Thesis title:

Preparing, measuring and capturing G-protein coupled receptor (GPCR) signalling complexes for future development of cell-free assay technologies

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SUMMARY

G-protein coupled receptors (GPCRs) are integral membrane proteins which represent primary cellular targets for intracellular signalling. Many of these receptors are altered in disease states and hence are the target for over 50% of marketed drugs. Despite their physiological importance, high-throughput, cell-free assays which measure functional or signalling activity are only recently being investigated. The current approach by the pharmaceutical industry to initially screen compounds for functionality is to use heterologous cell-based assay formats. The aim of this work was to reconstitute a cell-free GPCR signalling system on an appropriate platform (surface) as a prototype for future rapid drug screening and other applications. The proof-of-concept approach involved using the α_{2A}-adrenergic receptor (α_{2A}-AR) containing cell membrane preparations as the model GPCR, reconstituted with a set of heterotrimeric G-proteins; $G\alpha_{i1}$ and $\beta_1\gamma_2$ (the signal transducing complex being termed a "transductosome"). However, other receptors and G-proteins were also investigated. Receptors were initially obtained from natural (tissue) sources, however in the later stages they were expressed in a heterologous system (insect or mammalian expression system). G-proteins were expressed in *Spodoptera frugiperida* (*Sf9*) insect cells using the baculovirus expression system. Receptor expression was verified by radioligand binding assays and endogenous G-proteins were removed from membrane preparations using the chaotropic agent urea to allow for reconstitution with purified G-proteins. Signal transduction through the transductosome was measured using the [^{35}S]GTPγS binding assay. Receptor activated [^{35}S]GTPγS binding was used to determine functional reconstitution and to validate that the system was working in the normal physiological manner both on and off a surface (with surface attachment being via histidine attachment on the $G\alpha_{i1\,(6xHIS)}$ subunit). Using the captured (surface-attached) transductosomes, the IC_{50}

values for Rauwolscine, Yohimbine (potent α_2-AR antagonists), Prazosin (potent α_1-AR antagonist) and Propranolol (β-AR antagonist) displayed the appropriate rank order for this class of receptor. This cell-free, surface-attached signalling complex prototype may have use in the future development of drug screening and discovery assay technologies as well as other applications as an alternative to cell-based assays which are not readily amendable to miniaturisation, long term storage and therefore stable robust microarray formats.

DECLARATION:

'I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Olgatina Bucco (August 2005)

"Nobody climbs mountains for scientific reasons. Science is used to raise money for the expeditions, but you really climb for the hell of it." - Sir Edmund Hillary

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PUBLICATIONS AND ABSTRACTS ARISING FROM THIS THESIS

[#]Leifert WR, Aloia, AL, **Bucco, O**, Glatz, RV and McMurchie, EJ (2005) G-protein coupled receptors in drug discovery: nano-sizing using cell-free technologies and molecular biology approaches. *J Biomol Screen*, Dec 10 (8) pp. 765-779.

[#]Leifert WR, Aloia, AL, **Bucco, O** and McMurchie, EJ (2005) GPCR-induced dissociation of G-protein subunits: Gα and Gβγ subunit separation in early stage signal transduction. *Mol Mbrane Biol*, Nov-Dec 22 (6) pp. 507-517.

Bucco O, Leifert WR, Aloia AL, Burnard SL, Barritt GJ and McMurchie EJ (2004) G-Protein receptor transductosome capture for cell-free, rapid drug screening *The Australian Health and Medical Research Congress Proceedings*, Poster presentation. **This poster was awarded the Johnson & Johnson prize for best translational research at the conference.**

Bucco O, Leifert WR, Aloia AL, Burnard SL, Barritt GJ and McMurchie EJ (2004) G-Protein receptor transductosome capture for cell-free, rapid drug screening. *The Australian Society for Medical Research South Australian Division Annual Scientific Conference Proceedings*, Oral presentation.

Bucco O, Leifert WR, Aloia, AL, Burnard, SL, Barritt, GJ and McMurchie, EJ (2005) Designing novel cell-free drug screening platforms using captured G-protein coupled receptor 'transductosomes'. *BIO 2005 Innovation corridor poster session proceedings*. Poster presentation.

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[#] Full paper

ABBREVIATIONS

β_1AR- β-adrenergic receptor
α_{2A}AR- α_{2A} adrenergic receptor
[^{35}S]GTPγS- radioactive isotope [^{35}S] conjugated to GTPγS
AAGR- average annual growth rate
AC– adenylate cyclase
AlF_4^-- aluminium fluoride
AMP– adenosine monophosphate
Ang II– Angiotensin II
AT_{1A}– Angiotensin II type 1
ATP– adenosine triphosphate
Bp– base pairs
BRET- bioluminescence resonance energy transfer
BSA- bovine serum albumin
cAMP– cyclic adenosine monophosphate
CAMs– constitutively active receptor mutants
CHN- CSIRO Division Human Nutrition*
CHO– Chinese hamster ovary cells
CMC– critical micelle concentration
CMHT- CSIRO Division of Molecular and Health Technologies*
CSIRO- Commonwealth Scientific and Industrial Research Organisation
Da– daltons
DMF- dimethylformide
DMSO– dimethyl sulphoxide
DNA– deoxyribose nucleic acid
DTT– dithiothreitol
E.coli– Escherichia coli
EC_{50}– effective concentration at which half the maximum effect is achieved
FBS- foetal bovine serum
FPR- formyl peptide receptor
FRET- fluorescence energy transfer
Gα- G-protein alpha subunit
GAP– GTPase activating protein
GAPS-γ-aminopropylsilane
GDI- guanosine dissociation inhibitor
GDP- guanosine diphosphate
GEF- guanosine exchange factor
GF/C– glass fibre filter mats
GPCR- G-protein coupled receptor
G-protein– heterotrimeric guanine nucleotide (guanosine) binding protein
GTP– guanosine triphosphate
GTPγS– guanosine 5-O-(3-thiotriphosphate)
HPLC- high-performance liquid chromatography
HTS- high throughput screening
i.e.- in other words; that is to say that
IC_{50}- inhibitory concentration at 50% or also referred to as the molar concentration of an antagonist that reduces the response to an agonist by 50%.

* Formerly the CSIRO Division Health Sciences and Nutrition

ICYP- [^{125}I]-(-) Iodocyanopindolol
IMAC-immobilised metal affinity chromatography
IP_3- inositol trisphosphate
k_{assoc}- rate of association
k_{cat}- overall reaction rate of catalysis
K_d– dissociation constant
k_{dissoc}- rate of dissociation
k_{hydro}- rate of hydrolysis
MOI– multiplicity of infection
Ni(NTA)– nickel-nitrilotriacetic acid
NMR- nuclear magnetic resonance spectroscopy
PEG- polyethylene glycol
PEI- polythyleneimine
PFM–plaque forming units
PMSF- phenylmethylulfonyl
RAMPs- receptor activity modifying proteins
RGS- regulator of G-protein signalling
S/B- signal to background
S/N- signal to noise
SD– standard deviation
SDS- PAGE– sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM- standard error of the mean
*Sf*9- *Spodoptera frugiperda* 9
SPA- scintillation proximity assay
SPR- surface plasmon resonance
TIFR- total internal florescence refraction
UK-14304- synthetic adrenaline analogue