

Direct analysis of a GPCR-agonist interaction by surface plasmon resonance

Peter J. Harding · Timothy C. Hadingham ·
James M. McDonnell · Anthony Watts

Received: 9 February 2006 / Revised: 3 April 2006 / Accepted: 20 April 2006 / Published online: 18 May 2006
© EBSA 2006

Abstract Despite their clinical importance, detailed analysis of ligand binding at G-protein coupled receptors (GPCRs) has proved difficult. Here we successfully measure the binding of a GPCR, neurotensin receptor-1 (NTS-1), to its ligand, neurotensin (NT), using surface plasmon resonance (SPR). Specific responses were observed between NT and purified, detergent-solubilised, recombinant NTS-1, using a novel configuration where the biotinylated NT ligand was immobilised on the biosensor surface. This SPR approach shows promise as a generic approach for the study of ligand interactions with other suitable GPCRs.

Keywords GPCR · Neurotensin receptor · Surface plasmon resonance · Ligand-binding · High-throughput screening

Abbreviations

CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHS Cholesteryl hemisuccinate

DCM	Dichloromethane
DDM	Dodecyl- β -D-maltoside
EDTA	Ethylenediaminetetraacetate
GPCR	GTP-binding protein-coupled receptor
HPLC	High pressure liquid chromatography
MBP	<i>Escherichia coli</i> maltose binding protein
NT	Neurotensin
NTR/NTS	Neurotensin receptor
NTS-1A	MBP-rT43NTR-TrxA-H10
SPR	Surface plasmon resonance
TES	Triethylsilane
TFA	Trifluoroacetic acid

Introduction

G-protein coupled receptors (GPCRs), of which over 2000 have now been cloned, are a family of integral membrane proteins with seven transmembrane helices. GPCRs are involved in a wide range of physiological processes, including cell–cell communication, sensory transduction, neuronal transmission and hormonal signalling (Ji and Ji 1998; Iyengar et al. 2002). Consequently, GPCRs are of particular pharmacological importance and they are expected to comprise a large proportion of future drug targets (Terstappen and Reggiani 2001).

Neurotensin (NT) is an endogenous tridecapeptide neurotransmitter (N-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-C) found in the central nervous system and gastrointestinal tract and is responsible for the activation of the neurotensin receptor (NTS) family (Carraway and Leeman 1973). One such receptor, neurotensin receptor type 1

P. J. Harding · T. C. Hadingham · A. Watts (✉)
Biomembrane Structure Unit,
Department of Biochemistry,
University of Oxford, South Parks Road,
Oxford OX1 3QU, UK
e-mail: anthony.watts@bioch.ox.ac.uk

J. M. McDonnell
The Laboratory of Molecular Biophysics,
Department of Biochemistry, University of Oxford,
South Parks Road, Oxford OX1 3QU, UK

(NTS1) is a member of the GPCR superfamily (Tanaka et al. 1990). Central effects of NT include analgesia, inhibition of food intake, modulation of dopaminergic systems, modulation of pituitary hormone secretion and regulation of digestive function. It also acts as a trophic factor on human colon, pancreatic, prostate, and lung cancer cell lines (Vincent et al. 1999). Thus, NTS1 represents a putative target for the treatment of pain, obesity, schizophrenia, and cancer.

Surface plasmon resonance (SPR) allows the sensitive detection of molecular interactions in real time, without the use of labels (Van der Merwe 2000). The technique has proved to be extremely versatile, permitting the study of analytes ranging in size from hundreds of daltons to whole cell binding (McDonnell 2001). Application to receptor–ligand interactions has largely been limited to soluble ligand-binding domains (Kroger et al. 1999; De Crescenzo et al. 2000; Rich et al. 2002). Detailed analysis of ligand–receptor interactions for GPCRs has proved difficult, due to problems encountered in successful overexpression, purification and stabilisation of GPCRs, although solid state NMR approaches indicate a significant electrostatic contribution to receptor–ligand binding (Williamson et al. 2001, 2002; Watts 2005).

Immobilisation of rhodopsin, a GPCR, on the biosensor surface in a supported lipid bilayer has been achieved, allowing the kinetics of transducin activation to be observed (Heyse et al. 1998; Karlsson and Lofas 2002). A similar receptor immobilisation technique was applied to the chemokine receptors CXCR4 and CCR5 and active ligand binding was demonstrated, although with lower affinity than had previously been published (Stenlund et al. 2004) and more recently, ligand binding to α 2-adrenergic receptors has been demonstrated (Sen et al. 2005).

An alternative approach, the measurement of the direct binding of functionally active GPCR to immobilised ligand on the SPR chip, has not been successful until now. Since responses observed in SPR are proportional to the molecular weight of bound analyte, binding of the large receptor protein gives improved sensitivity when compared with analyses in the reverse orientation. This point is key, given the low molecular weight of many GPCR ligands. Here, SPR was used to study the kinetics of the NT–NTS1 interaction as a first example of this approach for a purified, detergent-solubilised GPCR, showing potential for high-throughput ligand screening for this class of receptors.

Materials and methods

Functionally active rat NTS-1 (rNTS-1) was expressed in *Escherichia coli* DH5 α as a fusion protein (termed NTS-1A) from the pRG/III-hs-MBPP-T43NTR1-TrxA-H10 plasmid (Tucker and Grisshammer 1996; Grisshammer et al. 1999; Williamson et al. 2002). The fusion protein consists of *E. coli* maltose binding protein (including its periplasmic targeting sequence) attached to the N-terminus of an N-terminally truncated form of rNTS-1 (rT43NTR), with thioredoxin and a His-10 tag, which aid stability and purification, attached to the C-terminus.

NTS-1A for SPR analysis was solubilised and purified on a large scale as previously described (White et al. 2004). A two-stage purification procedure, involving immobilised metal affinity chromatography (IMAC) followed by an NT affinity column, was carried out, with all steps at 4°C unless otherwise stated. Briefly, a 250 g bacterial pellet (40 mg scale preparation) was resuspended and disrupted by ultrasonication. Functionally active receptors were solubilised using a CHAPS (0.5%)/DDM (1%)/CHS (0.1%) detergent mix. NTS-1A was purified by IMAC using a 100 ml Ni-NTA column. This was followed by a 20 ml NT affinity column, which comprised biotinylated-NT immobilised on tetrameric avidin resin (Promega). NT affinity column eluate was stored at –80°C in NTS-1A storage buffer (50 mM Tris/HCl, pH 7.4, 30% glycerol (v/v), 50 mM NaCl, 0.5% CHAPS, 0.1% CHS, 0.1% DDM, 1 mM EDTA). A ^3H -NT (New England Nuclear) radioligand binding assay was used to quantify amounts of active receptor present. Non-specific binding was quantified in the presence of excess unlabelled NT. Separation of bound from free ligand was achieved using Microspin G-50 columns (GE Healthcare).

N-terminally-biotinylated NT (biotin-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) and ‘scrambled sequence’ NT (biotin-Leu-Tyr-Asn-Arg-Pro-Arg-Pro-Tyr-Leu-Glu-Ile-Lys-Glu) peptide ligands were produced by Fmoc solid phase peptide synthesis using an automated peptide synthesiser (Pioneer, Perspective Biosystems). Peptides were purified to > 95% homogeneity by reversed phase HPLC using a C18 Kromasil 150–10 12 ml semi-preparative column (Hichrom) by applying a gradient of acetonitrile containing 0.1% TFA. The presence of desired product was confirmed using ESI mass spectrometry.

Surface plasmon resonance analysis was carried out using a Biacore 3000 biosensor instrument (Biacore AB) at 25°C. All buffers used were filtered and

de-gassed though a 0.2 μm filter (Whatman). 250 RU of biotinylated NT in HBS-EP buffer (Biacore AP) was immobilised on a streptavidin-coated chip (Bio-sensor SA, Biacore). 250 RU of 'scrambled sequence' biotinylated NT was immobilised in a control flow cell in the same manner. Analyte used was purified, detergent-solubilised NTS-1A, buffer-exchanged into a glycerol-free 'SPR' buffer to reduce bulk shift effects (50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.5% CHAPS, 0.1% CHS, 0.1% DDM, 1 mM EDTA). Interaction experiments were carried out at a flow rate of 20 $\mu\text{l min}^{-1}$, with sample racks maintained at a constant 5°C throughout. Surface regeneration was achieved via two sequential 1 min injections of SPR buffer containing 1 M NaCl to dissociate bound receptor. BiaEvaluation Software v4.1 (Biacore AP) and Origin v6.1 (Microcal Software) were used for data processing.

Results and discussion

Functionally active rat NTS1 was expressed as the NTS-1A fusion protein, which consists of an N-terminally truncated NTS-1 with an *E. coli* maltose binding protein (MBP) attached to the N-terminus and thioredoxin and a deca-His tag attached to the C terminus (Tucker and Grisshammer 1996; Grisshammer et al. 1999; Williamson et al. 2002). NTS-1A for SPR analysis was solubilised and purified on a large scale using a two-stage purification procedure, involving immobilised metal affinity chromatography (IMAC) followed by an NT affinity column (White et al. 2004). An N-terminally-biotinylated NT peptide ligand was produced by solid phase synthesis to facilitate immobilisation on streptavidin-functionalised SPR chips. As a rigorous control for binding, a biotinylated 'scrambled sequence' peptide, but with the same 13 residues as NT, was synthesised to ensure the specificity of any interactions observed with NT.

Specific binding data for the NT–NTS1A interaction was obtained by subtracting the control peptide flow cell response from the immobilised NT flow cell response (Fig. 1a). Responses observed for the flow cell with scrambled control peptide immobilised were not significantly different from a blank flow cell (Fig. 1b), indicating a highly specific interaction of the solubilised receptor with the NT peptide. In the absence of glycerol the half life ($t_{1/2}$) of binding activity decreases from 400 to 50 hours at 4°C (Tucker and Grisshammer 1996). This decay was taken into account when calculating the concentration of active NTS-1A present at the time of the SPR experiment.

The amplitude of the observed response is lower than might be expected, given the immobilisation level of the 1,916 Da peptide ligand (250 RU) and the mass of the receptor being used as the analyte (100 kDa). It is possible that this is caused by occlusion of a proportion of the immobilised ligand, thereby preventing some analyte binding. However, a systematic study of alternative sensor surfaces which might address problems of ligand occlusion has not yet been carried out. Also, whilst the biotinylated NT used in

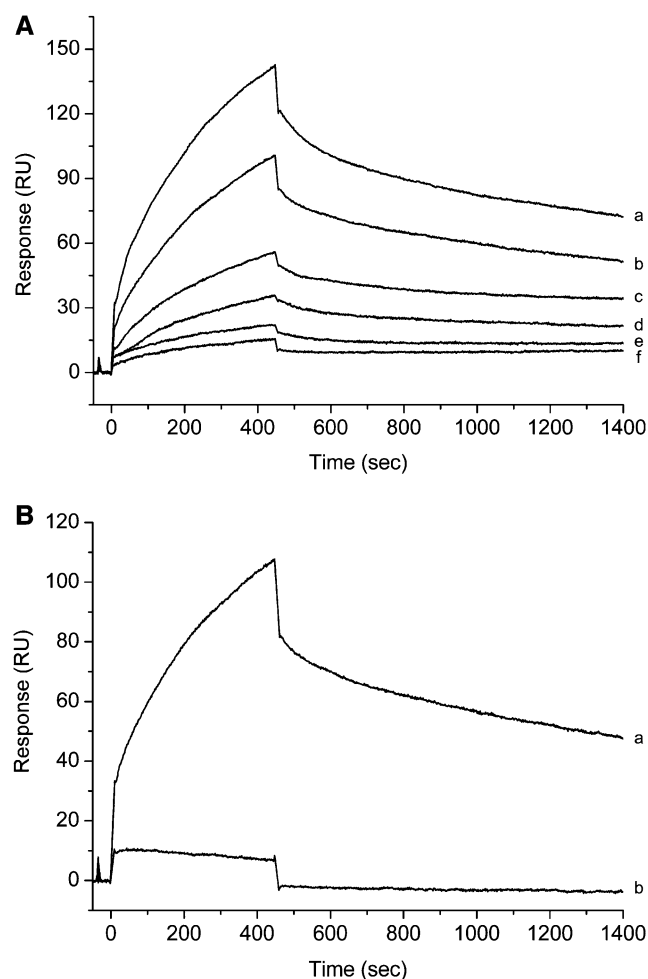


Fig. 1 SPR response curves for the NT/NTS-1A interaction using immobilised NT. **a** The interaction between detergent-solubilised NTS-1A and biotinylated NT. Various concentrations of NTS-1A [5.8 nM (a), 2.9 nM (b), 1.5 nM (c), 0.73 nM (d), 0.36 nM (e), 0.18 nM (f)], were injected at 20 $\mu\text{l min}^{-1}$ over 250 RU immobilised NT and control ligands. The control (scrambled peptide) response is subtracted and therefore only specific binding of NTS-1A to immobilised NT is shown. **b** Binding of detergent-solubilised NTS-1A to the biotinylated NT (a) and control peptide (b) flow cells at a concentration of 2.9 nM. The response obtained for a blank Sensor Chip SA flow cell has been subtracted from both curves, revealing negligible non-specific binding to the control peptide surface

this experiment already has seven residues between the point of immobilisation and the six C-terminal residues known to be involved in receptor binding, an increase in linker length might also relieve any ligand occlusion problems.

This preliminary SPR study has yielded the first SPR binding data using a full length, detergent-solubilised ligand-binding GPCR as the analyte. The use of the large receptor molecule as the analyte overcomes the problem of the small responses encountered in the reverse orientation, specifically the measurement of ligand binding to the immobilised receptor molecule on the chip surface. This technique will have useful applications to other suitable GPCR systems, not only providing insights into the kinetics and binding mechanisms involved in ligand binding, but also in the search for novel ligands and in the more rigorous optimisation of detergent solubilisation procedures than is currently possible using conventional techniques. The study of ligand–GPCR binding events will be crucial if an understanding of how ligand binding at the cell surface is coupled to downstream signalling is to be gained.

Acknowledgements We acknowledge the Medical Research Council (MRC), Biotechnology and Biological Sciences Research Council (BBSRC) and the UK Bionanotechnology IRC for funding this work. We thank GlaxoSmithKline (GSK), specifically Claus Spitzfaden, Martin Hibbs, Emma Jones, and Mark Hessey for allowing access to large scale fermentation, purification and SPR facilities. We would like to thank Dr. Simon Davis (Nuffield Department of Medicine, Oxford) for the use of his SPR machine.

References

- Carraway RE, Leeman SE (1973) Discovery of neurotensin. *J Biol Chem* 248:6854
- De Crescenzo G et al (2000) Real-time kinetic studies on the interaction of transforming growth factor R with the epidermal growth factor receptor extracellular domain reveal a conformational change model. *Biochemistry* 39:9466–9476
- Grisshammer R et al (1999) Improved purification of a rat neurotensin receptor expressed in *Escherichia coli*. *Biochem Soc* 27(5):899–903
- Heyse S et al (1998) Incorporation of rhodopsin in laterally structured supported membranes: observation of transducing activation with spatially and time-resolved surface plasmon resonance. *Biochemistry* 37:507–522
- Iyengar R et al (2002) G-protein pathways. *Methods Enzymol* 343, 344, 345: Parts A, B and C
- Ji TH, Ji I (1998) G Protein-coupled receptors. *J Biol Chem* 28(10):17299–17302
- Karlsson OP, Lofas S (2002) Flow-mediated on-surface reconstitution of G-protein coupled receptors for applications in surface plasmon resonance biosensors. *Anal Biochem* 300:132–138
- Kroger D et al (1999) Ligand binding to nicotinic acetylcholine receptor investigated by surface plasmon resonance. *Anal Chem* 71:3157
- McDonnell JM (2001) Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. *Curr Opin Chem Biol* 5:572–577
- Rich RL et al (2002) Kinetic analysis of estrogen receptor/ligand interactions. *Proc Natl Acad Sci USA* 99(13):8562–8567
- Sen S et al (2005) Functional studies with membrane-bound and detergent-solubilized $\alpha 2$ -adrenergic receptors expressed in Sf 9 cells. *Biochim Biophys Acta* 1712:62–70
- Stenlund P et al (2004) Capture and reconstitution of G protein-coupled receptors on a biosensor surface. *Anal Biochem* 316:243–250
- Tanaka K et al (1990) Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* 4(6):847–854
- Terstappen GC, Reggiani A (2001) In silico research in drug discovery. *Trends Pharmacol Sci* 22:23–26
- Tucker J, Grisshammer R (1996) Purification of a rat neurotensin receptor expressed in *Escherichia coli*. *Biochem J* 317:891–899
- Van der Merwe PA (2000) Surface plasmon resonance in protein–ligand interactions: a practical approach. Oxford University Press, Oxford
- Vincent JP et al (1999) Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* 20:302–309
- Watts A (2005) Solid state NMR in drug design and discovery for membrane embedded targets. *Nat Rev Drug Discov* 4:555–568
- White JF et al (2004) Automated large-scale purification of a G protein-coupled receptor for neurotensin. *FEBS Lett* 564:289–293
- Williamson PTF et al (2001) Characterization and assignment of uniformly labeled NT(8–13) at the agonist binding site of the G-protein coupled neurotensin receptor. In: Kühne SR, de Groot HJM (eds) Focus on structural biology, vol 1. Perspectives on solid state NMR in biology. Kluwer, Dordrecht, pp 191–202
- Williamson PTF et al (2002) Probing the environment of neurotensin whilst bound to the neurotensin receptor by solid state NMR. *FEBS Lett* 518:111–115