug discovery and development. *Trends Biotechnol.* **18**, 64–77.
- Boucard, A. A., Wilkes, B. C., Laporte, S. A., Escher, E., Guillemette, G. and Leduc, R. (2000) Photolabeling identifies position 172 of the human AT₁ receptor as a ligand contact point: receptor-bound angiotensin II adopts an extended structure. *Biochemistry* **39**, 9662–9670.
- Coulie, B., Matsuura, B., Dong, M., Hadac, E. M., Pinon, D. I., Feighner, S. D., Howard, A. D. and Miller, L. J. (2001) Identification of peptide ligand-binding domains within the human motilin receptor using photoaffinity labeling. *J. Biol. Chem.* **276**, 35518–35522.
- Greenberg, Z., Bisello, A., Mierke, D. F., Rosenblatt, M. and Chorev, M. (2000) Mapping the bimolecular interface of the parathyroid hormone (PTH)-PTH1 receptor complex: spatial proximity between Lys²⁷ (of the hormone principal binding domain) and Leu²⁶¹ (of the first extracellular loop) of the human PTH1 receptor. *Biochemistry* **39**, 8142–8152.
- Kenakin, T. (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.* **24**, 346–354.

- 17 Boucard, A. A., Sauve, S. S., Guillemette, G., Escher, E. and Leduc, R. (2003) Photolabelling the rat urotensin II/GPR14 receptor identifies a ligand-binding site in the fourth transmembrane domain. *Biochem. J.* **370**, 829–838
- 18 Fraker, P. J. and Speck, Jr, J. C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril. *Biochem. Biophys. Res. Commun.* **80**, 849–857
- 19 Lanctot, P. M., Leclerc, P. C., Escher, E., Leduc, R. and Guillemette, G. (1999) Role of N-glycosylation in the expression and functional properties of human AT1 receptor. *Biochemistry* **38**, 8621–8627
- 20 Dauber-Osguthorpe, P., Roberts, V. A., Osguthorpe, D. J., Wolff, J., Genest, M. and Hagler, A. T. (1988) Structure and energetics of ligand binding to proteins: *Escherichia coli* dihydrofolate reductase–trimethoprim, a drug–receptor system. *Proteins* **4**, 31–47
- 21 Carotenuto, A., Grieco, P., Campiglia, P., Novellino, E. and Rovero, P. (2004) Unraveling the active conformation of urotensin II. *J. Med. Chem.* **47**, 1652–1661
- 22 Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E. et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289**, 739–745
- 23 Clement, M., Martin, S. S., Beaulieu, M. E., Chamberland, C., Lavigne, P., Leduc, R., Guillemette, G. and Escher, E. (2005) Determining the environment of the ligand binding pocket of the human angiotensin II type I (hAT1) receptor using the methionine proximity assay. *J. Biol. Chem.* **280**, 27121–27129
- 24 Boucard, A. A., Roy, M., Beaulieu, M. E., Lavigne, P., Escher, E., Guillemette, G. and Leduc, R. (2003) Constitutive activation of the angiotensin II type 1 receptor alters the spatial proximity of transmembrane 7 to the ligand-binding pocket. *J. Biol. Chem.* **278**, 36628–36636
- 25 Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L. and Khorana, H. G. (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science* **274**, 768–770
- 26 Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P. and Bourne, H. R. (1996) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature* **383**, 347–350
- 27 Onan, D., Hannan, R. D. and Thomas, W. G. (2004) Urotensin II: the old kid in town. *Trends Endocrinol. Metab.* **15**, 175–182
- 28 Reference deleted
- 29 Patacchini, R., Santicoli, P., Giuliani, S., Grieco, P., Novellino, E., Rovero, P. and Maggi, C. A. (2003) Urotensin II: an ultrapotent urotensin II antagonist peptide in the rat aorta. *Br. J. Pharmacol.* **140**, 1155–1158
- 30 Song, W., McDonald, J., Camarda, V., Calo, G., Guerrini, R., Marzola, E., Thompson, J. P., Rowbotham, D. J. and Lambert, D. G. (2006) Cell and tissue responses of a range of Urotensin II analogs at cloned and native urotensin II receptors: evidence for coupling promiscuity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **373**, 148–157
- 31 Dong, M., Hadac, E. M., Pinon, D. I. and Miller, L. J. (2005) Differential spatial approximation between cholecystokinin residue 30 and receptor residues in active and inactive conformations. *Mol. Pharmacol.* **67**, 1892–1900
- 32 Dong, M., Hosohata, K., Pinon, D. I., Muthukumaraswamy, N. and Miller, L. J. (2006) Differential spatial approximation between secretin and its receptor residues in active and inactive conformations demonstrated by photoaffinity labeling. *Mol. Endocrinol.* **20**, 1688–1698
- 33 Brkovic, A., Hattenberger, A., Kostenis, E., Klabunde, T., Flohr, S., Kurz, M., Bourgault, S. and Fournier, A. (2003) Functional and binding characterizations of urotensin II-related peptides in human and rat urotensin II–receptor assay. *J. Pharmacol. Exp. Ther.* **306**, 1200–1209
- 34 Grieco, P., Carotenuto, A., Campiglia, P., Marinelli, L., Lama, T., Patacchini, R., Santicoli, P., Maggi, C. A., Rovero, P. and Novellino, E. (2005) Urotensin-II receptor ligands: from agonist to antagonist activity. *J. Med. Chem.* **48**, 7290–7297
- 35 Camarda, V., Song, W., Marzola, E., Spagnol, M., Guerrini, R., Salvadori, S., Regoli, D., Thompson, J. P., Rowbotham, D. J., Behm, D. J. et al. (2004) Urotensin-II induced calcium release in cells expressing recombinant UT receptors. *Eur. J. Pharmacol.* **498**, 83–86
- 36 Boivin, S., Guilhaudis, L., Milazzo, I., Oulyadi, H., Davoust, D. and Fournier, A. (2006) Characterization of urotensin-II receptor structural domains involved in the recognition of U-II, URP, and urantide. *Biochemistry* **45**, 5993–6002
- 37 Mannstadt, M., Luck, M. D., Gardella, T. J. and Juppner, H. (1998) Evidence for a ligand interaction site at the amino-terminus of the parathyroid hormone (PTH)/PTH-related protein receptor from cross-linking and mutational studies. *J. Biol. Chem.* **273**, 16890–16896
- 38 Zang, M., Dong, M., Pinon, D. I., Ding, X. Q., Hadac, E. M., Li, Z., Lybrand, T. P. and Miller, L. J. (2003) Spatial approximation between a photolabile residue in position 13 of secretin and the amino terminus of the secretin receptor. *Mol. Pharmacol.* **63**, 993–1001
- 39 Perodin, J., Deraet, M., Auger-Messier, M., Boucard, A. A., Rihakova, L., Beaulieu, M. E., Lavigne, P., Parent, J. L., Guillemette, G., Leduc, R. and Escher, E. (2002) Residues 293 and 294 are ligand contact points of the human angiotensin type 1 receptor. *Biochemistry* **41**, 14348–14356
- 40 Tan, Y. V., Couvineau, A., Van Rampelbergh, J. and Laburthe, M. (2003) Photoaffinity labeling demonstrates physical contact between vasoactive intestinal peptide and the N-terminal ectodomain of the human VPAC1 receptor. *J. Biol. Chem.* **278**, 36531–36536
- 41 Sachon, E., Bolbach, G., Chassaing, G., Lavielle, S. and Sagan, S. (2002) C γ H2 of Met¹⁷⁴ side chain is the site of covalent attachment of a substance P analog photoactivable in position 5. *J. Biol. Chem.* **277**, 50409–50414
- 42 Gether, U. and Kobilka, B. K. (1998) G protein-coupled receptors. II. Mechanism of agonist activation. *J. Biol. Chem.* **273**, 17979–17982
- 43 Strnad, J. and Hadcock, J. R. (1995) Identification of a critical aspartate residue in transmembrane domain three necessary for the binding of somatostatin to the somatostatin receptor SSTR2. *Biochem. Biophys. Res. Commun.* **216**, 913–921
- 44 Schwartz, T. W., Frimurer, T. M., Holst, B., Rosenkilde, M. M. and Elling, C. E. (2006) Molecular mechanism of 7TM receptor activation: a global toggle switch model. *Annu. Rev. Pharmacol. Toxicol.* **46**, 481–519

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