

MOL #65151

Ligand supported purification of the Urotensin-II receptor

Ann T. Du¹, Döne Onan¹, Diem T. Dinh, Michael J. Lew, James Ziogas, Marie-Isabel Aguilar, Leonard K. Pattenden, Walter G. Thomas

Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia (A.T.D. and M.I.A.); Baker IDI Heart and Diabetes Institute, Melbourne, Australia (D.O. and D.T.D.); Department of Pharmacology, University of Melbourne, Melbourne, Australia (M.J.L. and J.Z.); Health Innovations Research Institute, RMIT University, Melbourne, Australia (L.K.P.); School of Biomedical Sciences, The University of Queensland, Queensland, Australia (W.G.T.).

MOL #65151

RUNNING TITLE PAGE

Running title: GPCR Ligand Supported Purification

Address Correspondence To:

Prof. Walter G. Thomas

The School of Biomedical Sciences

The University of Queensland

St Lucia, Queensland, 4072 Australia

Phone: +61-7-3365-4656

Fax: +61-7-3365-1040

Email: w.thomas@uq.edu.au

Manuscript Information:

Number of Text Pages: 22

Number of Figures: 8

Number of Tables: 0

Number of References: 40

Number of words in Abstract: 246

Number of words in Introduction: 747

Number of words in Discussion: 1454

Non-standard Abbreviations:

Urotensin-II, U-II; Urotensin-II receptor, UT; Angiotensin II, Ang II; Type 1A Angiotensin receptor,

AT_{1A}; G protein-coupled receptor, GPCR; *n*-dodecyl β -D-maltoside, DDM; Bio-U-II, biotinylated U-II.

MOL #65151

ABSTRACT

A crucial limitation for structural and biophysical analysis of G protein-coupled receptors (GPCRs) is the inherent challenge of purifying and stabilizing these receptors in an active (agonist-bound) conformation. Peptide ligands, such as the vasoactive, cyclic hormone Urotensin-II (U-II), may provide new purification tools, via high affinity, pseudo-irreversible binding suitable for ligand-based affinity purification. We show that the U-II receptor (UT) is resistant to desensitization due to low phosphorylation and diminished endocytosis. UT also displays an unusual proclivity to remain active with vasoconstriction sustained despite extensive washout of the ligand. To exploit these properties for ligand supported purification, we modified the U-II ligand by attaching a biotin moiety and spacer arm to the N-terminus, creating a novel affinity ligand (Bio-U-II) to interface with streptavidin media. Bio-U-II bound to UT with analogous pharmacological properties to the unmodified U-II ligand (high affinity, pseudo-irreversible binding). The pre-binding of Bio-U-II to UT (before exposure to detergent) facilitated specific capture of UT by stabilizing the receptor structure during solubilization with detergent. Solubilization of UT with the most compatible detergent, *n*-dodecyl β -D-maltoside, was dependent on the critical micelle concentration and $G\alpha_{q/11}$ protein was co-purified with captured Bio-U-II-UT complexes. Furthermore, captured Bio-U-II-UT complexes were resistant to dissociation at elevated temperatures, suggesting that UT is relatively thermostable, making it an ideal candidate for future structural and biophysical studies. This work demonstrates the utility of pseudo-irreversible ligands to support the purification of a GPCR during detergent extraction, resulting in the first successful purification of the UT.

MOL #65151

INTRODUCTION

Conventional paradigms of GPCR activation predict that the active conformation (R* state) is selected (or induced) by ligand binding, and couples to G proteins, which initiate intracellular signaling. These signals are terminated by receptor phosphorylation mediated by GPCR kinases and recruitment of arrestins, preventing further interaction with G proteins and promoting receptor internalization (Oakley et al., 2001). For GPCRs, the balance that exists between receptor activation and deactivation (desensitization) determines the strength and duration of a given stimulus.

Despite recent advances in delineating the mechanism of GPCR activation, our understanding of the conformational landscape that underpins GPCR activation is underdeveloped. This is primarily because GPCRs (particularly the R* forms) are generally recalcitrant to purification and are inherently unstable during membrane extraction. The problems associated with purifying GPCRs are multi-factorial – a) they are expressed at low levels; b) it is difficult to select and stabilize the R* state(s); c) the detergent extraction and separation processes destabilize important hydrophobic interactions. The result of destabilizing these interactions is that large quantities of GPCRs become denatured or irreversibly inactivated, leading to poor purification yields (reviewed in (Chiu et al., 2008)). This is reflected by the lack of structural information on the active conformation of GPCRs. Moreover, most current GPCR structures are stabilized in an inactive (R) conformation (reviewed in (Topiol and Sabio, 2009)), except for an intermediary form of the rhodopsin receptor (Salom et al., 2006). Recently, Scheerer et al., crystallized the complex between an 11-mer synthetic peptide of the G α_t C-terminus and opsin, showing slight structural shifts in both the receptor and G protein fragment (Scheerer et al., 2008). While the study provides clues to the mode of GPCR activation, the structure may not be indicative of an active state as the interaction between GPCR and G protein is likely to be more complex. Indeed, NMR in combination with molecular modeling has been used to demonstrate structural rearrangement of TM6 and 7 via disruption of a salt bridge in ECL2 which may play a role in receptor activation (Bokoch et al., 2010).

MOL #65151

One approach to overcome barriers associated with GPCR instability is to screen for receptor mutations that increase protein thermal stability (Magnani et al., 2008; Serrano-Vega et al., 2008; Shibata et al., 2009; Standfuss et al., 2007; Warne et al., 2008), however, this mutation approach can also yield a conformer that is inactive (Standfuss et al., 2007; Warne et al., 2008).

As an alternative, or perhaps as a supplement to mutagenesis, we reasoned that some receptor-ligand pairings might be amenable to affinity chromatography, using resins functionalized to capture a related affinity-tag linked to the receptor's ligand. This approach to GPCR purification, termed "ligand supported purification", can exploit essentially irreversible GPCR-ligand pairings, where the ligand is modified and serves to place the GPCR in the R* state. Detergents are then applied to solubilize the plasma membrane and the ligand supports the GPCR structure during extraction and purification by dampening the denaturing effects of the detergent, with the goal of purifying the GPCR to a solid support capture matrix in the active R* state.

The cyclic peptide hormone Urotensin-II (U-II) is one of the most potent vasoconstrictors in a variety of species (Douglas et al., 2000). The rat U-II receptor (UT) is a $G\alpha_{q/11}$ -coupled receptor that activates phospholipase C β 1 to generate the second messengers, inositol triphosphate and diacylglycerol, which release calcium from intracellular stores and activate protein kinase C, respectively (Proulx et al., 2008).

The U-II/UT pairing has features that make it a prime candidate for ligand supported purification: a) we confirm here that the UT/U-II interaction is high affinity, being essentially irreversible (Douglas et al., 2000) with long-lasting effects – both at a cellular level and *in vivo* (Camarda et al., 2002); b) the N-terminus of the U-II peptide is reportedly redundant for high affinity binding (Coy et al., 2002), allowing modification of the ligand with the affinity group; c) we show that the U-II bound UT is resistant to the common regulatory mechanisms of phosphorylation, arrestin binding and internalization that normally

MOL #65151

lead to desensitization of receptor signals, suggesting the receptor may exist in a more restricted set of active conformations. Together, these properties indicate that the UT has a strong propensity to remain in an active conformation at the cell surface for prolonged periods of time, increasing the likelihood of purifying a GPCR in the active R* state. We report here the application of ligand supported purification with a biotinylated U-II ligand (Bio-U-II), enabling the capture of stable ligand-receptor complexes.

MOL #65151

MATERIALS AND METHODS

Materials

Cell culture, transfection reagents and Protein A agarose beads were purchased from Invitrogen. The rat U-II and Bio-U-II peptides were synthesized by GL Biochem (Shanghai) Ltd. Angiotensin II (Ang II) was synthesized by Auspep Pty Ltd. Peptides were iodinated with an [¹²⁵I]-tracer by ProSearch International Australia (specific activity ~1000Ci/mmol). Detergents, protease inhibitors and the streptavidin resin, silanizing agent (Sigmacote[®]) were purchased from Sigma Aldrich. Goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were purchased from BioRad. Rabbit polyclonal anti-ERK 2, rabbit polyclonal G $\alpha_{q/11}$ and goat anti-rat IgG-HRP were purchased from Santa Cruz Biotechnology. Other antibodies used were mouse monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology), mouse monoclonal anti-HA antibody (12CA5) and rat monoclonal anti-HA High Affinity (3F10, Roche Applied Science). Western blots were developed with Supersignal West Pico Chemiluminescent substrate (Pierce). The biotin assay kit was purchased from Biacore (GE Healthcare).

Organ Bath Studies

Sprague Dawley rats (240g-400g) were anaesthetized with 20% O₂/80% CO₂, decapitated, and thoracic aorta removed, placed in oxygenated Krebs's solution (118mM NaCl, 4.5mM KCl, 0.45mM MgSO₄, 1.03mM KH₂PO₄, 25mM NaHCO₃, 11.1mM glucose, 2.5mM CaCl₂), cleared of connective tissue and cut into 3mm rings. The aortic rings were placed in organ baths containing 7ml Krebs's solution (37°C) bubbled with 95% O₂/5% CO₂ to maintain pH 7.4. The rings were stretched to a tension of 20mN and equilibrated for 60min. A reference contraction to 80mM KCl was followed by treatment with either U-II (30nM) or Ang II (100nM). Changes in tension were recorded using a Grass FTO3 isometric transducer connected to a MacLab recording system.

Expression plasmids

MOL #65151

The UT was cloned as previously described (Tzanidis et al., 2003). For efficient immunoprecipitation, three HA epitope tags were inserted after the start codon of the UT using standard PCR-based mutagenesis. All constructs were verified by direct DNA sequencing, and expression was monitored by radioligand binding and immunoprecipitation. Expression plasmids for the HA-tagged Ang II Type 1A receptor (AT_{1A}) were as described (Thomas et al., 1998). β -arrestin constructs were kindly provided by Dr RJ Lefkowitz and Dr MG Caron.

Rational Bio-U-II design

The UT binding pocket for the U-II peptide was modeled using Bovine Rhodopsin (PDB accession: 1U19) as a template. The PDB accession 2F01 was used to view the binding pocket of streptavidin for biotin. Swiss-Pdb Viewer v3.7 was used for the rational design of Bio-U-II as outlined in Supplementary Tables 1 and 2 and Supplementary Fig. 1.

Cell culture and transfection

HEK293, CHO-K1 or COS-1 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and passaged with trypsin into 12-well plates (150,000 cells/well) or onto 100mm culture dishes (1,800,000 cells/dish). Cells were maintained at 37°C in air enriched with 5% CO₂. Cells were transiently transfected with 0.6 μ g DNA per well (in a 12-well plate) using Lipofectamine™ reagent with expression vectors containing DNA upon reaching 70-80% confluence. For transfection in 100mm culture dishes, volumes for all reagents were increased by a factor of twelve. Transfection was stopped by replacing the transfection mixture with DMEM supplemented with 10% FBS.

Receptor Phosphorylation

MOL #65151

Receptor immunoprecipitation and phosphorylation was performed as previously described for the AT_{1A} (Thomas et al., 1998). 12CA5 anti-HA mouse monoclonal was used for immunoprecipitation and 3F10 high affinity anti-HA rat monoclonal was used for Western blotting.

Radioligand binding assays

Binding experiments with [¹²⁵I]-U-II and [¹²⁵I]-Bio-U-II were carried out 48h after transfection. Cells transfected with UT in 12-well plates were washed twice with phosphate buffered saline (PBS), followed by binding 30pM of the radioligand at room temperature for 5h in U-II binding buffer (20mM Tris-HCl pH 7.4, 100mM NaCl, 5mM MgCl₂, 10mM D-glucose and 0.1% BSA). Following binding, cells were washed three times with 1ml U-II binding buffer to remove unbound ligand and harvested with 1ml lysis buffer (0.25% SDS, 0.25M NaOH). Non-specific binding was determined in the presence of 1μM unlabeled U-II.

Competitive binding assays were carried out on cells transfected with UT. Either U-II or Bio-U-II was added to samples at varying final concentrations (0-3μM). Radio-iodinated U-II (30pM) was added to samples and incubated (5h, 4°C). Cells were then washed with U-II binding buffer and harvested with lysis buffer.

Quantitative Internalization assay

Cells expressing UT or AT_{1A} were washed twice with PBS (4°C) and incubated with receptor binding buffer containing 120pM of [¹²⁵I]-U-II (5h, 4°C). Unbound [¹²⁵I]-U-II was removed by washing with ice-cold PBS. Warm receptor binding buffer (37°C) was added to cells and incubated at 37°C for specified times. Receptor internalization was terminated by washing with ice-cold PBS and surface-bound [¹²⁵I]-U-II stripped using acid wash solution (50mM acetic acid, 150mM NaCl) with two 30min incubations. Internalized [¹²⁵I]-U-II was harvested using lysis buffer. Receptor internalization was calculated as the

MOL #65151

percentage of radioactivity in the cell lysate relative to the total counts. Data was corrected for non-specific internalization determined in the absence of a 37°C incubation step.

Pseudo-irreversible binding assay

For acid wash experiments, cells were incubated with 100,000cpm of either [¹²⁵I]-U-II, [¹²⁵I]-Bio-U-II or [¹²⁵I]-Ang II for (5h, 4°C) as described in the radioligand binding assay method. Acid wash solution was added to cells for 3min, removed and then retained for analysis. Several samples were subjected to a second 3min acid wash to analyze further dissociation of the radioligand. Cells were then washed with U-II binding buffer and lysed with solubilization buffer to determine the level of radioligand associated with receptor.

Analysis of radioligand binding data

All radioligand binding experiments were repeated in triplicate with each data point carried out in duplicate or triplicate. Radioactivity was quantified by gamma counting. Analysis of binding data was carried out with GraphPad Prism 4.0 (GraphPad Software Inc.).

Biotin assay

Accessibility of the biotin moiety within the Bio-U-II was determined using a Biacore 3000 instrument. The format chosen was an indirect assay with anti-biotin antibodies and a biotin sensor chip. The effective concentration of the Bio-U-II peptide was determined by comparing the response to a calibration curve. The calibration curve was prepared with a 1:2 dilution of biotin in HBS-EP buffer (10mM HEPES, 150mM NaCl, 3.4mM EDTA, 0.005% (v/v) Tween-20) with a constant amount of anti-biotin antibody (50 – 1.59ng/ml) as provided in a biotin assay kit. Given that the quantification range of the biotin assay is 2.0-70ng/ml the concentration of biotin within Bio-U-II chosen for analysis was 25ng/ml and samples were similarly prepared diluted in HBS-EP buffer as for the biotin calibration standards. A total of 70µl of

MOL #65151

each sample was injected over the biotin chip at a rate of 40 μ l/min. Responses were evaluated using GraphPad Prism 4.0 and analysis was corrected for purity of the ligand.

ERK1/2 assay

Transfected CHO-K1 cells cultured in a 12-well plate were serum starved with DMEM containing antibiotics 12h prior to experimentation. Cells were stimulated with U-II or Bio-U-II of varying final concentrations (0-100nM) and incubated for 10min (37°C). Following incubation, cells were washed twice with PBS (4°C) and lysed with radio-immunoprecipitation assay buffer (50mM Tris-HCl, pH 7.4, 100mM NaCl, 2mM EDTA, 50mM NaF, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10mM sodium pyrophosphate, 1 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 μ g/ml pepstatin). Samples were centrifuged at 14,000xg (15min, 4°C), supernatants were retained and analyzed with SDS-PAGE followed by transfer. Western blotting was carried out using anti-phospho-p44/42 MAPK antibody and anti-mouse HRP-conjugated antibody. Western blots were then stripped and re-probed with anti-ERK 2, washed and probed with anti-rabbit HRP-conjugated antibody.

Purification of the UT

Membrane preparations were generated from cells cultured in six 100mm dishes and were transfected with UT and serum starved 16h prior to experimentation. Cells were then washed twice with PBS (4°C). Hypotonic solution (50mM HEPES pH 7.5, 1mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin) was added to each plate and the cells lifted by scraping. Cells were pooled and homogenized using a Kinematica AG Polytron PT3000 (10,000rpm, 4x5s, 4°C). The homogenate was centrifuged at 800xg (10min, 4°C) with slow deceleration. The supernatant was decanted and was further centrifuged at 30,000xg (30min, 4°C) to separate the membrane pellet from the cytosolic fraction. The cytosolic fraction was decanted while the membrane pellet was resuspended in solubilization buffer without detergent (50mM HEPES pH 7.5, 250mM NaCl, 2mM EDTA, 1mM Na₃VO₄, 1mM NaF, 1mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin) and homogenized (10,000rpm, 4x5s, 4°C).

MOL #65151

The crude membrane suspension was incubated in the presence or absence of 1mM of Bio-U-II with agitation (18h, 4°C). Membranes were evenly aliquoted into pre-siliconized tubes containing solubilization buffer with detergent, making the final volume up to the desired concentration. Suspensions were solubilized by vortexing (4x5s, 4°C) followed by gentle agitation on an inverted rocker (20min, 4°C). Samples were centrifuged at 1,000xg (5min, 4°C) to separate solubilized material from the insoluble material.

The supernatant was incubated with streptavidin resin – these samples were placed on an inverted rocker (20min, 4°C) to allow interaction between Bio-U-II and streptavidin. Samples containing streptavidin and receptor were centrifuged at 500xg (2min, 4°C) to separate captured receptors from the uncaptured flow-through.

Material that was not captured to the streptavidin resin was collected into tubes containing 20µl of protein A agarose beads and 2µg of anti-HA (12CA5) mouse hybridoma antibody for immunoprecipitation and samples were incubated overnight with rocking (4°C). Samples were centrifuged at 8,000xg (1min, 4°C) and the supernatant was removed. To the remaining beads, 1xurea-based sample buffer (63mM Tris-HCl pH 6.8, 2% SDS, 10% β-mercaptoethanol, 6M urea and 20% glycerol) was added to each sample to solubilize protein. Samples were then heated at 65°C for 15min.

Receptor material captured to the streptavidin resin was reduced in 1 x urea-based sample buffer and heated (15min, 65°C). The receptor protein within all samples (captured to the streptavidin resin and uncaptured material within the flow-through) were resolved through SDS-PAGE and silver stained or Western blotted using anti-HA high affinity rat monoclonal antibody (3F10) and anti-rat HRP-conjugated antibody.

MOL #65151

For the identification of UT-G $\alpha_{q/11}$ complexes, streptavidin-captured samples were analyzed by SDS-PAGE and Western blotted with rabbit anti-G $\alpha_{q/11}$ antibody and anti-rabbit HRP-conjugated antibody.

Thermostability testing of the UT

The UT receptor was solubilized and captured as described in 'Purification of the UT.' Samples of the streptavidin resin containing captured UT were then heated to 30°C, 37.5°C, 45°C, 52.5°C or 60°C for 30min. The heat-treated samples were then centrifuged at 500xg (1min) to separate captured receptors from dissociated ligand-receptor complexes. The streptavidin resin was then washed with solubilization buffer at the temperature corresponding to the specific heat treatment by buffer addition, centrifugation at 500xg (1min) and removal of the supernatant. The streptavidin-captured samples were analyzed by SDS-PAGE and Western blotted with rat anti-HA antibody.

MOL #65151

RESULTS

U-II induces sustained vasoconstriction

U-II reportedly binds to UT pseudo-irreversibly, resulting in unusually sustained cellular responses leading to potent vasoconstriction (Douglas et al., 2000). To confirm this, we compared the vasoconstriction of thoracic aortic rings to U-II and Ang II, which both activate $G\alpha_{q/11}$ -coupled signaling pathways.

U-II induced vasoconstriction of rat aortic rings is slow to develop (maximal at 30min, $273\pm 13\%$ of 80mM KCl) and prolonged, remaining 50% of maximal 2h after stimulation (Fig. 1). This contrasts with the rapid and transient responses to Ang II, which activates the $G\alpha_{q/11}$ -coupled AT_{1A} . Contraction to Ang II was maximal at 5min ($171\pm 17\%$ of 80mM KCl) and returned to near basal at 12min (Fig. 1).

UT is resistant to classical desensitization mechanisms

We next examined the capacity of the UT to be phosphorylated and internalized following U-II stimulation. The rat UT possesses numerous potential phosphorylation sites – 8 of 15 amino acids in the central region of the carboxyl-terminus are serine/threonine residues, indicating that the UT might be regulated by canonical phosphorylation, arrestin binding and internalization (Oakley et al., 2001).

A UT containing three HA tags at the N-terminus was constructed to examine UT phosphorylation (note, introduction of three HA tags did not affect receptor expression, binding affinity or U-II-mediated accumulation of inositol phosphates; not shown).

Cells expressing either the UT or the AT_{1A} (as a comparative control) were labeled with $^{32}P_i$ and stimulated with ligands for the times indicated. Stimulation of the UT for up to 60min resulted in

MOL #65151

negligible phosphorylation of the receptor (Fig. 2A). This refraction to phosphorylation was also observed in HEK293 and COS-1 cells (data not shown), suggesting that the lack of receptor phosphorylation is not cell-specific. In contrast, the AT_{1A} was robustly phosphorylated following Ang II stimulation (Fig. 2A).

Confocal microscopy was used to examine the interaction and trafficking of β -arrestin-1/2-GFP in response to activation of UT expressed in HEK293 cells. The trafficking of β -arrestins following activation of the UT was very weak and most arrestin protein remained cytoplasmic (Fig. 2B). Even after 60min of U-II stimulation, some weak accumulation of β -arrestin-1/2 in membrane-localized pits was evident, but deep core vesicles did not form (Fig. 2B). In contrast, β -arrestin-1/2 rapidly trafficked to agonist-activated AT_{1A} with almost all cytoplasmic β -arrestins recruited to the cell membrane by 5min and trafficked strongly with AT_{1A} into deep-core vesicles by 60min (Fig. 2B).

The kinetics of UT internalization was measured by determining acid-resistant binding of [¹²⁵I]-U-II to CHO-K1 cells expressing UT (Fig. 2C). Internalization of UT was slow ($t_{1/2}$ =15min) reaching only 41±2% at 60min stimulation, whereas activated AT_{1A} internalized rapidly ($t_{1/2}$ =3.3min) and robustly (73±4% at 20min, Fig. 2C). This slower and weaker internalization was also observed in HEK293 cells expressing UT (data not shown).

The prolonged activity of U-II together with the resistance of UT to classic desensitization mechanisms (namely, phosphorylation, arrestin recruitment and internalization) shows that the UT preferentially adopts an active conformation at the cell surface. These properties were then exploited for ligand-based purification to stabilize the active state of UT.

Bio-U-II design

The rat U-II peptide was modified to include an N-terminal biotin tag separated from the U-II sequence by a GSSG spacer arm. The length of the spacer arm was determined by modeling the key binding

MOL #65151

residues of the UT onto their analogous location in the Rhodopsin (1U19) crystal structure (Supplementary Table 1 and Fig. 1). Calculations were based upon the distance required to clear both the receptor binding pocket and streptavidin binding pocket (PDB entry 2F01) – this ensures that a) the resin and receptor do not sterically clash and b) the modified ligand can provide a bridge between the receptor and resin. Based on these calculations (Supplementary Table 2), the rat U-II peptide was modified with three repeating GSSG peptide motifs and a biotin affinity group attached to an 8-carbon polylinker group (referred to as Bio-U-II) (Fig. 3). Details of the molecular modeling are more fully described in the supplementary section.

Bio-U-II interacts with both streptavidin and UT

A surface plasmon resonance assay was used to investigate whether an anti-biotin antibody binds to Bio-U-II in the same way as free biotin. It was found that anti-biotin antibodies bind to Bio-U-II within $\pm 3.0\%$ of the expected value of free biotin – as corrected based on the purity of Bio-U-II (80.47%) (Fig. 4A), demonstrating that the biotin moiety is accessible to binding partners.

U-II and Bio-U-II have similar functionality

Binding assays were carried out to ensure that Bio-U-II retains capacity to bind to UT expressed in HEK293 cells. The affinity of Bio-U-II was characterized through its ability to competitively displace an [125 I]-U-II radio-label in comparison to unlabeled U-II. The addition of U-II displaced the specific binding of [125 I]-U-II with an IC_{50} value of 1.0nM, whereas unlabeled Bio-U-II displaced [125 I]-U-II with an IC_{50} value of 2.8nM (Fig. 4B). Thus, the affinities of U-II and Bio-U-II for the UT were comparable.

For successful ligand supported purification, it is essential that Bio-U-II retains the ability to bind UT pseudo-irreversibly (Douglas et al., 2000). The pseudo-irreversible binding nature of both the U-II and Bio-U-II was tested by the ability of an acid solution to strip the ligand from its binding site (Fig. 4C). After binding radio-labeled [125 I]-U-II and [125 I]-Bio-U-II, a 3min acid wash only removed 10% and 8%

MOL #65151

of the ligand, respectively (Fig. 4C). Even when cells were subjected to a second 3min acid wash only 24% and 17% of the total available ligand was removed. In contrast, binding [¹²⁵I]-Ang II to AT_{1A} was removed to near completion by an acid wash under the same conditions (91% removal after the first acid wash and 96% removal upon two 3min washes) (Fig. 4C). These results show that unlike Ang II, Bio-U-II is able to bind strongly and specifically to UT, and Bio-U-II demonstrates similar pseudo-irreversible binding behavior compared to the parent U-II peptide.

Confirmation of receptor activation by Bio-U-II was tested using phosphorylation of ERK1/2. Stimulation of UT-expressing CHO-K1 cells with the Bio-U-II peptide at 0.1nM was sufficient to elicit phosphorylation of ERK1/2 (Fig. 4D) indicating Bio-U-II remains a potent agonist.

Purification of the UT with detergents

To test the concept of ligand supported purification with Bio-U-II, five representative detergents were screened for the purification of the UT. The detergents used for screening were: CTAB (cationic), sodium cholate (anionic), dodecyl maltoside (DDM) (non-ionic – alkyl glucoside), digitonin (non-ionic – steroid derivative) and CHAPS (zwitterionic).

Fig. 5 shows the extraction of the UT using pre-bound Bio-U-II at 10x the critical micelle concentration (CMC) for a panel of detergents. The UT-Bio-U-II complex was captured onto a streptavidin matrix and analyzed by Western blotting for the HA epitope tag on the receptor. Fig. 5A illustrates insoluble material following detergent ‘solubilization’ and shows that different detergents had varying solubilization efficiencies for the UT. Fig. 5B shows that, of the representative detergents screened, DDM was the only detergent which allowed solubilization and specific capture of the UT to the streptavidin resin. As demonstrated in Fig. 5C, a significant amount of solubilized UT remained uncaptured at this CMC and was detected in the flow-through and analyzed by immunoprecipitation of the HA epitope tag.

MOL #65151

The CMC dependence of purification was further investigated with pre-binding of Bio-U-II with DDM at 2x, 5x and 10x the CMC (Fig. 6). Ligand supported purification of the UT was found to be successful at all three detergent concentrations but was most effective at 5x the CMC (Fig. 6).

An experiment comparing pre-binding of Bio-U-II against pre-solubilization was carried out to determine the effectiveness of pre-solubilization during ligand supported purification (Fig. 7). It was found that pre-binding produced greater yields of UT in comparison to pre-solubilization (lanes PB and PS, Fig. 7A) for 5xCMC DDM. Furthermore, a single wash of the streptavidin beads with captured receptors using different DDM concentrations (2x, 5x and 10xCMC) did not affect purification in pre-bound or pre-solubilized samples. Once it was established that the ligand-receptor complex was not susceptible to dissociation after one wash, multiple washes were incorporated at 5xCMC of DDM. After three washes, ligand-receptor complexes dissociated somewhat in samples prepared by pre-solubilization (Fig. 7A, lanes denoted PS) while pre-bound samples were unaffected (Fig. 7A, see lanes denoted PB). Under the optimized conditions, we estimate that about one third of the solubilized receptor can be captured on the resin and that this can be efficiently separated from the non-bound receptor by extensive washing (Fig. 7B).

Fig. 7C shows a silver stained SDS-PAGE gel of pre-bound UT or control samples (washed three times with 5xCMC DDM). There was a significant enrichment of the protein bands in the pre-bound samples compared to those in the NL control (Fig. 7C, right panel). Because the UT was released from the streptavidin resin through boiling instead of elution, both samples show contaminating proteins, which were non-specifically released throughout. The corresponding Western blot for both samples has been included for molecular weight comparison (Fig. 7C, left panel).

MOL #65151

G $\alpha_{q/11}$ protein remains associated to captured UT-Bio-U-II complexes

Pre-bound UT samples captured to the streptavidin resin were probed with anti-G $\alpha_{q/11}$ antibody to determine whether G $\alpha_{q/11}$ was co-purified with the UT (Fig. 7D). After three washes with 5xCMC DDM, G $\alpha_{q/11}$ was found to be complexed to UT-Bio-U-II (Fig. 7D, lane PB). In contrast, G $\alpha_{q/11}$ was not detected in control samples that were not captured with Bio-U-II (Fig. 7D).

Thermostability of the UT

Fig. 8 demonstrates the capture of the UT to streptavidin resin followed by incubation at different temperatures (4°C, 30°C, 37.5°C, 45°C, 52.5°C or 60°C) for 30min. At 4°C (standard purification conditions), sample capture was greatest, yet, even at higher temperatures (30°C to 60°C), the amount of UT captured to streptavidin was maintained and only slightly reduced.

MOL #65151

DISCUSSION

The inherent difficulties in purifying active R* conformations of GPCRs prompted the question whether a ligand-based approach can yield purified R* GPCRs. Here, we introduce a biotin-tagged version of the peptide hormone U-II that allows ligand supported purification of its cognate GPCR, the UT. Our approach to ligand supported purification involved a ligand that was modified for affinity chromatography without markedly perturbing receptor binding or activation, and exploited essentially irreversible receptor/ligand interaction. These features are associated with a number of GPCR/ligand pairings, such as the relaxin RXPF1/2 (Callander et al., 2009) and endothelin ET_A receptors (Waggoner et al., 1992), making them ideal candidates for ligand supported purification. Preferably, the receptor should have a stable, yet restricted, set of conformers, resistance to the regulatory processes of phosphorylation and β -arrestin binding and internalization.

Although UT desensitization has been a contentious issue (Giebing et al., 2005; Proulx et al., 2008; Proulx et al., 2005), we show here that the UT is refractory to classical GPCR desensitization mechanisms and propose this relates to its sustained contractile actions. It appears the binding of U-II to the UT induces a receptor conformation capable of activating G $\alpha_{q/11}$, which is not readily phosphorylated nor bound by β -arrestins. Furthermore, we found UT has a relatively long internalization half-life ($t_{1/2}$ = 15min), which is similar to previous reports in COS-7 cells (Proulx et al., 2005). For U-II, presumably the initial interaction with the receptor promotes a structure that activates G $\alpha_{q/11}$, but does not permit subsequent docking events that promote additional conformation(s) for phosphorylation and desensitization. This may reflect the strong, pseudo-irreversible nature of U-II binding (Douglas et al., 2000). These properties are ideal for ligand supported purification as they show that agonist stimulated UT preferentially adopts an active R* conformation that is not readily removed from the cell surface.

MOL #65151

Two different approaches were investigated for purifying the UT with Bio-U-II. (1) Solubilizing the UT with detergents followed by purification through binding with the affinity ligand, or (2) pre-binding the Bio-U-II and then solubilizing the UT with detergents. The rationale for co-purifying GPCRs with detergents and a pre-bound ligand is that the ligand places the receptor in the active state and may remain bound during purification.

GPCRs have been previously purified with pre-solubilization, either by immobilizing the ligand to a solid support (Couvineau et al., 1990; Hazum, 1990) or by binding the ligand to the solubilized receptor (Hagiwara et al., 1992; Ohtaki et al., 1998; Santos-Alvarez and Sanchez-Margalet, 2000). Few studies have used pre-binding during ligand supported purification with a biotinylated ligand (Brown and Schonbrunn, 1993; Desarnaud et al., 1992; Eppler et al., 1992; Zysk et al., 1996), although, in each case, the authors have demonstrated that pre-binding is superior to pre-solubilization (Brown and Schonbrunn, 1993; Desarnaud et al., 1992; Eppler et al., 1992; Zysk et al., 1996). Indeed, the results presented here demonstrate that pre-binding with Bio-U-II produced a greater yield of UT compared to pre-solubilization with Bio-U-II. Furthermore, pre-bound complexes appear to be more stable, being more resistant to detergent effects upon washing. It has been suggested poor recovery of functional receptors upon purification is due either to denaturation upon solubilization (Brown and Schonbrunn, 1993) or the dissociation of the GPCR-G protein complex (Brown and Schonbrunn, 1993; Eppler et al., 1992) – both possibilities lead to reduced yield and produce unstable receptors or low affinity receptors (Brown and Schonbrunn, 1993; Eppler et al., 1992). Indeed, in the case of somatostatin, pre-binding of the affinity ligand was required because of the instability and inactivation of somatostatin upon detergent purification (Brown and Schonbrunn, 1993; Eppler et al., 1992).

Evidence that G proteins may be co-purified with GPCRs using ligand supported purification has been suggested to indicate that the purified receptor adopts an active R* conformation (Brown and Schonbrunn, 1993; Eppler et al., 1992; Santos-Alvarez and Sanchez-Margalet, 2000; Zysk et al., 1996).

MOL #65151

Brown and Schonbrunn (1993) showed that somatostatin remains sensitive to guanine nucleotides and exploited the co-capture of G proteins using GDP to dissociate receptor-ligand complexes during elution (causing a notable decrease in agonist affinity), which purified the receptor in an inactive conformation (Brown and Schonbrunn, 1993). We show that $G\alpha_{q/11}$ protein is co-captured with UT-Bio-U-II complexes suggesting that the R^* state has been isolated. However, we do not rule out the possibility that the $G\alpha_{q/11}$ protein is bound to an intermediary form of UT which is separate from its active R^* conformation (i.e. capable of signaling). Indeed, studies using fluorescence or bioluminescence resonance energy transfer show compelling evidence that pre-coupled GPCR-G protein complexes exist in the absence of agonist stimulation (Gales et al., 2006; Nobles et al., 2005). Hence, further testing is required to more fully determine if the UT is in an active R^* conformation or if $G\alpha_{q/11}$ is pre-coupled to UT.

The UT-U-II system is interesting because of the duration and magnitude of U-II-mediated responses as well as the atypical regulation of UT – indicating that it may be conformationally restricted compared to other GPCRs of known structure, (Rhodopsin (structures reviewed in (Palczewski et al., 2000; Teller et al., 2001; Topiol and Sabio, 2009)), $\beta_{1/2}$ -adrenergic (Cherezov et al., 2007; Warne et al., 2008) and A_{2A} adenosine receptors (Jaakola et al., 2008)). Indeed, there is only 19% sequence homology between rat UT and bovine Rhodopsin, making a structure of UT of broad interest to GPCR structural biologists and modelers. Of note, these crystallized GPCRs are in an *inactive* (R) conformation.

Structural and biophysical studies of GPCRs are a challenge due to their instability upon detergent extraction. Recent GPCR crystallography successes has been attributed to significant protein engineering such as C-terminal truncation (Cherezov et al., 2007; Jaakola et al., 2008; Murakami and Kouyama, 2008; Park et al., 2008; Warne et al., 2008), fusion protein generation (Cherezov et al., 2007; Jaakola et al., 2008; Rasmussen et al., 2007; Rosenbaum et al., 2007) and introduction of thermo-stabilizing mutations (Standfuss et al., 2007; Warne et al., 2008). However, these changes are not representative of a wild-type receptor in an active conformation. C-terminal truncations and formation of fusion proteins act to remove

MOL #65151

what are believed to be highly flexible regions of GPCRs which may impede the crystallization process, while thermo-stabilizing mutations improve receptor stability in detergents by potentially locking the GPCR in a single conformation.

In the case of the β_1 -adrenergic receptor (Magnani et al., 2008; Serrano-Vega et al., 2008; Warne et al., 2008) and adenosine A_{2a} receptor (Magnani et al., 2008; Serrano-Vega et al., 2008), alanine-scanning mutagenesis was conducted on the full length protein to identify amino acid residues which altered the receptor's thermostability as determined by heating the GPCR followed by radioligand binding (Magnani et al., 2008; Serrano-Vega et al., 2008; Warne et al., 2008). Interestingly, Serrano-Vega *et al.* (2008) showed that pre-incubation of an agonist with subsequent detergent-solubilization of the thermostable β_1 -adrenergic receptor increased the T_m by 23°C compared to non-ligand bound wild-type receptor (Magnani et al., 2008; Serrano-Vega et al., 2008; Warne et al., 2008).

In a similar approach, we captured UT to streptavidin using a pre-bound ligand, and examined the thermostability as a measure of UT retention on the resin. Although there is a reduction (~50%) in specific capture of Bio-U-II-UT between 4°C and the tested temperatures, UT was strongly retained on the streptavidin resin between 30°C and 60°C. The resistance to ligand-receptor dissociation at these temperatures suggests that UT has good thermostable properties when pre-bound to Bio-U-II. This may be attributed to four factors: a) the inherent high-stability of the receptor-ligand interaction which forms a pseudo-irreversible complex and remains active for extended periods; b) the selection of a stable conformation of UT; c) a naturally compact intracellular third loop; and d) the retention of G protein on the receptor which may contribute to global as well as localized stability. For these reasons, the application of ligand supported purification to the UT provides an exciting possibility to study a GPCR system which does not need to be modified to obtain structural information on the active conformation.

MOL #65151

In conclusion, we report the first successful purification of the UT which is physically associated with $G\alpha_{q/11}$ protein. Although ligand supported purification can be applied to other GPCRs for protein stabilization during detergent extraction and potentially tailored for a wider range of GPCRs (such as the relaxin RXPF1 and RXPF2 (Callander et al., 2009) and endothelin ET_A (Waggoner et al., 1992) receptors, which also display pseudo-irreversible binding), we attribute appreciable yields of UT to the long-lasting, pseudo-irreversible binding of U-II, the resistance of UT to classic desensitization mechanisms and selection of a stable receptor conformation. Using this study as a basis, we have embarked upon scaled expression and purification of the UT to allow large-scale production of purified UT in the active R^* state for biophysical and structural studies.

MOL #65151

ACKNOWLEDGMENTS

β -arrestins were kindly provided by Dr RJ Lefkowitz and Dr MG Caron.

The use of animal tissue was approved by the University of Melbourne, Animal Experiment Ethics Committee.

MOL #65151

REFERENCES

Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi HJ, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L and Kobilka BK (2010) Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature* **463**(7277):108-112.

Brown PJ and Schonbrunn A (1993) Affinity purification of a somatostatin receptor-G-protein complex demonstrates specificity in receptor-G-protein coupling. *J Biol Chem* **268**(9):6668-6676.

Callander GE, Thomas WG and Bathgate RA (2009) Prolonged RXFP1 and RXFP2 signaling can be explained by poor internalization and a lack of beta-arrestin recruitment. *Am J Physiol Cell Physiol* **296**(5):C1058-1066.

Camarda V, Rizzi A, Calo G, Gendron G, Perron SI, Kostenis E, Zamboni P, Mascoli F and Regoli D (2002) Effects of human urotensin II in isolated vessels of various species; comparison with other vasoactive agents. *Naunyn Schmiedebergs Arch Pharmacol* **365**(2):141-149.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**(5854):1258-1265.

Chiu ML, Tsang C, Grihalde N and MacWilliams MP (2008) Over-expression, solubilization, and purification of G protein-coupled receptors for structural biology. *Comb Chem High Throughput Screen* **11**(6):439-462.

Couvineau A, Voisin T, Guijarro L and Laburthe M (1990) Purification of vasoactive intestinal peptide receptor from porcine liver by a newly designed one-step affinity chromatography. *J Biol Chem* **265**(22):13386-13390.

MOL #65151

Coy DH, Rossowski WJ, Cheng BL and Taylor JE (2002) Structural requirements at the N-terminus of urotensin II octapeptides. *Peptides* **23**(12):2259-2264.

Desarnaud F, Marie J, Larguier R, Lombard C, Jard S and Bonnafous JC (1992) Protein purification using combined streptavidin (or avidin)-Sephacrose and thiopropyl-Sepharose affinity chromatography. *J Chromatogr* **603**(1-2):95-104.

Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar NV, Ohlstein EH and Willette RN (2000) Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol* **131**(7):1262-1274.

Eppler CM, Zysk JR, Corbett M and Shieh HM (1992) Purification of a pituitary receptor for somatostatin. The utility of biotinylated somatostatin analogs. *J Biol Chem* **267**(22):15603-15612.

Gales C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H and Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat Struct Mol Biol* **13**(9):778-786.

Giebing G, Tolle M, Jurgensen J, Eichhorst J, Furkert J, Beyermann M, Neuschafer-Rube F, Rosenthal W, Zidek W, van der Giet M and Oksche A (2005) Arrestin-independent internalization and recycling of the urotensin receptor contribute to long-lasting urotensin II-mediated vasoconstriction. *Circ Res* **97**(7):707-715.

Hagiwara H, Nagasawa T, Lodhi KM, Kozuka M, Ito T and Hirose S (1992) Affinity chromatographic purification of bovine lung endothelin receptor using biotinylated endothelin and avidin-agarose. *J Chromatogr* **597**(1-2):331-334.

Hazum E (1990) Purification of gonadotropin releasing hormone receptors using the avidin-biotin technique. *J Chromatogr* **510**:233-238.

MOL #65151

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP and Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* **322**(5905):1211-1217.

Magnani F, Shibata Y, Serrano-Vega MJ and Tate CG (2008) Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor. *Proc Natl Acad Sci U S A* **105**(31):10744-10749.

Murakami M and Kouyama T (2008) Crystal structure of squid rhodopsin. *Nature* **453**(7193):363-367.

Nobles M, Benians A and Tinker A (2005) Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci U S A* **102**(51):18706-18711.

Oakley RH, Laporte SA, Holt JA, Barak LS and Caron MG (2001) Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. *J Biol Chem* **276**(22):19452-19460.

Ohtaki T, Ogi K, Masuda Y, Mitsuoka K, Fujiyoshi Y, Kitada C, Sawada H, Onda H and Fujino M (1998) Expression, purification, and reconstitution of receptor for pituitary adenylate cyclase-activating polypeptide. Large-scale purification of a functionally active G protein-coupled receptor produced in Sf9 insect cells. *J Biol Chem* **273**(25):15464-15473.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**(5480):739-745.

Park JH, Scheerer P, Hofmann KP, Choe HW and Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454**(7201):183-187.

Proulx CD, Holleran BJ, Lavigne P, Escher E, Guillemette G and Leduc R (2008) Biological properties and functional determinants of the urotensin II receptor. *Peptides* **29**(5):691-699.

MOL #65151

Proulx CD, Simaan M, Escher E, Laporte SA, Guillemette G and Leduc R (2005) Involvement of a cytoplasmic-tail serine cluster in urotensin II receptor internalization. *Biochem J* **385**(Pt 1):115-123.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI and Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**(7168):383-387.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC and Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* **318**(5854):1266-1273.

Salom D, Lodowski DT, Stenkamp RE, Le Trong I, Golczak M, Jastrzebska B, Harris T, Ballesteros JA and Palczewski K (2006) Crystal structure of a photoactivated deprotonated intermediate of rhodopsin. *Proc Natl Acad Sci U S A* **103**(44):16123-16128.

Santos-Alvarez J and Sanchez-Margalet V (2000) Affinity purification of pancreastatin receptor-Gq/11 protein complex from rat liver membranes. *Arch Biochem Biophys* **378**(1):151-156.

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP and Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**(7212):497-502.

Serrano-Vega MJ, Magnani F, Shibata Y and Tate CG (2008) Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proc Natl Acad Sci U S A* **105**(3):877-882.

Shibata Y, White JF, Serrano-Vega MJ, Magnani F, Aloia AL, Grisshammer R and Tate CG (2009) Thermostabilization of the neurotensin receptor NTS1. *J Mol Biol* **390**(2):262-277.

Standfuss J, Xie G, Edwards PC, Burghammer M, Oprian DD and Schertler GF (2007) Crystal structure of a thermally stable rhodopsin mutant. *J Mol Biol* **372**(5):1179-1188.

MOL #65151

Teller DC, Okada T, Behnke CA, Palczewski K and Stenkamp RE (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**(26):7761-7772.

Thomas WG, Motel TJ, Kule CE, Karoor V and Baker KM (1998) Phosphorylation of the angiotensin II (AT1A) receptor carboxyl terminus: a role in receptor endocytosis. *Mol Endocrinol* **12**(10):1513-1524.

Topiol S and Sabio M (2009) X-ray structure breakthroughs in the GPCR transmembrane region. *Biochem Pharmacol* **78**(1):11-20.

Tzanidis A, Hannan RD, Thomas WG, Onan D, Autelitano DJ, See F, Kelly DJ, Gilbert RE and Krum H (2003) Direct Actions of Urotensin II on the Heart. Implications for Cardiac Fibrosis and Hypertrophy. *Circ Res* **93**:246-253.

Waggoner WG, Genova SL and Rash VA (1992) Kinetic analyses demonstrate that the equilibrium assumption does not apply to [¹²⁵I]endothelin-1 binding data. *Life Sci* **51**(24):1869-1876.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**(7203):486-491.

Zysk JR, Gaylinn BD, Lyons CE, Johnson B, Eppler CM, Baumbach WR and Thorner MO (1996) Purification of the growth hormone releasing hormone receptor with a C-terminal, biotinylated affinity ligand. *Biochem Biophys Res Commun* **221**(1):133-139.

MOL #65151

FOOTNOTES

This work was supported by National Health and Medical Research Council of Australia project grants [Grant 268925], [Grant 251646] and [Grant 334049]; D.O. was the recipient of a PhD scholarship from the Baker Heart Research Institute.

Correspondence to: Prof. Walter G. Thomas, The School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland, 4072 Australia, Tel: +61-7-3365-4656, Fax: +61-7-3365-1040, e-mail: w.thomas@uq.edu.au

¹ Both authors contributed equally to this work.

MOL #65151

FIGURE LEGENDS

Fig. 1. The contractile action of U-II is sustained. Rat aortic strips were exposed to U-II (30nM) or Ang II (100nM) and tension measured every 30s and normalized to that of 80mM KCl. The traces are averages (n=4 for U-II, n=2 for Ang II).

Fig. 2. Regulation of the UT. (A) CHO-K1 cells expressing UT or AT_{1A} were assayed for phosphorylation, following incubation with U-II (100nM) or Ang II (100nM) for indicated times. A representative blot is shown. (B) Cellular trafficking of β -arrestins upon agonist stimulation. The trafficking β -arrestin-1/2-GFP upon stimulation with agonist in HEK293 cells expressing UT or AT_{1A} (as indicated) was followed using confocal microscopy. (C) Internalization was measured in CHO-K1 cells expressing UT (closed circles, solid line) or AT_{1A} (open circles, dashed line), loaded with radio-labeled agonist (5h at 4°C) and incubated at 37°C for indicated times (n=6).

Fig. 3. The Bio-U-II peptide. A biotin moiety was linked to the U-II peptide (green) via a spacer sequence.

Fig. 4. Characterization of Bio-U-II binding and stimulation. (A) The biotin-streptavidin interaction was examined using Biacore analysis. A standard curve of known concentrations of biotin is shown. Two samples containing expected biotin concentrations of 25ng/ml yielded 1295 response units, which corresponds to 20.8ng/ml when corrected for the purity of Bio-U-II (80.5%). This shows that functionalized biotin (Bio-U-II) deviates by only 3% in recognition by anti-biotin antibody compared to free biotin. (B) A competitive displacement assay was carried out to determine the affinity of Bio-U-II for the UT in comparison to U-II. The IC₅₀ value of U-II displacing [¹²⁵I]-U-II was 1.0nM, the IC₅₀ value of Bio-U-II displacing [¹²⁵I]-U-II was 2.8nM, therefore, Bio-U-II has a similar ability to displace [¹²⁵I]-U-II. (n = 3). (C) An acid strip protocol (1 x 3 min or 2 x 3 min) was carried out on HEK293 cells expressing

MOL #65151

either the UT or AT_{1A} in which [¹²⁵I]-U-II, [¹²⁵I]-Bio-U-II or [¹²⁵I]-Ang II was bound. Control cells were not subjected to acid treatment to represent 100% of ligand binding. Binding of [¹²⁵I]-U-II and [¹²⁵I]-Bio-U-II to UT was resistant to wash out, whereas acid treatment of AT_{1A} led to almost complete removal of [¹²⁵I]-Ang II from the cell surface (n=3). (D) Bio-U-II dose-dependently stimulated UT to promote ERK1/2 phosphorylation (pERK); tERK is total ERK as a loading control.

Fig. 5. Solubilization and purification of UT using Bio-U-II and a variety of detergents. The UT was pre-bound to Bio-U-II (+) or without Bio-U-II (-). Report points were taken at the solubilization and capture stages, immunoprecipitation was used to detect the uncaptured flow-through. Shown is (A) Insoluble material; sodium cholate and CHAPS were efficient solubilizers. In contrast, CTAB, DDM and digitonin appear to be less effective. (B) Solubilized material captured by streptavidin resin; DDM allows specific capture of UT using Bio-U-II, whereas without ligand (DDM/-) yielded no detectable receptor. Other detergents screened did not allow specific capture of the receptor. (C) Immunoprecipitated flow-through (not captured by the resin); note, all detergents were used at 10x above the CMC, except CHAPS (2x CMC).

Fig. 6. Extraction efficiency of DDM was tested at 2x, 5x and 10x above the CMC. UT was pre-bound to Bio-U-II (+) or unbound (-) and captured on streptavidin beads at different CMC values.

Fig. 7. Testing the stability of Bio-U-II-UT complexes. Dissociation of the Bio-U-II/UT complex was monitored upon multiple washes of the streptavidin beads with DDM 5xCMC (NL = no ligand; PB = pre-bound ligand; PS = pre-solubilized receptor). (A) UT captured by streptavidin using Bio-U-II: pre-bound samples (PB) were unaffected by washing whereas pre-solubilized (PS) Bio-U-II-UT complexes dissociated upon extended washing. (B) Immunoprecipitated flow-through (not captured by resin): UT was present in the flow-through when the resin was not washed. Upon washing, small amounts of UT were released, with smaller amounts being released with each subsequent wash. (C) Samples were silver

MOL #65151

stained (left panel), the protein bands in pre-bound samples (PB) were more intense than those in the no-ligand control (NL). The corresponding Western blot (right panel) is a molecular weight comparison and shows the capture of the UT to the streptavidin resin. (D) $G\alpha_{q11}$ (42kDa) was co-purified with Bio-U-II-UT complexes in pre-bound (PB) samples that were washed with DDM 5xCMC as detected by Western blotting.

Fig. 8. UT purified by ligand supported purification is thermostable. The effect of temperature on the interaction between the pre-bound (PB) Bio-U-II ligand and UT captured on the streptavidin matrix. A no-ligand control (NL) is also shown for each temperature.

Figure 1

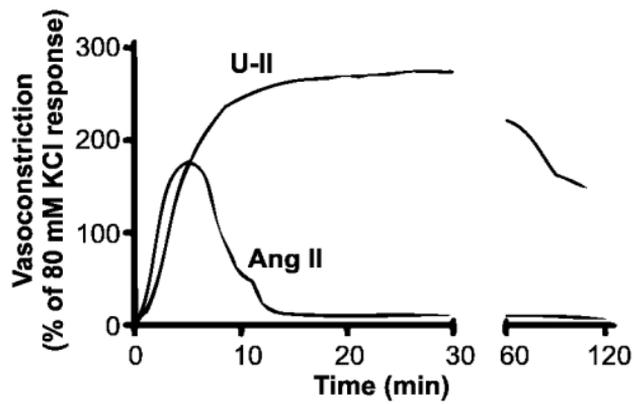


Figure 2

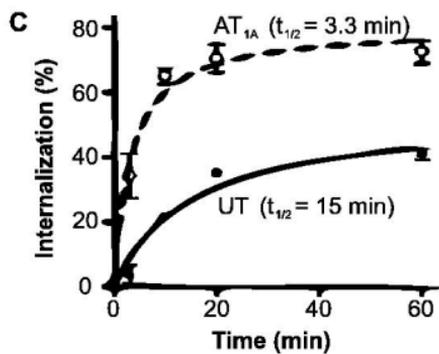
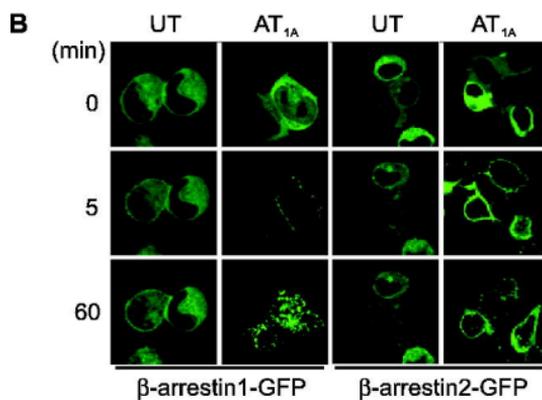
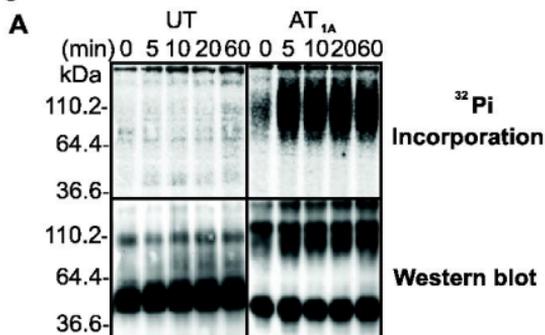


Figure 3

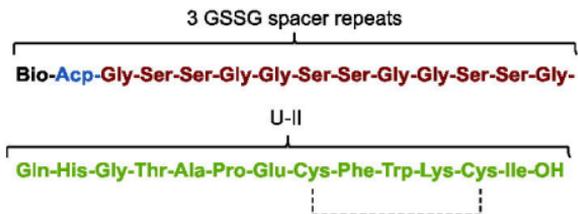


Figure 4

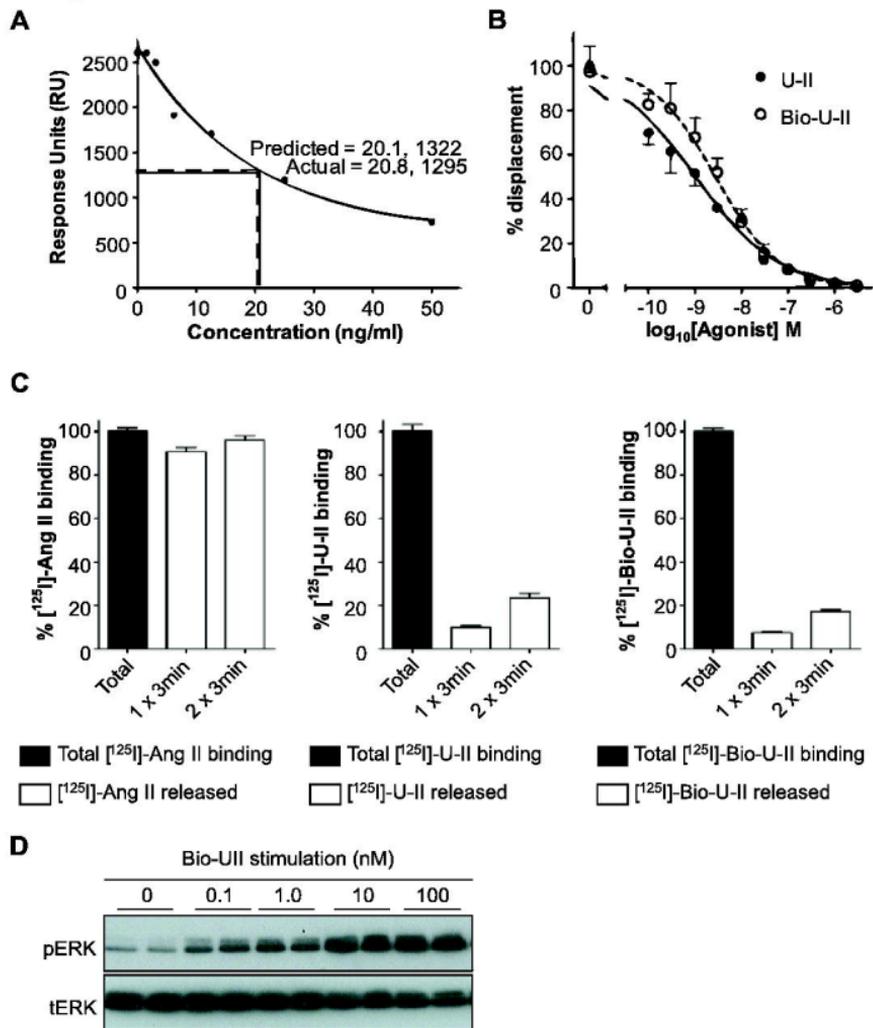
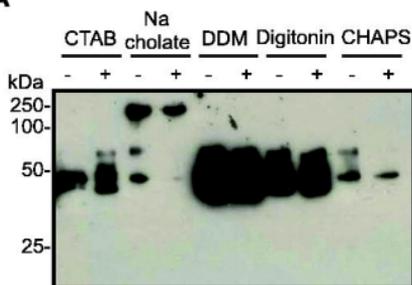
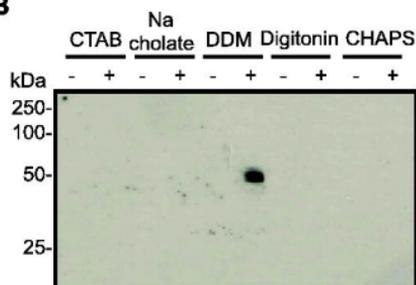


Figure 5

A



B



C

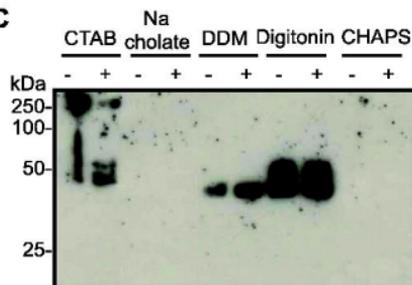


Figure 6

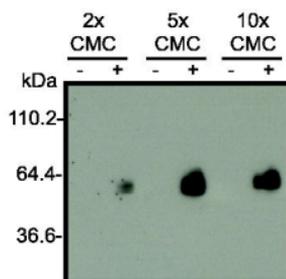
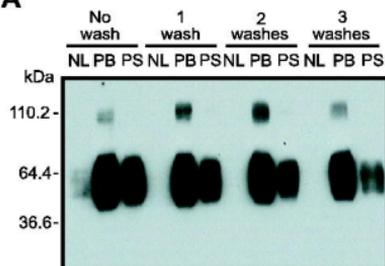
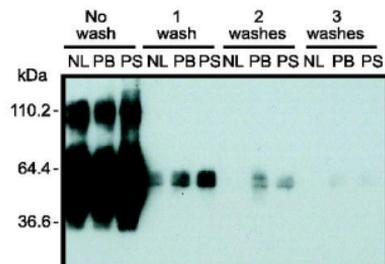


Figure 7

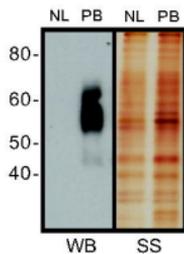
A



B



C



D

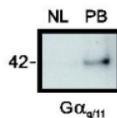


Figure 8

