PROGRAM TIMETABLE

Molecular Pharmacology of G Protein-Coupled Receptors 2003

7:30 – 8:25 am REGISTRATION

8:25 – 8:30 am WELCOME

First Session ***Receptor activation and signaling I***

Chair: Arthur Christopoulos

8:30 – 9:15 am **Steve Nahorski**

"Visualising GPCR activated phosphoinositide and Ca^{2+} signalling in single cells"

9:15 – 10:00 am **Lakshmi Devi**

"G protein coupled receptor dimerization: Implications for a role in signaling and drug development"

10:00 – 10:45 am **John Northup**

"G protein subunit interactions with G protein-coupled receptors"

10:45 – 11:15 am MORNING TEA/POSTER VIEWING

Second Session ***Receptor regulation***

Chair: Roger Summers

11:15 am – 12:00 pm **Eamonn Kelly**

"Protein kinase regulation of mGluR1 splice variants"

12:00 – 12:45 pm **Phil Robinson**

"Phosphorylation of endocytic proteins in synaptic vesicle endocytosis"

12:45 – 1:00 pm **Ross Bathgate**

"The relaxin peptide family and their novel G protein-coupled receptors"

1:00 – 2:00 pm LUNCH/POSTER VIEWING

Third Session ***Class II G protein-coupled receptors***

Chair: Patrick Sexton

2:00 – 2:45 pm Marc Laburthe

"The VPAC1 receptor, a prototypical class II GPCR: Molecular basis of its phenotype"

2:45 – 3:30 pm Larry Miller

"The secretin receptor: a prototypic member of the class II family of GPCRs"

3:30 – 4:00 pm AFTERNOON TEA/POSTER VIEWING

Fourth Session ***Receptor activation and signaling II***

Chair: Bob Graham

4:00 – 4:45 pm Susan Steinberg

"Beta-adrenergic signalling in cardiac membrane microdomains"

4:45 – 5:30 pm Brian Kobilka

"Ligand-induced conformational changes in the beta-2 adrenoceptor"

5:30 – 6:25 pm Ruben Abagyan

2003 VCCRI PRINCESS' LECTURE

(Sponsored by the Victor Chang Cardiac Research Institute)

"Computational structural genomics and GPCRs"

6:25 – 6:30 pm CLOSING REMARKS

6:30 – 8:00 pm DRINKS/MIXER

First session:

Receptor activation and signaling I [8:30 – 10:45 am]

Chair: Arthur Christopoulos

8:30 – 9:15 am **Steve Nahorski**

"Visualising GPCR activated phosphoinositide and Ca^{2+} *signalling in single cells"*

9:15 – 10:00 am **Lakshmi Devi**

"G-protein coupled receptor dimerization: Implications for a role in signaling and drug development"

10:00 – 10:45 am **John Northup**

"G-protein subunit interactions with G-protein-coupled receptors"

Visualising GPCR Activated Phosphoinositide and Ca^{2+} Signalling in Single Cells

S.R. Nahorski, M.S. Nash, K.W. Young, J.M. Willets, R.A.J. Challiss

Dept. of Cell Physiology & Pharmacology, University of Leicester, LEICESTER. LE1 9HN. UK.

Over the last two decades our understanding of the nature and regulation of GPCRs that preferentially couple through $Gq_{/11}$ to activate phospholipase Cβ has dramatically increased. We now know that this phosphoinositide signalling cascade is present in virtually all cells and more GPCRs use this transduction mechanism than any other. Moreover, the use of Ca^{2+} sensitive dyes has allowed imaging of single cell intracellular Ca^{2+} and revealed a diversity of patterns including oscillations and waves and these spatio-temporal responses add greatly to the versatility of Ca^{2+} signalling. However, until recently it has not been possible to visualise the upstream messengers, IP_3 and diacylglycerol in real-time in individual cells and this has greatly hindered our understanding of this fundamental signalling pathway.

Here we will describe our attempts to use translocating fluorescent biosensors to track various aspects of GPCR-phosphoinositide signalling in simple recombinant cells and in primary hippocampal neurones to illustrate how they have clarified our understanding of this signalling pathway. Most of our studies have utilised the transient transfection of constructs to express fusion proteins of various signalling domains and e-GFP. In particular, we have extensively used the PH-domain of PLCδ1 (PH-PLCδ-eGFP) to detect changes in PIP_2 hydrolysis and IP_3 accumulation. The translocation of this biosensor from the plasma membrane to the cytosol can be detected by confocal microscopy and in both model cells and primary neurones it detects IP_3 accumulation rather than PIP_2 depletion **[1]**.

We have used these approaches to address several issues of GPCR phospholipase C-Ca^{2+} signalling and we will review recent published evidence that Ca^{2+} oscillations following the activation of certain GPCRs (e.g mGluR5) appear to be driven by oscillations of IP_3 **[2-5]**. New data will also be presented on the use of these biosensors in primary hippocampal neurones.

Primary hippocampal neurones prepared from new born rats were transfected at 5 DIV with PH-PLCδ-EGFP and imaged at >12 days DIV. Imaging from the cell soma or dendrites, M_1 muscarinic $G_q/_{11}$-mediated generation of IP_3 could be visualized. These responses were markedly enhanced when the cultures were induced to display synchronous synaptically-driven networks revealed by Ca^{2+} spikes following Ca^{2+} entry via ligand- and voltage-sensititive Ca^{2+} channels. Moreover, only under these conditions could the M_1 receptors mobilise intracellular Ca^{2+} stores in these neurones. We believe that these data indicate how the integration of GPCR and ionotropic-stimulated Ca^{2+} signalling in neurones amplify IP_3 generation that may overcome spatial or metabolic barriers to Ca^{2+} store release in differentiated cells. It seems probable that these and other related imaging technologies will transform our appreciation of GPCR signalling in primary cells/tissues.

1. Nahorski S.R., Young K.W., Challiss R.A.J. and Nash M.S. (2003) Visualizing phosphoinositide signalling in single neurons gets a green light. Trends in Neuroscience. 26, 444-452

2. Nash M.S., Young K.W., Challiss R.A.J. and Nahorski S.R. (2001) Receptor-specific messenger oscillations. *Nature* 413, 381-2

3. Nash M.S., Young K.W. Willars, G.B., Challiss R.A.J. and Nahorski S.R. (2001) Single cell imaging of graded Ins(1,4,5)P_3 production following G-protein-coupled-receptor activation. *Biochem. J.* 356, 137-142.

4. Nash, M.S., Schell, M.J., Atkinson, P.J., Johnston, N.R., Nahorski, S.R. and Challiss, R.A.J. (2002) Determinants of metabotropic glutamate receptor-5-mediated Ca^{2+} and inositol 1,4,5-trisphosphate oscillation frequency: receptor density *versus* agonist concentration. J. Biol. Chem. 277, 35947-35960.

5. Young K.W., Nash M.S., Challiss R.A.J. and Nahorski S.R. (2003) Role of Ca^{2+} feedback on single cell inositol 1,4,5-trisphosphate oscillations mediated by G-protein-coupled receptors. J. Biol. Chem. 278, 20753-20760.

G-protein coupled Receptor dimerization: Implications for a role in signaling and drug development.

Lakshmi A. Devi

Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, USA.

Opioid receptors belong to the family of G-protein coupled receptors characterized by their seven transmembrane domains. The activation of these receptors by narcotic analgesics or by endogenous opioid peptides leads to the activation of inhibitory G-proteins followed by the activation of multiple signal transduction pathways. A number of investigations have suggested that opioid receptor types interact with each other. Previous studies using receptor selective antagonists, antisense oligonucleotides or animals lacking opioid receptors have suggested that these interactions modulate receptor activity. We examined opioid receptor interactions (homotypic and heterotypic) using biochemical, biophysical and pharmacological techniques. Recently, using receptor type-selective antibodies directed against the N-terminal region of opioid receptors we were able to immunoisolate interacting complexes from heterologous cells as well as endogenous tissue. Furthermore, we were able to explore changes in the conformation of interacting receptors upon agonist binding with these antisera. Finally, we find that these receptor-receptor interactions modulate receptor function as evidenced by *in vitro* and *in vivo* assays. Taken together these studies support the notion that receptor-receptor association is an universal phenomenon and provides a mechanism for cross-talk between members of the G-protein coupled receptor family.

G-Protein Subunit Interactions with G-Protein-Coupled Receptors

John K. Northup

Laboratory of Cellular Biology, NIDCD, National Institutes of Health, Bethesda, Maryland 20892, USA

The activation of specific heterotrimeric G-proteins initiates a cascade of intracellular events mediating the signaling responses of G-protein-coupled receptors (GPCRs) to agonist ligands. In order to examine the molecular details of this initial biochemical event in GPCR function, we have devised methods for the *in situ* reconstitution of recombinant expressed receptors with recombinant G-protein subunits. Extraction of plasma membrane-enriched fractions with 7M urea from cells expressing a high abundance of a variety of receptors with family 1 structure yields membranes more than 95% depleted of active G-protein retaining 100% of receptor binding for antagonist but not agonist ligands. These membranes serve a source of uncoupled receptors for the addition of fractions of purified, defined G-protein subunits to examine ligand activation. These procedures have been successfully applied in our laboratory to $5HT_{1A}$, $5HT_{2C}$, M1-muscarinic, α_2-adrenergic, CB_1- and CB_2-cannabinoid, V_{1a}-vassopressin, ocytocin, GRP, neuromedin B and the subtype3 bombesin receptors. Detailed examination of the 5HT, CB and bombesin receptors have revealed a number of conserved features of G-protein interaction as assessed by agonist-stimulated GTP-binding. First, the ligand-stimulated GTPγS binding reactions conform quite well to the expecations of a simple Michaelis-Menton enzyme reaction with the rate of the receptor-catalyzed activation of G-protein dependent upon fractional occupancy of the agonist. *In situ* reconstitution provides an assay system with no spare receptors in which maximal rates of G-protein activation at saturating ligand can be used to compare agonist efficacies directly. Second, all of these receptors have measurable "basal" activities. For the $5HT_{2C}$ and CB_1 receptors, several antagonist compounds are revealed to be inverse agonists by these assays. Lastly, each receptor we have examined displays a unique profile of K_m and V_{max} values for defined G-protein subunits, suggesting that this approach can provide a quantitative assessment of G-protein specificity for each GPCR.

To complement these biochemical studies, we have developed a biophysical approach to examine GPCR-G-protein subunit binding directly using surface plasmon resonance detection (SPR). To date these methods have been applied only to rhodopsins, but the methods in principle should apply to any GPCR. Using rhodopsins immobilized via extracellular carbohydrate binding to covalently-linked concanavalin A, we can measure the kinetic and thermodynamic properties of the independent and synergistic interactions of Gαβγ and Gβγ subunits. First, as found for the *in vitro* activation of GTPγS binding to Gα, SPR analysis shows that lipid modifications of Gα and Gγ are essential for binding to rhodopsin. Even with identically lipid-modified subunits, SPR reveals that Gβγ dimers with distinct primary structures display dramatically different rates of association and dissociation from bovine rhodopsin. Also, Gα subunits with differing rates of rhodopsin-catalyzed GTPγS binding display dramatically different dissociation rates from bovine rhodopsin that correlate with the rate of rhodopsin activation. Lastly, the dissociation of Gα from rhodopsin, as well as the overall rate of turnover for guanine nucleotide exchange, is independent of guanine nucleotide. These data suggest a mechanism for GPCR activation of G-protein in which the rate of catalysis is limited by the dissociation of an "empty" Gα from the receptor-Gαβγ complex. We are currently modifying our procedures to facilitate the examination of other family 1 GPCR structures by these techniques in order to develop a more complete understanding of the mechanism(s) of GPCR function.

Second Session:

Receptor regulation [11:15 am – 1:00 pm]

Chair: Roger Summers

11:15 am – 12:00 pm Eamonn Kelly

"Protein kinase regulation of mGluR1 splice variants"

12:00 – 12:45 pm Phil Robinson

"Phosphorylation of endocytic proteins in synaptic vesicle endocytosis"

12:45 – 1:00 pm Ross Bathgate

"The relaxin peptide family and their novel G protein-coupled receptors"

Protein kinase regulation of mGluR1 splice variants

Stuart Mundell, Giordano Pula, Peter Roberts and Eamonn Kelly

Department of Pharmacology, University of Bristol, School of Medical Sciences, U.K.

The group I metabotropic glutamate receptors (mGluRs) are $G_{q/11}$-coupled GPCRs consisting of mGluR1, mGluR5 and their respective C-terminus splice variants. The mGluR1 splice variants include mGluR1a and mGluR1b, which have long and short C-terminus tails, respectively. Another important difference is that mGluR1a is constitutively active, whereas mGluR1b is not. Along with other GPCRs, group I mGluRs appear to undergo regulatory processes such as desensitization and internalization. Here we have investigated the role of both G protein-coupled receptor kinases and second messenger-dependent kinases such as PKC and PKA in the regulation of mGluR1a and mGluR1b. For the experiments described here, the splice variants were HA epitope-tagged in the N-terminus and transiently expressed in HEK293 cells.

We have found that the glutamate-induced regulation of mGluR1a is both GRK- and PKC/CaMKII-dependent, whereas that of mGluR1b is only GRK-dependent. To investigate the molecular basis of this phenomenon, we assessed the ability of GRK2 and arrestin-2 to associate with these receptors in co-immunoprecipitation experiments. Glutamate increased the association of GRK2 and arrestin-2 with both splice variants. However intriguingly, we found that inhibition of PKC/CaMKII blocked the glutamate-induced association of GRK2 with mGluR1a but not mGluR1b. Thus the glutamate-induced association of GRK2 with mGluR1a requires PKC/CaMKII activation.

Activation of heterologous $G_{q/11}$-coupled GPCRs (e.g. muscarinic receptors with carbachol) in the cells also promoted the desensitization and internalization of agonist-unoccupied mGluR1a and mGluR1b. This time, however, PKC/CaMKII inhibition blocked the carbachol-induced regulation of both splice variants. In co-immunoprecipitation experiments, carbachol increased the association of GRK2 and arrestin-2 with mGluR1a but not mGluR1b. The carbachol-induced association of GRK2 with mGluR1a was also inhibited by an inverse agonist at mGluR1a. This means that muscarinic receptor activation triggers the association of GRK2/arrestin-2 with mGluR1a, but only when the latter is in an active conformation.

In our hands, mGluR1 activation with glutamate does not increase cAMP levels in the cells. However, activation of PKA with forskolin or an agonist at a G_s-coupled receptor, inhibits the glutamate- and carbachol-stimulated desensitization and internalization of mGluR1a and mGluR1b. Most interestingly, PKA activation also inhibits the glutamate-induced association of GRK2/arrestin-2 with mGluR1a and mGluR1b, as well as the carbachol-induced association of GRK2/arrestin-2 with mGluR1a.

Taken together, these results indicate that PKC/CaMKII activation is necessary for the agonist-induced association of GRK2/arrestin-2 with mGluR1a, whereas PKA activation blocks this association. These may represent new mechanisms for GPCR regulation by second messenger-dependent protein kinases. On the other hand GRK2/arrestin-2 association with mGluR1b does not require PKC/CaMKII activity. However, PKA activation is able to inhibit glutamate-stimulated GRK2/arrestin association with this short splice variant. Our future experiments will focus on whether PKC/CaMKII and PKA directly phosphorylate the splice variant C-terminal tails, and whether it is this that regulates GRK2 interaction with the receptors.

Phosphorylation of endocytic proteins in synaptic vesicle endocytosis

Phillip J Robinson

Cell Signalling Unit, Children's Medical Research Institute, Westmead, NSW, Australia

Exocytosis is the release from nerve terminals of neurotransmitters which are packaged in small synaptic vesicles (SVs). Within 1 second, synaptic vesicle endocytosis (SVE) retrieves the empty SV to replenish the small vesicle pool within nerve terminals. SVE is thus important for sustaining synaptic transmission. Receptor-mediated endocytosis (RME) is the internalisation of activated growth factor receptors and GPCRs from the surface of cells, to initially contribute to their signalling and to ultimately terminate signalling. The molecular machinery underlying SVE and RME are essentially identical, but they have quite distinct triggers and modulatory factors. SVE is activated by a calcineurin-mediated dephosphorylation event that is stimulated by depolarization-dependent calcium (Ca^{2+}) influx in neurons. Eight endocytic proteins, collectively called dephosphins, are dephosphorylated by calcineurin in nerve terminals. They include dynamin I, synaptojanin, amphiphysin, epsin, and AP180. Each dephosphin is implicated at different stages in SVE. In non-neuronal cells, phosphorylation plays a major role in regulation of RME, but the kinases and phosphatases involved are poorly understood at this stage.

The dephosphins are constitutively phosphorylated in resting nerve terminals, and their rephosphorylation after the termination of SVE is essential for subsequent rounds of endocytosis. The protein kinases that mediate the essential rephosphorylation step are still undetermined. Protein kinase C (PKC) has been proposed to be a dephosphin kinase, since it phosphorylates dynamin I *in vitro* on Ser-795 and PKC inhibitors block SVE. Dynamin I has a C-terminal proline rich domain (PRD) containing numerous binding motifs for SH3 domains. The interaction of the dynamin I PRD with SH3 domains of amphiphysin or endophilin is essential for SVE. The PRD is also the site of endogenous dynamin I phosphorylation. Cyclin-dependent kinase 5 (cdk5) is an attractive candidate for the endogenous dynamin I kinase in nerve terminals, since the PRD contains multiple Ser-Pro or Thr-Pro motifs that are potential targets for cyclin-dependent kinases. In the adult brain cdk5 and its activator protein, p35 are both enriched in nerve terminals. Our aim was to determine whether cdk5 is the dynamin I kinase and if it is required for maintenance of SVE.

Using MALDI-TOF mass spectrometry we found that cdk5 phosphorylates dynamin I on residues Ser-774 and Ser-778 *in vitro*. Phospho-dynamin I was isolated from intact nerve terminals and the endogenous phosphorylation sites *in vivo* were found to be the same. This rules out PKC as the endogenous kinase. The cdk5 antagonist roscovitine blocked phosphorylation of dynamin I, but not of amphiphysin or AP180, and inhibited SVE. In neuronal cell lines and primary cultures of neurons from brain, the expression of dominant-negative cdk5 abolished dynamin phosphorylation in axons and nerve terminals and inhibited SVE. We developed a morphological assay of SVE in synaptosomes by use of electron microscopy. Cdk5 inhibitors had no effect on nerve terminal morphology after a single stimulation, but blocked SV replenishment after repetitive rounds of stimulation which depletes constitutive phosphorylation. The experiments have two important outcomes. Firstly, we have revealed that cdk5 is the first identified dephosphin kinase in nerve terminals and is essential for SVE. Secondly, we have revealed the presence of a second dephosphin kinase that is responsible for the phosphorylation of amphiphysin and AP180 during SVE. It will be important to determine whether similar phosphorylation events play a role in the control of dynamin II-mediated RME.

The relaxin peptide family and their novel G protein-coupled receptors

Ross A.D. Bathgate, Daniel Scott, Ping Fu, Sharon Layfield, Tania Ferraro, John D. Wade, Satoko Sudo*, Jin Kumagai*, Aaron JW Hsueh* and Geoffrey W. Tregear.

Howard Florey Institute, University of Melbourne, Victoria 3010, Australia, * Department of Obstetrics and Gynecology, Stanford University Medical Centre, Stanford, USA.

Insulin-like peptide 3 (INSL3; relaxin-like factor, RLF), relaxin-1 and relaxin-3 are all members of the insulin/relaxin family of peptide hormones. They have distinct patterns of expression and sites of action. INSL3 is primarily a product of the testicular Leydig cells and is essential for testicular descent. Relaxin-1 is essential for the development of the reproductive tract during pregnancy and for normal tissue collagen turnover. H2 relaxin is the human relaxin-1 equivalent, H1 relaxin is primate specific. Relaxin-3 (human H3, mouse M3) was recently identified by our group and is a specific neuropeptide. Research into these peptides has been limited by the inability of researchers to identify specific receptors. However, recently porcine relaxin-1 was shown to activate two orphan leucine-rich repeat-containing G protein-coupled receptors (LGR), LGR7 and LGR8.

Data from in vitro and in vivo experiments clearly indicate that the LGR7 is the relaxin-1 receptor, however the LGR8 is actually the receptor for INSL3. Using cells transfected with human LGR7 and LGR8 together with numerous relaxin and INSL3 peptides and specific relaxin binding and activation (cAMP) assays we have studied their ligand binding and activation specificity. The LGR7 receptor demonstrated high affinity for relaxin peptides but only bound INSL3 with very low affinity. In contrast the LGR8 receptor showed highest affinity for INSL3, but showed differential binding specificity for relaxin peptides. Importantly, H3 and pro-M3 relaxin as well as rat relaxin-1 did not stimulate cAMP production at concentrations up to 10µM. These data demonstrate relaxin-3 is a LGR7 specific ligand and that there are species differences in relaxin-1 LGR8 receptor specificity. Therefore although LGR8 is undoubtedly the INSL3 receptor, the biology of INSL3 action and hence testis descent may be complicated in some species by the actions of relaxin-1. Interestingly, relaxin-3 has recently been demonstrated to bind and activate two other novel G-protein coupled receptors, GPCR 135 & GPCR 142. These receptors do not bind other relaxin family members.

The LGR receptor family includes the glycoprotein hormone receptors LHR, FSHR and TSHR as well as three other orphan receptors LGR4-6. The LGR7 and LGR8 receptors form a subclass of this family with a distinct hinge-like region of the receptor and a unique LDL receptor-like domain at their N-terminus. We have been utilizing various chimeric LGR7 and LGR8 receptors as well as native splice variants of the LGR7 and LGR8 to study the determinants of ligand binding and activation of these two novel receptors. Chimeras of LGR8 with an LGR7 LDL receptor-like domain demonstrate INSL3 binding but no activation. Furthermore, a native splice variant of the LGR8 receptor which is missing the LDL domain, also binds INSL3 and H2 relaxin but does not show signaling. These data indicate that the LDL receptor-like domain is essential for receptor signaling and the leucine-rich repeats (LRRs) are the sites of primary ligand binding. More recent data indicates that the transmembrane exoloops may also have a role in secondary receptor binding and subsequent signaling. We therefore now have a basic model of LGR7/8 function that involves ligand binding to a primary site in the LRRs followed by a secondary binding site in the TM-domains. The LDL receptor-like domain is then essential for ligand directed activation.

Further studies on these unique receptors will increase our understanding of the mechanisms of action of these peptide hormones as well as their clinical potentials.

Third Session:

Class II G protein-coupled receptors [2:00 – 3:30 pm]

Chair: Patrick Sexton

2:00 – 2:45 pm **Marc Laburthe**

“The VPAC1 receptor, a prototypical class II GPCR: Molecular basis of its phenotype”

2:45 – 3:30 pm **Larry Miller**

“The secretin receptor: a prototypic member of the class II family of GPCRs”

The VPAC1 receptor, a prototypical class II GPCR: Molecular basis of its phenotype

Marc Laburthe, Alain Couvineau, Yossan Var Tan

French Institute of Health and Medical Research, INSERM U410, Laboratory of Neuroendocrinology and Cell Biology of the Digestive Tract, Faculty of Medicine Xavier Bichat, 75018 Paris, France (email: laburthe@inserm.bichat.fr)

Class II G protein-coupled receptors (GPCR) for peptides (VIP, PACAP, PTH, secretin, glucagon, calcitonin etc…) display original common properties including a large N-terminal ectodomain (> 120 amino acid residues) with 6 strictly conserved cysteins, a N-terminal leader sequence, multiple consensus N-glycosylation sites, high sequence homologies within the transmembrane domains and the N-terminal ectodomain and a complex gene organization with > 12 introns (Laburthe et al, Ann. N. Y. Acad Sci., 805: 94-109, 1996). The human VPAC1 receptor for the neuropeptides VIP (vasoactive intestinal peptide) and PACAP (pituitary adenylate cyclase activating peptide) has served as a prototypical class II G protein-coupled receptor (GPCR) for understanding the structure-phenotype relationship of this class of GPCR (Laburthe et al, 2002). i) Site-directed mutagenesis shows that the N-terminal ectodomain plays a predominant, though not exclusive, role in VIP binding (Laburthe et al, Receptors Channels 8: 137-153, 2002). A 3D-structure model of this domain indicates the presence of a putative binding groove (Lins et al, J. Biol. Chem. 276 : 10153-10160 2001) and fits with the existence of 3 S-S bonds between conserved cysteins. The cystein pairing (C1-C3; C2-C5; C4-C6) represents a new signature of class II GPCR. Photoaffinity labeling of VPAC1 receptor with ^{125}I-[Bpa^{22}-VIP] followed by receptor digestions demonstrates physical proximity between the biologically crucial central part of VIP (Nicole et al, J. Biol. Chem., 275 : 24003-24012, 2000) and the putative binding groove within the N-terminal ectodomain of the receptor (Tan et al, J. Biol. Chem. 278 : 36531-35536 2003); ii) Selectivity filters (Couvineau et al, J. Biol. Chem., 271: 12795-12800, 1996; Du et al, J. Biol. Chem., 277 : 37016-37022, 2002) have been characterized. They restrict access to VIP-related peptides (secretin, PHI) and are made of small clusters of amino acids present at the junction between extracellular domains and transmembrane segments; iii) Cytoplasmic domains required for activation of adenylyl cyclase have been identified (Couvineau et al, J. Biol. Chem., 278 : 24759-24766 2003). Two charged amino acids in intracellular loop 3 and proximal C-terminal tail are crucial. They are strictly conserved in all class II GPCRs. Therefore, it appears that the VPAC1 receptor follows a paradigm reminiscent of, but not identical to, that of class I GPCR; iv) Receptor activity modifying proteins seem to play an important role in the expression of class II GPCR phenotypes. Recent data from P. Sexton's group indicate that RAMP2 augments VPAC1 receptor signaling with respect to phosphoinositide hydrolysis with no change in cAMP response (Christopoulos et al, J. Biol. Chem., 278: 3293-3297 2003). Altogether, these observations document the molecular basis of the VPAC1 receptor phenotype and more generally shed light on the functioning of class II GPCR for peptides.

The secretin receptor: a prototypic member of the class II family of GPCRs

Laurence J. Miller, Maoqing Dong, Zhijun Li*, and Terry P. Lybrand*

Mayo Clinic, Cancer Center and Department of Molecular Pharmacology and Experimental Therapeutics, Scottsdale, AZ 85259 and *Vanderbilt University, Department of Chemistry and Center for Structural Biology, Nashville, TN 37232

Consistent with its historical position as representing the first hormone and launching modern endocrinology, secretin also has a prominent position in our understanding of receptors. The secretin receptor was the first member of the class II family of G protein-coupled receptors that was cloned. It has since become clear that this is prototypic of the entire family, binding and being activated by a moderately large natural peptide ligand with a diffuse pharmacophoric domain. The long and complex, disulfide-bonded amino-terminal tail domain of this receptor plays a critically important role in ligand recognition. This domain includes three intradomain disulfide bonds, but no such bonds to the body of the receptor. By using receptor mutagenesis and photoaffinity labeling approaches, we have built a series of structural constraints that can be built into a working three-dimensional model of this receptor. The amino-terminal tail domain provides a platform for secretin binding that includes a broad array of residue-residue approximations with the docked peptide ligand that span the entire pharmacophoric region of this hormone. Key sites of labeling are located within the distal 36 residues of the tail and the region of the tail just outside of the first transmembrane segment. The amino-terminus of secretin is directed toward the top of the sixth transmembrane segment of the receptor, possibly providing a tether to transmit force from the binding platform to the stable confluence of helices representing the body of this receptor. Much remains to be established for the structure of the helical confluence for the class II family of GPCRs, based on conserved differences in sequence of this group from that of the rhodopsin family in which a crystal structure now exists. Other features of secretin receptor structure, function, and regulation are fully analogous to those features of the class I family of GPCRs. These include biochemical and cellular mechanisms of desensitization, such as agonist-stimulated receptor phosphorylation and receptor internalization and trafficking. A particularly interesting variant of the secretin receptor was recently described, representing a misspliced form of this receptor that is common on pancreatic carcinomas, acting as a dominant-negative inhibitor of normal receptor function. Since the secretin receptor normally acts to inhibit cell growth, this inhibition of function is growth-promoting and may have pathologic importance. In exploring the molecular basis of this effect, it was shown that the secretin receptor can form dimers on the cell surface and that the misspliced form of this receptor that is not able to bind secretin or signal in response to secretin can also form complexes with the wild type receptor. As the structure of this physiologically important receptor is further characterized and refined, and as we learn more about its mechanisms of action and regulation, these insights should help in the development and refinement of new receptor-active drugs with a variety of novel therapeutic applications.

Fourth Session:

Receptor activation and signaling II [4:00 – 6:30 pm]

Chair: Bob Graham

4:00 – 4:45 pm Susan Steinberg

"Beta-adrenergic signalling in cardiac membrane microdomains"

4:45 – 5:30 pm Brian Kobilka

"Ligand-induced conformational changes in the beta-2 adrenoceptor"

5:30 – 6:25 pm Ruben Abagyan

2003 VCCRI PRINCESS' LECTURE
(Sponsored by the Victor Chang Cardiac Research Institute)

"Computational structural genomics and GPCRs"

Beta-adrenergic signaling in cardiac membrane microdomains

Susan F. Steinberg, MD

Columbia University, NYC

β-Adrenergic receptors (β-ARs) provide an important source of inotropic support, but also induce abnormalities in cardiomyocyte growth, energy utilization, and calcium regulation that accelerate the natural history of heart failure. The cardiac actions of catecholamines generally have been attributed to the predominant β_1-AR that couples to G_s and cAMP accumulation. However, cardiomyocytes co-express β_2-ARs, which gain importance in heart failure (when β_1-ARs are down-regulated). β_2-ARs provide an ancillary mechanism for inotropic support and also couple to signaling pathways that influence cardiomyocyte growth and/or survival during ischemic stresses. β_1- and β_2-ARs have very similar signaling phenotypes when heterologously expressed in undifferentiated cell lines. However, the signaling phenotypes for native β_1- and β_2-ARs in highly-differentiated cells such as cardiomyocytes are quite distinct. Cardiomyocyte β_1- and β_2-ARs exhibit important differences in their coupling to the cAMP/PKA pathway as well as to effectors not traditionally identified as targets of β-ARs (such as components of MAPK cascades and the PI3-K/AKT signaling pathway). We have attributed certain aspects of signaling specificity to the distinct spatial distributions of β_1- and β_2-ARs in cardiomyocyte membrane subdomains. Specifically, β_2-ARs are confined to caveolae (or lipid raft) membranes, in association with the bulk of the adenylyl cyclase enzyme, in resting cardiomyocytes; β_2-ARs egress from caveolae upon activation. In contrast, β_1-ARs partition between caveolae and other membrane subdomains at rest and don't detectably traffic when activated. The presentation will focus on recent progress defining the role of β-AR compartmentation to distinct membrane subdomains as a mechanism to bias receptor coupling to distinct subsets of downstream effectors.

Ligand-induced conformational changes in the beta2-adrenoceptor

Brian Kobilka

Department of Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305

G protein coupled receptors (GPCRs) constitute the largest family of receptors in the human genome and are important targets for drug discovery. The beta 2 adrenoceptor is a prototypical Family A GPCR that mediates physiologic responses to adrenaline and noradrenaline. In cells, the function of the beta 2 adrenoceptor can be modulated by a spectrum of synthetic ligands including full agonists (maximal activation), partial agonists (submaximal activation), neutral antagonists (no biological effect), and inverse agonists (inhibit basal activity). I will discuss biochemical and biophysical approaches to map the conformational changes that occur following the binding of these different classes of ligands. I will also talk about efforts to characterize dynamic properties of the beta 2 adrenoceptor structure (the existence of distinguishable conformational states and the transitions between states) in the presence of various drugs.

Computational structural genomics and GPCRs

Ruben Abagyan, PhD

Department of Molecular Biology, The Scripps Research Institute

Advanced modeling by homology techniques can be employed to generate 3D models for most of the interesting new gene family members. This opens many new opportunities for structural structure based functional annotation and quick identification of lead compounds via flexible docking and virtual ligand screening.

Pocket identification. We have developed two new techniques to assign rational drug design using crystallographic structures or models by homology. The binding pockets can be automatically identified even if no the native ligand is unknown. This algorithm has been tested on over 10,000 complexes. We have also built a comprehensive database of protein pockets and clustered them into families. The algorithm identifies the ligand binding pocket in Rhodopsin and Bacteriorhodopsin unambiguously.

Receptor flexible docking. A deformed ligand binding pocket in a model by homology can be refined by explicit global optimization of one or several known ligands and surrounding receptor side-chains. Using the Rhodopsin structure as a GPCR model we demonstrated that even after complete randomization of the pocket side chains, the new receptor-flexible docking procedure restores the correct conformation of the pocket as well as accurately predicts the conformation of the retinal.

De-orphanization. The ICM docking and scoring procedure was applied to find the native ligand from a collection of all known biological substrates. For bovine Rhodopsin, our automated computer screening of over 7000 substrates from the KEGG database scored the ligand within 1.5% of the best docking energies, and for Bacteriorhodopsin within 0.2%. This virtual screening procedure in conjunction with pocket identification and modeling may be used to predict substrates of orphan GPCRs.

Poster Presentations

Poster Presentations

[Only the presenting author's name is listed]

1. **Vimesh Avlani** *Probing the molecular basis of allosteric modulator action at the human M_2 muscarinic acetylcholine receptor*

2. **Natalie Broxton** *Ligand directed coupling of the α_{1A}-adrenoceptor to multiple signaling pathways*

3. **Diem Dinh** *Helix I of β–arrestin is not involved in post-endocytic trafficking*

4. **Michelle Glass** *Differential activation of G-proteins by endocannabinoids*

5. **Renate Griffith** *Molecular dynamics studies on a model of the α_{1b}-adrenergic receptor: The effects of water, adrenaline and site directed mutagenesis*

6. **Michelle Halls** *Characterisation of sites on the LGR7 (relaxin) and LGR3 (insulin3) receptors required for binding and signal transduction*

7. **Debbie Hay** *The role of hydrophilic residues in transmembrane helix 2 of the $CGRP_1$ receptor (CL/RAMP1) in signal transduction*

8. **Michael Lew** *Modulation of tachyphylaxis at the angiotensin AT1 receptor in rat mesenteric arteries*

9. **Julia Nevzorova** *Signalling mechanisms mediating β_2-adrenoceptor stimulated glucose transport in L6 skeletal muscle myotubes*

10. **Done Onan** *Atypical regulation of the urotensin II receptor*

11. **Vi Pham** *Direct identification of interacting sites between calcitonin antagonists and the human calcitonin receptor*

12. **Brooke Purdue** *Molecular characterization of a human calcitonin receptor polymorphism*

13. **Hongwei Qian** *Activation-deficient type 1 angiotensin receptors display arrestin-based class switching*

14. **Peter Reik** *Residue correlations between positions in the helices of the olfactory receptors*

15. **Daniel Scott** *A truncated splice variant of the relaxin receptor, LGR7, is a functional relaxin antagonist*

16. **Nicola Smith** *Angiotensin II receptor transactivation of the EGF receptor: role of G protein coupling and metalloprotease activation in cardiac hypertrophy*

17. **Nanda Tilakaratne** *Receptor activity modifying protein (RAMP) effects on the calcitonin receptor (CTR) are modulated by heterotrimeric G_α-proteins*

18. **Madhara Udawela** *Molecular characterisation of receptor activity modifying proteins (RAMPs)*

19. **Tim Werry** C*oupling to* $G\alpha_q$*-mediated signaling downstream of* $5HT_{2C}$ *receptors in Chinese hamster ovary cells is sufficient for ERK1/2 phosphorylation*

Poster #1

Probing the molecular basis of allosteric modulator action at the human M_2 muscarinic acetylcholine receptor

Vimesh A. Avlani, Patrick M. Sexton and Arthur Christopoulos

Howard Florey Institute and Dept. Pharmacology, The University of Melbourne

Many G protein-coupled receptors (GPCRs) possess allosteric binding sites, which are domains that are topographically distinct from the classic, "orthosteric", site recognised by the receptor's endogenous ligand(s). When occupied by "allosteric modulator" ligands, GPCR allosteric sites can change the receptor's conformation and hence its interaction with endogenous or exogenous ligands that bind to the orthosteric site. Targeting of allosteric binding sites in drug development has a number of potential therapeutic advantages: 1) the sites are often less well conserved across receptor subtypes than the orthosteric site (offering greater potential for subtype selectivity), 2) allosteric modulators can be either positive (enhancers) or negative (inhibitors) with respect to their effect on endogenous ligand binding and function, and 3) drug action at these sites may maintain the spatial and temporal aspects of physiological signalling. Muscarinic acetylcholine receptors (mAChRs) are prototypic examples of GPCRs with allosteric binding sites. There are 5 mAChR subtypes and the development of receptor-specific drugs acting at the orthosteric site has not met with great success. M_2 mAChRs are particularly important in smooth muscle contraction and have been implicated in memory and learning; as such they are potential targets for development of drugs to treat bladder dysfuntion and Alzheimer's dementia. Previous studies of the M_2 mAChR identified the sequence ^{172}EDGE^{175} of the 2nd extracellular loop to be important for the selectivity of the modulator, gallamine. More recent studies have identified additional residues that contribute to the selectivity of other modulators. However, most mutational analyses of mAChR modulator binding sites to date have led to the hypothesis that the affinity of a given modulator is tightly regulated by specific receptor epitopes, whereas the cooperativity between allosteric and orthosteric receptor sites is a global property of the receptor.

In this study, the EDGE sequence of the M_2 mAChR was mutated to QNGQ, eliminating the negative charge of this sequence, while maintaining the essential side chain structure of the amino acids. In addition, we have also mutated K^{19} in the N-terminal tail to A, as 3D-homology modelling of the receptor in our laboratory predicted that this residue could form a salt bridge with E^{173} within the EDGE sequence. The effect of the mutations on the allosteric modulators gallamine, C_7/3-phth or alcuronium was assessed against the binding of the orthosteric antagonist [^{3}H]-N-methyl scopolamine in CHO cell membranes, using a 96-well plate assay format. Parameters of binding affinity and cooperativity for each of the allosteric drugs were determined. For gallamine, the QNGQ mutant exhibited a small decrease in affinity, while C_7/3-phth exhibited decreased negative cooperativity. Interestingly, alcuronium exhibited a complete reversal from positive to negative cooperativity. In contrast, no alteration was noted in the properties of WIN 62,577, a novel benzimidazole believed to interact at a second allosteric site on mAChRs. For the K^{19}A mutant no difference was seen with any of the allosteric ligands examined. In more recent experiments using a 24-probe cell harvester, the magnitude of shift in binding parameters for the QNGQ mutant was less dramatic, although they followed the same trend seen in the high throughput assay format, suggesting that control of the level of membrane/receptor in the assay is important with respect to the absolute value of parameters generated. Collectively, these data provide direct evidence for the existence of two separate allosteric binding sites for prototypical modulators *vs* WIN 62, 577, as well as demonstrating the importance of a small epitope for both the strength and nature of the cooperative interactions in the M_2 mAChR. Furthermore, the difference in allosteric binding parameters observed for the compounds between different assay formats has important practical implications for high throughput drug discovery based on around allosteric modulators of GPCRs.

Poster #2

Ligand directed coupling of the α_{1A}-adrenoceptor to multiple signaling pathways

NM Broxton, BA Evans, RJ Summers

Molecular Pharmacology Laboratory, Dept of Pharmacology, Monash University, Vic 3800

The α_1-ARs are G-protein coupled receptors (GPCR) that are activated by noradrenaline and adrenaline to stimulate coupling to the α subunit of G_q/G_{11} G-proteins. This activates phospholipase C (PLC), increases intracellular levels of inositol (1,4,5) trisphosphate (IP_3) and Ca^{2+} together with diacylglycerol (DAG) (Hieble et al 1995). However, studies with various tissues that endogenously express α_1-ARs and cells expressing recombinant receptors have demonstrated that α_1-ARs are able to couple to multiple second messenger pathways including phospholipase A_2 (PLA_2), phospholipase D (PLD), adenylate cyclase, extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) (reviewed in Graham et al 1996). The current study examines the signalling pathways activated by the α_{1A}-AR expressed in CHO-K1 cells and identifies that some agonists are able to preferentially activate particular signalling pathways. Agonist stimulation of the α_{1A}–AR in the cytosensor microphysiometer increased the extracellular acidification rate (ECAR) (pEC_{50}(n): noradrenaline 8.1±0.8(4), methoxamine 7.6±0.3(4), A61603 8.3±0.1(4), oxymetazoline 8.9±0.6(4) and clonidine 7.8±0.2(4)). Coupling of α_{1A}-AR to G_q/G_{11} and PLC was demonstrated for these agonists using a [^{3}H]PI depletion assay (Millan et al 2001). In contrast to the ECAR and [^{3}H]PI depletion responses only noradrenaline (pEC_{50}(n):5.2±0.02(4)), methoxamine (4.8±0.13(3)) and A61603 (7.8±0.06(3)) stimulated cAMP production whereas clonidine and oxymetazoline had no discernible effect. The α_{1A}–AR also coupled to the $G_{i/o}$ pathway since in the cytosensor, responses to noradrenaline (28.5±2.4 vs 40.5±2.7, $P<0.0001$), methoxamine (19.4±1 vs 34.5±2.6, $P<0.0001$) and A61603 (27.4±1.8 vs 36.7±2.7, $P<0.0001$) were significantly increased following PTX treatment. However, there was no significant effect of PTX on the response to oxymetazoline ($P=0.15$). cAMP production in response to noradrenaline (40.5±4.5 vs 18.3±6.7 $P<0.0001$) and methoxamine (36.6±8.0 vs 19.1±6.2 $P<0.005$) was decreased by PTX, whereas the lack of effect of oxymetazoline on cAMP accumulation was unaffected by PTX. These studies show that α_{1A}–AR when stimulated by noradrenaline, methoxamine or A61603 can couple effectively not only to G_q/G_{11} but also to G_s and $G_{i/o}$. In contrast, the agonists oxymetazoline and clonidine can produce effective signalling through G_q/G_{11} but not G_s or $G_{i/o}$ suggesting ligand-directed coupling at α_{1A}–AR (Kenakin, 2002).

Hielble JP et al (1995) Pharmacol. Rev. 47: 267-270.
Graham RM et al (1996) Circ. Res. 78: 737-49
Millan MJ et al (2001) J Pharmacol Exp Ther. 297: 876-87
Kenakin T (2002) Annu Rev Pharmacol Toxicol. 42: 349-79

Poster #3

Helix I of βarrestin is not involved in post-endocytic trafficking

Diem T. Dinh, Hongwei Qian, Walter G. Thomas

Baker Medical Research Institute, Melbourne, Australia

The non-visual arrestins, βarrestin1 and βarrestin2, are ubiquitously expressed and modulate the activity of hundreds of seven-transmembrane-spanning, G protein coupled receptors (GPCRs), including the type 1 angiotensin II (AngII) receptor (AT_1). βarrestins bind to activated and phosphorylated GPCRs, promoting receptor internalisation and preventing further interaction of receptors with G-proteins, thereby terminating signalling. βarrestin contains only one helix, an amphipathic and potentially amphitropic (membrane binding) helix, termed helix I. To investigate the role of this helix, which is conserved in βarrestin1 (T^{98}RLQERLIKKL^{108}) and βarrestin2 (T^{99}RLQDRLLKKL^{109}), we mutated hydrophobic (L^{100}, L^{104}, L^{108} to non-hydrophobic alanines; βarrestin1 numbering) and positively charged residues (R^{99}, R^{103}, K^{106}, K^{107} to uncharged glutamines) in GFP tagged versions of βarrestin1/2. We also engineered a construct where helix I of βarrestin1/2 was fused at the c-terminus of eGFP (eGFP-helix I). Using confocal microscopy, wild-type and mutant GFP-labelled βarrestin1 and 2, as well as eGFP-helix I are evenly distributed throughout the cytoplasm of Human Embryonic Kidney (HEK) cells in the absence of AngII. Following agonist stimulation, wild-type βarrestin1 and 2 are rapidly translocated from the cytoplasm to the cell surface and then redistributed into deep core endocytic vesicles in response to the AT_1 receptor activation. In contrast, βarrestin1 and 2 mutants are translocated to the cell membrane, but remain at the surface even after 1 hour of receptor activation and fail to traffic into vesicles. Furthermore, eGFP-helix I of both βarrestin1 and 2 remains present in a predominantly diffuse, cytoplasmic distribution, suggesting that the helix alone is not sufficient to target membrane anchoring. Interestingly, these mutants fully support receptor internalisation in HEK cells and mouse embryonic fibroblasts from βarrestin1 and 2 knockout. Taken together, these data suggest that helix I is not amphitropic, instead it is implicated in βarrestins trafficking to deep core vesicles. Moreover, βarrestin mutants do not act as dominant-negatives and despite blockade of βarrestin trafficking at the cell membrane, receptor internalisation was not affected.

Poster #4

Differential Activation of G-proteins by Endocannabinoids

Katherine Blake-Palmer[2], John Northup[3] and Michelle Glass[1,2]

[1]Department of Pharmacology and [2]Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand [3]National Institute of Deafness and Other Communication Disorders, 5 Research Court, Rockville MD 20850

We have previously demonstrated agonist-selective G protein signalling for synthetic ligands of the CB_1 cannabinoid receptor using an in-situ reconstitution approach and G-proteins purified from bovine brain (Glass & Northup, 1999, Mol. Pharmacol. 56, 1362-1369).

Homogenous myristoylated $G\alpha_{oA}$ and $G\alpha_{i1}$ subunits produced in E. coli were examined for equivalence to brain derived G-proteins. Recombinant $G\alpha_{i1}$, in the presence of recombinant $G\beta_1\gamma_2$, saturated the CB_1 receptor identically to the previously reported saturation for the native membrane-derived bovine brain Gi trimer. Recombinant $G\alpha_{oA}$, however, saturated the CB_1 receptor with a K_m of 29±4 nM, some 2.3-fold higher affinity than found previously for the bovine brain G_o protein.

We determined the efficacies for G-protein signalling of four putative endogenous cannabinoid ligands, anandamide, noladin ether, virodhamine and 2-arachidonyl glycerol (2AG) using recombinant G protein subunits α_{oA}, α_{i1} and $\beta_1\gamma_2$. For the CB_1 receptor, anandamide, noladin ether and 2AG activated $G\alpha_{i1}$ with similar efficacy as the synthetic full agonist HU210, while virodhamine functioned as partial agonist with about 30% of the efficacy of HU210. Anandamide, but not the other ligands, displayed a different efficacy for CB_1 receptor activation of $G\alpha_{oA}$, activating at only 64% of the efficacy of HU210. As was found for $G\alpha_{i1}$ 2AG, and noladin ether were full agonists and virodhamine was a partial agonist for the activation of $G\alpha_{oA}$. As CB_2 was previously shown to have only a low affinity interaction with Go, only interactions with $G\alpha i$ were examined. Anandamide and virodamine were partial agonists while 2AG and noladin ether were full agonists in the activation of $G\alpha_{i1}$. As the activation of $G\alpha_{i1}$ by anandamide was more efficacious than that observed with brain Gi, we compared the ability of 2AG and anandamide to activate $G\alpha_{i2}$, agonist activation of both $G\alpha_{i1}$ and $G\alpha_{i2}$ was examined and found to be identical.
Palmitoyl ethanolamide (PEA), which is occasionally cited for activity at CB receptors was also examined. PEA neither activated nor competed for the activation of these G proteins by CB receptors.

These data demonstrate that anandamide, but not the other endocannabinoids show agonist-selective G protein activation through the CB1 receptor. Furthermore, they demonstrate a graded response of activation of both the CB1 and CB2 receptors, by the different endocannabinoids, ranging from weak partial agonism (e.g. virodhamine) to full agonism (e.g. 2AG).

Poster #5

Molecular Dynamics Studies On A Model Of The α_{1b}-Adrenergic Receptor: The Effects Of Water, Adrenaline And Site Directed Mutagenesis

Geoffry N. DeIuliis and Renate Griffith

School of Environmental and Life Sciences, University of Newcastle, Callaghan, Australia

G-protein coupled receptors (GPCRs) are cell surface receptors, which consist of seven transmembrane α-helical columns (TMHs), three intracellular loops, three extracellular loops, an intracellular C-terminal and an extracellular N-terminal. It is proposed that in order for the receptor to associate with the G-protein inside the cell upon extracellular activation by the effector molecule (*i.e.* adrenaline), "rigid-body" movement of one or more of the TMHs is needed[1,2] and not necessarily a change in secondary structure[2]. In certain mutant forms of the receptors, the activated state is achieved without the binding of the effector molecule, and these constitutively active receptors also show enhanced affinity and efficacy of the effector.

We constructed a homology model of the GPCR, α_{1b}-adrenergic receptor (α_{1b}-AR), based on a recently published crystal structure of another GPCR, rhodopsin[3]. This crystal structure captures the inactive conformation of rhodopsin, and it is therefore assumed, that our model represents an inactive state of the α_{1b}-AR. This is consistent with the observation of a salt bridge constraint between helices three and six, which is proposed to be crucial for keeping GPCRs in the inactive conformation[2].

After conducting minimisation studies on this homology model, we confirmed that this method alone could not predict the active conformations of this protein. Even the addition of the agonist adrenaline, as well as explicit water molecules, could not force a conformational change through minimisation. Molecular dynamics simulations are needed to overcome the energy barriers to achieve the internal movement required to change the receptor into an activated state.

This paper will present comparisons of a range of dynamics simulation experiments, including different simulation lengths, the inclusion of adrenaline, explicit interior water molecules, the effects of changing the dielectric constant and of mutagenesis.

1. Chen, S., Xu, F. L. M., Graham, R. M. *Biochemistry*. **2002**, 41, 588-596
2. Fanelli, F., Meziani C., Scheer, A., Cotecchia, S., De Benedetti, P. G. *Methods:* **1998,** 14, 307-317
3. Okada T., Fujiyoshi Y., Silow M., Navarro J., Landau E.M., Shichida Y., *Proc Nat Acad Sci*., **2002**, 99, 5982-5987.

Poster #6

Characterisation of sites on the LGR7 (relaxin) and LGR3 (insulin3) receptors required for binding and signal transduction

ML Halls (1), CP Bond (1), RA Bathgate (2) & RJ Summers (1)

(1) Dept of Pharmacology, Monash University, VIC 3800, (2) Howard Florey Institute, VIC 3052

Relaxin (RLX) is a two-chain peptide, structurally related to insulin. Recently RLX was found to be the ligand of two orphan leucine-rich repeat-containing GPCRs named LGR7 and LGR8 (Hsu *et al.*, 2002). LGR7 has since been identified as the RLX receptor (Hsu *et al.*, 2002), while LGR8 has been determined the insulin3 (Insl3) receptor (Kumagai *et al.*, 2002). The high degree of structural homology (60% sequence identity) between the two receptors and observed ligand cross-reactivity has allowed generation of receptor chimeras (LGR7/8 and LGR8/7) that have been expressed in HEK293T cells as well as the parent receptors to further elucidate binding and signalling characteristics.

Whole cell binding assays were performed in a 96-well plate format, using [^{33}P]-hRLX2 (human gene 2 RLX) as a ligand to characterise receptor properties in HEK293T cells transiently and stably expressing the receptors. Saturation binding studies revealed similar characteristics for LGR7, LGR7/8 and LGR8/7 (pK_D values for transiently expressing cells [stably expressing cells]: 9.69±0.29 [9.5±0.15], 9.56±0.18 and 9.52±0.06 respectively, n=4-6), whereas [^{33}P]-hRLX2 identified two binding sites at the LGR8 receptor [9.2±0.3 and 8.3±0.1 respectively, n=5]. Competition for [^{33}P]-hRLX2 for binding by RLX analogues revealed different rank orders of potency at each of the four receptors. LGR7/8 more closely resembled LGR7 while LGR8/7 resembled the native LGR8 receptor, suggesting that the ectodomain is the major determinant of receptor behaviour, although the transmembrane region does have a minor contribution. Additionally, differences in the rank order of ligand affinity were observed for the two LGR8 binding sites, with not all analogues able to interact with both sites. The binding studies suggest localisation of the two LGR8 binding sites: a low affinity site on the transmembrane region, and a high affinity site on the ectodomain.

cAMP accumulation studies confirmed the importance of both the transmembrane and ectodomains for optimal signal transduction, and reiterated the two-site binding properties of LGR8. All RLX analogues except Insl3 stimulated the LGR7 receptor to produce cAMP. At the LGR8 receptor, all analogues except rat RLX (rRLX) induced cAMP accumulation. The chimeras again exhibited characteristics that reflected their respective receptor-ectodomain origins. However, Insl3 stimulation of receptor chimeras in transient HEK293T cells produced an interesting pattern of cAMP accumulation when compared to the native LGR8 (transients) (pEC_{50} 8.05±0.36, Max (%Forskolin) 15.17±1.13, n=4). At LGR7/8, Insl3 produced cAMP (pEC_{50} 8.88±0.35, Max (%Forskolin) 5.07±1.35, n=4), while LGR8/7 coupling to cAMP production was decreased (pEC_{50} 8.86±0.37, Max (%Forskolin, n=4) 10.77±1.97) compared to the LGR8 receptor (both $P<0.05$ versus LGR8). This suggested that Insl3 required both binding sites at LGR8 to produce the optimal cAMP accumulation response. This study indicates that RLX but not Insl3 can interact with the LGR7 receptor, supporting the concept that LGR7 is the RLX receptor. RLX also appears to interact at two distinct sites on the Insl3 receptor (LGR8), although not all RLX analogues can interact with both sites.

References

HSU, S.Y., NAKABAYASHI, K., NISHI, S., KUMAGAI, J., SHERWOOD, O.D. & HSUEH, A.J. (2002). Activation of orphan receptors by the hormone relaxin. *Science*, **295**, 671-674.

KUMAGAI, J., HSU, S.Y., MATSUMI, H., ROH, J., FU, P., WADE, J.D., BATHGATE, R.A.D. & HSUEH, A.J. (2002). INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *The Journal of Biological Chemistry*, **277**, 31283-31286.

Poster #7

The role of hydrophilic residues in transmembrane helix 2 of the $CGRP_1$ receptor (CL/RAMP1) in signal transduction

[1,4]A. C. Conner, [1,2]D. L. Hay, [1]S. G. Howitt, [3]D. M. Smith, [4]M. Wheatley & [1]D. R. Poyner

[1]Life and Health Sciences, Aston University, Birmingham, B4 7ET, UK; [2] ICSM, London, UK; [3]CVGI Group, AstraZeneca, Alderley Edge, Cheshire, UK, [4]School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

The $CGRP_1$ receptor is an atypical family-B G-protein coupled receptor (GPCR) consisting of two components; calcitonin receptor-like receptor (CL or CRLR) and receptor activity modifying protein 1 (RAMP1). Both are needed for receptor function (McLatchie *et al.,* 1998). Several hydrophilic residues are found within transmembrane (TM) helix two of CL. Such residues are commonly found to be important for receptor function. In this study, each hydrophilic residue in TM2 was individually mutated to alanine and the effects on CGRP-stimulated cyclic AMP production studied.

Point mutations in human CL were introduced by the Stratagene Quick-Change mutagenesis method. Transfection of CL and RAMP1 into Cos-7 cells, construction of dose-response curves to human αCGRP (10^{-12}M to 10^{-7}M), cyclic AMP measurements and radioligand binding to cell membranes using ^{125}I CGRP were as described previously (Hay *et al.,* 2003; Howitt *et al*., 2003). Curve fitting to obtain pEC_{50} and maximum effect (E_{max}) relative to wild type (WT) was by PRISM Graphpad. Mutant and WT pEC_{50}s were compared by paired Student's t-tests; B_{max} and E_{max} values were compared to 100% (WT) by Mann-Whitney tests.

H155A and T169A reduced the E_{max} (Table 1). T169A and H155R significantly reduced the CGRP pEC_{50}. Despite altered receptor function, neither H155A nor T169A had significantly altered B_{max} values (120 ± 6 and 72 ± 18 respectively, WT 100%).

Table 1. Effects of mutants on cyclic AMP production

Name	n	EC50 WT	EC50 mutant	Emax (% control)
T153A	3	10.57±0.28	10.27±0.35	64±12
H155A	7	10.39±0.27	9.82±0.04	36±12*
H155R	4	10.53±0.22	9.48±0.13**	102±10
K156A	4	9.58±0.36	9.26±0.21	61±16
N157A	5	9.96±0.32	10.32±0.32	112±23
S161A	3	10.38±0.29	10.56±0.10	81±9
N165A	5	9.97±0.45	9.79±0.58	108±13
S166A	4	10.76±0.28	10.51±0.26	65±23
T169A	5	10.22±0.44	9.05±0.36***	69±5*
H172A	4	10.47±0.27	9.87±0.43	86±9

** $P < 0.01$, *** $P < 0.001$ paired t-test; * $P < 0.05$ Mann-Whitney test. Values are means ± s.e.m.

These data suggest that H155 and T169 are required for optimal signal transduction in the $CGRP_1$ receptor. These residues are predicted to be on the same face of helix 2 and may make contacts with helix 3, a helix which is implicated in many models of GPCR activation (Lu *et al.*, 2002).
This work was supported by the Wellcome Trust and an MRC studentship to DLH.

Hay, D.L. *et al*., (2003). *Br. J. Pharmacol*, 140, 477-486.
Howitt, S.G. *et al*., (2003). *Br. J. Pharmacol*, 138, 325-332.
Lu, Z.L., *et al.,* (2002). *Trends Pharmacol Sci*. 23, 140-146.
McLatchie, L.M. *et al*., (1998). *Nature*, 393, 333-339.

Poster #8

Modulation of tachyphylaxis at the angiotensin AT1 receptor in rat mesenteric arteries

Ben Wheaton, Michael Lew & James Ziogas.

Dept. Pharmacology, The University of Melbourne, Victoria, 3010.

Responses to the potent vasoconstrictor angiotensin II (AngII) mediated by the AT1 receptor are subject to tachyphylaxis such that responses to repeated AngII applications decrease in magnitude. Signalling through the AT1 receptor is complex but most of its vasoconstrictor effects are mediated by the Gq/11 - phospholipase C pathway. The AT1 receptor is known to transactivate the EGF receptor (EGFR; a receptor tyrosine kinase) and activate the mitogen activated protein kinases ERK1 and ERK2 via MEK. The goal of the current study was to determine whether this transactivation plays a role in tachyphylaxis. Pressurised rat mesenteric arteries (353±8 microns in diameter) were exposed to two AngII cumulative concentration response curves with a 60 minute interval and the magnitude of the second response (measured as change in diameter) after various pretreatments was used as a measure of tachyphylaxis. The tachyphylaxis (19±5 microns after AngII pretreatment vs 101±14 microns after no pretreatment) was not a result of tissue fatigue as repeated phenylephrine curves produced sustained maximal responses of around 192±12 microns. No heterodesensitisation occurred between angiotensin II and phenylephrine. It was found that the selective EGF kinase inhibitor AG1478 (5 microM), and the MEK inhibitor U0126 (10 microM) prevented or greatly reduced tachyphylaxis (2nd curve maxima of 106±14 and 69±3 microns respectively; both n=6) when present only during the initial curve. This led to the idea that whatever caused tachyphylaxis happened during the first curve and involved transactivation of the EGF receptor and a role for MEK. With this in mind, the initial angiotensin II curve was replaced with a 10 minute EGF exposure (10 nM) to test whether EGFR stimulation alone was enough inhibit subsequent AngII responses. However the subsequent AngII curve was not significantly smaller than control (82±14 microns, n=6 $p>0.05$). These results imply that while transactivation of EGFR is necessary for tachyphylaxis, perhaps other factors such as AT1 receptor occupation and activation might also be important.

Poster #9

Signalling mechanisms mediating β_2-adrenoceptor stimulated glucose transport in L6 skeletal muscle myotubes

J Nevzorova (1), T Bengtsson (2), BA Evans (1) & RJ Summers (1)

(1) Department of Pharmacology, Monash University, VIC 3800. (2) Wenner-Gren Institute Stockholm University, S-106 91 Stockholm, Sweden

Agonist mediated activation of β_2-adrenergic receptors (AR) results in increases in glucose transport (GT) in the rat skeletal muscle cell line L6[1]. Following activation β_2-AR is phosphorylated by PKA and G protein receptor kinases, resulting in the switch of coupling from Gs to Gi protein, and activation of other signalling pathways, such as MAPK and PI3K (Daaka et al 1997; Zhu et al 2001). This study examines the role of cyclic AMP and Gs/Gi coupling in the β_2-AR mediated GT. Cyclic AMP levels in L6 cells were increased by isoprenaline (all β-ARs) and zinterol (β_2-ARs) in a concentration-dependent manner ($pEC_{50} = 7.8 \pm 0.1$ and 9.1 ± 0.1 respectively, n=3). The cyclic AMP response to isoprenaline was inhibited by the β_2-AR antagonist ICI118551 (10^{-8} M), but not by CGP20712A (10^{-8} M), suggesting that as for GT, cyclic AMP generation in L6 cells is mediated by β_2-ARs. GT was increased by cyclic AMP analogues 8-bromo cyclic AMP (% max response 204 ± 13) and dibutyryl cyclic AMP (206 ± 18) at 1mM, and a direct Gs protein activator cholera toxin at 1µg/ml (147 ± 2). However GT stimulated by 0.1µM zinterol was not inhibited by PKA inhibition. Zinterol stimulated GT was inhibited by pertussis toxin, indicating involvement of Gi protein. The results suggest that β_2-AR mediated GT requires increases in cyclic AMP, and appears to involve a Gi component.

Daaka, Y et al (1997) Nature 390, 88-91

Nevzorova, J., Bengtsson, T., Evans, BA. & Summers, RJ. *Brit. J. Pharmacol.,* 2002, **137**:9-18.
Zhu, WZ et al (2001) PNAS 98, 1607-1612

Poster #10

Atypical regulation of the urotensin II receptor

Done Onan [1,2] and Thomas W.G[1].

[1]Baker Heart Research Institute, [2]Department of Biochemistry and Molecular Biology, Monash University, Victoria Australia.

Urotensin II (U II), a cyclic peptide with potent vasoconstrictor properties, functions through GPR14, an orphan G protein-coupled receptor (GPCR) now referred to as the urotensin (UT) receptor[1]. The vasoconstriction response of U II in the rat thoracic aorta is sustained[1], whereas angiotensin II-mediates a brief response that is rapidly desensitised by the classical regulatory mechanisms of phosphorylation and internalisation. These processes require two regulatory protein families, GPCR kinases and the β-arrestins. The molecular mechanisms involved in the regulation of the UT receptor are unknown. The aim of this study was to determine whether the UT receptor is regulated in a classical manner, by phosphorylation and internalisation. We found that U II-mediated phosphorylation and internalisation of the UT receptor was very weak in comparison to the rapidly desensitised angiotensin II receptor, AT_{1A}. Furthermore and in contrast to the classically regulated AT_{1A}, U II stimulation did not traffic the regulatory protein β-arrestin. These results indicate that the UT receptor is poorly regulated by classical regulatory mechanisms. The coexpression of GPCR kinases and β-arrestins did not improve UT receptor phosphorylation or internalisation, further corroborating the lack of regulation theory. We propose that the lack of regulation may account for the sustained and potent vasoconstriction properties of U II.

1- Ames, R. S., H. M. Sarau, et al. (1999). "Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14." Nature **401**(6750): 282-6.

Poster #11

Direct identification of interacting sites between calcitonin antagonists and the human calcitonin receptor

Vi Pham[1], John D. Wade[1], Maoqing Dong[2], Laurence J. Miller[2] & Patrick M. Sexton[1]

[1]Howard Florey Institute, The University of Melbourne, Parkville, Vic 3010 and [2]Mayo Clinic Scottsdale, AZ 85259, USA

Calcitonins (CTs) are 32 amino acid polypeptide hormones whose most recognised action is the inhibition of osteoclast-mediated bone resorption [1]. CTs, through their action on osteoclasts and to a lesser extent in the kidney, are widely used clinically in the treatment of bone-related disorders such as osteoporosis, Paget's disease and hypercalcemia of malignancy [1,2]. However the molecular basis for CT binding to its receptor, a class II G protein-coupled receptor, is not well defined. Therefore the current study focuses on determining how CT interacts with its receptor. Salmon CT (sCT) was chosen as the template molecule due to its wide clinical use, and its high affinity and efficacy at all calcitonin receptors (CTRs) [1,2]. To directly identify sites of proximity between CT and its receptor, we carried out photoaffinity labeling studies followed by protein digestion and mapping of the radiolabeled photoconjugated receptor.

Three analogues of the antagonist sCT(8-32), containing a single photolabile *p*-benzoyl-L-phenylalanine (Bpa) residue in position 8, 14 or 19, have been used to demonstrate spatial proximity between the ligand and the receptor. The [Bpa^{19}]sCT(8-32) analogue cross-linked to the receptor at, or near, the equivalent cross-linking site of the full length peptide, within the fragment C^{134}-K^{141}. The [Bpa^{8}]sCT(8-32) and [Bpa^{14}]sCT(8-32) peptides displayed digestion patterns that were distinct from that of the [Bpa^{19}] analogue. For the [Bpa^{8}] peptide, cyanogen bromide (CNBr) digestion of the native receptor generated a predominant band of ~4.2-kDa, which did not further shift after deglycosylation with endoglycosidase-F (endo-F). This was consistent with cross-linking of the peptide to either the receptor fragment D^{50}-M^{59} or L^{368}-M^{376}. In contrast, CNBr digestion of the radiolabeled Bpa^{14}-receptor conjugate gave rise to a 21-kDa band, which was shifted to 12-kDa upon endo-F deglycosylation. Therefore the [Bpa^{14}] peptide cross-linked within the receptor fragment Q^{60}-M^{133} in the N-terminus. The sites of cross-linking for both the [Bpa^{8}] and [Bpa^{14}] analogues will be further resolved using receptor mutants with modified cleavage sites. These data provide further constraints on the interface between CT and its receptor and provide the basis for understanding the molecular mechanisms of ligand binding.

References

1. Sexton, P.M., Findlay, D.M. and Martin, T.J., Calcitonin, *Curr. Med. Chem.*, 6 (1999) 1067-1093.
2. Pondel, M., Calcitonin and calcitonin receptors: bone and beyond, *Int. J. Exp. Pathol.* 81 (2000) 405-422.

Poster #12

Molecular characterization of a human calcitonin receptor polymorphism

Brooke W. Purdue, Nanda Tilakaratne and Patrick M. Sexton

Howard Florey Institute, The University of Melbourne, Victoria, 3010.

The peptide hormone calcitonin (CT) is a potent inhibitor of bone resorption. In the 3' region of the human CT receptor (hCTR) coding sequence (nt 1377), a naturally occurring polymorphism exists, which results in a Leu→Pro shift in the intracellular C-terminal tail of the receptor protein[i]. Epidemiological studies indicate that the frequency of these alleles differs between population groups. Correlation of hCTR genotype with osteoporotic indices in these studies revealed that patients homozygous for leucine 447 had lower lumbar spine bone mineral density (BMD) in comparison to either proline homozygotes[ii], or heterozygotes[iii]. Subdivision of patients into normal and osteoporotic[2] or those with and without fractures[3], indicated that heterozygotes had lower fracture risk than leucine homozygotes[3], and that the leucine homozygote may be more represented in the osteoporotic population[2].

In the present study, the pharmacology of the polymorphic variants was examined for the two most commonly expressed human CTR isoforms (termed a and b, which differ by the absence or presence, respectively, of a 16 amino acid insert in intracellular domain 1 of the receptor). In transiently transfected HEK-293 cells, pIC_{50} values for competition binding of ^{125}I-salmon CT (^{125}I-sCT) by sCT were similar for the hCTRa isoform leucine and proline variants; 7.8 ± 0.3 (n=4) and 8.3 ± 0.1(n=4), respectively. There was, however, a difference between the b isoform leucine and proline variants; 9.0 ± 0.6 (n=3) and 8.3 ± 0.2 (n=3), respectively. In parallel there was a decrease in affinity for human CT (7.0 ± 0.1(n=2)) for the leucine variant compared to the proline receptor variant (7.8 ± 0.1 (n=4)). In these studies the pIC_{50} values likely reflect affinities for the inactive receptor and may suggest that the hCTRb leucine variant is uncoupled from G proteins to a greater extent in the 293 cells. No difference in ligand affinity between the b isoform variants was seen when COS-7 cells were transfected with the same plasmid DNA. However, in COS-7 cells, the hCTRb-proline variant displayed an increased basal activation of cAMP, while the leucine variant did not. This proline variant also underwent internalization at a much slower rate, which could be sped up by overexpression of beta arrestins in these cells. HEK-293 cells express much higher levels of endogenous β-arrestin proteins than COS-7 cells. Overall this data suggests that the presence of the proline polymorphism induces a structural change that decreases association of the receptor with accessory regulatory proteins. This is more prominent in the b isoform and may be due to tighter coupling to G proteins in this receptor isoform.

[1] Nakamura, et al (1997) *Hum Genet* **99,** 38-41
[2] Masi L, et al (1998) Biochem Biophys Res Commun **245,** 622-6.
[3] Taboulet J, Frenkian M, Frendo JL, Feingold N, Jullienne A, de Vernejoul MC (1998) Hum Mol Genet **7**, 2129-33.

Poster #13

Activation-deficient type 1 angiotensin receptors display arrestin-based class switching

Hongwei Qian[1], Aylin C. Hanyaloglu[2], Diem Dinh[1], Luisa Pipolo[1], Karin Eidne[2], Walter G. Thomas[1]

[1]Baker Medical Research Institute, Melbourne, Australia. [2]Western Australian Institute for Medical Research (WAIMR) and Keogh Institute for Medical Research, Sir Charles Gairdner Hospital, University of Western Australia, Perth, Australia

G protein-coupled receptors are classified as *class A* or *class B* based on differential recruitment and trafficking of βarrestin1 and βarrestin2. The type 1 angiotensin II (AngII) receptor (AT_1) is a *class B* receptor, engaging both arrestin isoforms following activation and translocating with them to deep core endocytic vesicles. Activation-deficient mutants of the AT_1 receptor ([D74E]AT_1 and [Y215F]AT_1) exhibit markedly altered AngII-induced βarrestin trafficking and display the hallmarks of *class A* receptors – they preferentially recruit βarrestin2, which does not traffic to deep core vesicles, but disperses back to the cytoplasm. Paradoxically, [D74E]AT_1 and [Y215F]AT_1 receptors are robustly phosphorylated by AngII, yet they are weakly internalised and bind βarrestin1 and βarrestin2 poorly, as assessed by co-immunoprecipitation and bioluminescence resonance energy transfer analysis. Thus, receptor phosphorylation is necessary, but not sufficient, for recruitment and high affinity binding of arrestins and their subsequent translocation to endocytic vesicles. This represents a unique example of arrestin-based *class* switching by a G protein-coupled receptor and suggests that the active state of the receptor is a primary determinant of this phenomenon.

Poster #14

Residue Correlations between Positions in the Helices of the Olfactory Receptors

Peter Riek and Robert Graham

Victor Chang Cardiac Research Institute, St Vincent's Hospital, Darlinghurst, Australia 2010

The mammmalian olfactory G-protein-coupled receptors (GPCRs) are a large subfamily of the Class 1 GPCR superfamily. Seven alignment tables have been developed incorporating each of the individual transmembrane regions. These tables have been used to determine the residue distribution at each of the positions within each transmembrane region. In addition, these tables have been used in the development of methods to investigate the significance between reside subtype and proportions at one position compared with the occurrences and levels at another intra-helical or inter-helical position. A residue combination unique to a sub-group comprising 13% of the sequences in the alignment table has been found: H4.52 containing arginine with H5.58 containing aspartate. This combination has not been found in the non-olfactory Class 1 GPCRs. Other strong correlations between inter-helical positions have been found within this subfamily. When these positions are compared with the equivalent positions within the structure of bovine rhodopsin, they are located on faces of the helices that are in contact with adjacent helices. Results arising from the use of these methods will aid in the building of models for the GPCR superfamily and, by extension, to other families of polytopic proteins.

Poster #15

A truncated splice variant of the relaxin receptor, LGR7, is a functional relaxin antagonist

DJ Scott, S Layfield, GW Tregear, RAD Bathgate.

The Howard Florey Institute, The University of Melbourne, Victoria, 3010

Relaxin is an extracellular matrix-remodeling hormone that is functionally important in reproductive tissues, brain, kidney and heart. The relaxin receptor was identified in parallel with the receptor for INSL3, a hormone closely related to relaxin that mediates testes descent. Failure of testes descent was observed in male mice missing the leucine-rich repeat containing, GPCR 8 (LGR8) gene. LGR8 was subsequently demonstrated to be the INSL3 receptor, whereas a closely related GPCR, LGR7 was found to be the relaxin receptor. LGR7 and LGR8 contain a unique LDL receptor-like domain (LDL domain) at their N-termini. The LGR8-Short splice variant is missing exon 2, encoding this LDL domain. LGR8-Short binds INSL3 with high affinity but is unable to signal through cAMP dependent pathways as LGR8 does, inferring functional importance to the unique LDL domain. We have identified a novel exon 3-deleted LGR7 splice variant. Due to a premature stop codon, this variant primarily encodes the LGR7 LDL domain. Homologous variants, collectively named LGR7-Truncate, were identified in mouse, rat, pig and human. Using quantitative real time PCR, LGR7-Truncate mRNA levels were detected to be as high as LGR7 mRNA in pregnant mouse uterine tissues. However, in the cerebral cortex, where LGR7 mRNA levels are highest, LGR7-Truncate mRNA was undetectable. LGR7-Truncate sequences were cloned into pcDNA3.1/Zeo vector with an N-terminal FLAG tag, and transfected into HEK293T cells. LGR7-Truncate conditioned media from these cells was used to treat LGR7 expressing 293T cells in the presence or absence of relaxin. In these experiments, LGR7-Truncate conditioned media inhibited relaxin induced intracellular cAMP accumulation in LGR7 expressing cells. LGR7-Truncate was purified from the conditioned media using an ANTI-FLAG M1 affinity gel. This purified product was able to completely block relaxin induced cAMP accumulation in LGR7 expressing cells. This *in vitro* evidence suggests that LGR7-Truncate is a functional antagonist of the relaxin receptor, furthermore LGR7-Truncate is the first identified relaxin antagonist. The differential expression and interspecies conservation of LGR7-Truncate suggests this variant has a functional role *in vivo*. If this is the case, the LGR7/LGR7-Truncate system will be a novel mode of GPCR regulation. Further studies are aimed at elucidating the efficacy of LGR7-Truncate and at proving LGR7-Truncate is an endogenously produced regulator of LGR7 activity.

Poster #16

Angiotensin II receptor transactivation of the EGF receptor: role of G protein coupling and metalloprotease activation in cardiac hypertrophy

Nicola J. Smith, Rebecca A. Lew, Ross D. Hannan & Walter G. Thomas

Baker Heart Research Institute, Victoria, Australia

Although critical for cardiovascular homeostasis, increased levels of Angiotensin II (Ang II) or its G protein-coupled receptor (AT_1R) have been associated with both hypertension and cardiac hypertrophy. While the exact mechanism of Ang II-mediated hypertrophy is unknown, we have previously demonstrated that stimulation of AT_1R-infected neonatal cardiomyocytes causes both extracellular signal-regulated protein kinase (ERK1/2) activation and cellular growth in an epidermal growth factor receptor (EGFR)-dependent manner [1]. We have examined the role of Ang II-mediated metalloprotease (MMP) activation and cleavage of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in ERK1/2 phosphorylation and hypertrophy. Ang II-induced ERK1/2 activation and cardiomyocyte hypertrophy was attenuated by the non-specific MMP inhibitor, BB94, but only partially blocked by more specific inhibitors of MMP-2, -3, -8 and –9, suggesting more than one protease is involved. HB-EGF inhibition by heparin attenuated both Ang II- and HB-EGF-mediated ERK1/2 activation and hypertrophy. Additionally, we have examined the structural/functional aspects of the AT_1R that facilitate EGFR-dependent transactivation, utilising G protein-uncoupled mutants (D74E, Y215F), a carboxyl terminal-truncated receptor (TK325) and a single residue mutant (Y319A) implicated in EGFR transactivation [2]. Interestingly, and contrary to current literature, our data suggest that AT_1R uncoupled mutants are unable to cause robust, EGFR-dependent, ERK1/2 activation. Using an alkaline phosphatase-tagged HB-EGF reporter construct (HB-EGF-AP) co-transfected with the AT_1R, specific Ang II-mediated cleavage of HB-EGF was demonstrated in both cardiomyocytes and COS-7 cells. Taken together, these results provide further evidence for the role of MMP mobilisation and HB-EGF cleavage in EGFR-dependent transactivation. G protein coupling appears necessary for AT_1R mediated HB-EGF and ERK1/2 activation, and may be important in Ang II-induced hypertrophy in cardiomyocytes.

1. Thomas, W.G., et al., *Adenoviral-directed expression of the type 1A angiotensin receptor promotes cardiomyocyte hypertrophy via transactivation of the epidermal growth factor receptor.* Circ Res, 2002. **90**(2): p. 135-42.

2. Seta, K. and J. Sadoshima, *Phosphorylation of tyrosine 319 of the angiotensin II type 1 receptor mediates angiotensin II-induced trans-activation of the epidermal growth factor receptor.* J Biol Chem, 2003. **278**(11): p. 9019-26.

Poster #17

Receptor activity modifying protein (RAMP) effects on the calcitonin receptor (CTR) are modulated by heterotrimeric G_α-proteins

Nanda Tilakaratne[1], Kaarin Smyth[1], Maria Morfis[1], George Christopoulos[1], Arthur Christopoulos[2] and Patrick M. Sexton[1]

[1]Howard Florey Institute and [2]Department of Pharmacology, The University of Melbourne, Parkville, VIC 3010

The calcitonin receptor (CTR) – a G-protein coupled receptor (GPCR), mediates the cellular actions of calcitonin (CT) – a hormone that regulates osteoclastic bone resorption. High affinity for calcitonin, but relatively low affinities for the related peptides – amylin and calcitonin gene-related peptide (CGRP) are the distinctive features of CTR pharmacology. However, on association with members of the receptor activity modifying protein (RAMP) family, the CTR acquires a high-affinity amylin receptor phenotype (1). The pharmacological properties of the CTR/RAMP-derived products vary, depending on the CTR isoform (human CTR_a or $hCTR_b$ which differ from each other by the absence or presence, respectively, of a 16-amino acid insert in the 1st intracellular loop) involved, the RAMP variant (RAMP1, - 2 or -3) and the host cell environment (2). The latter finding and the evidence that $hCTR_a$ and $hCTR_b$ differ in their ability to couple with different G_α subtypes (3), prompted the investigation of the role of G_α subtypes and their expression levels in determining CTR/RAMP interaction. ^{125}I-rat amylin (^{125}I-rAMY) binding was assessed in COS-7 cells co-tranfected with $hCTR_a$, one of the RAMPs and individually each of the G_α subtypes ($G_{\alpha s}$, $G_{\alpha i2}$, $G_{\alpha o}$ or $G_{\alpha q}$). RAMP1-induced ^{125}I-rAMY binding (mean ± s.e., 11.1 ± 0.8 pM/10^6 cells) was increased to 15.1±1.2 pM/10^6 cells in $G_{\alpha s}$ transfected cells. Enhanced $G_{\alpha i2}$, $G_{\alpha o}$ or $G_{\alpha q}$ expression, all resulted in a loss of ^{125}I-rAMY binding (9.2 ± 1.9, 7.8 ± 1.8 and 8.8 ± 1.2 pM/10^6 cells, respectively). In COS-7 cells, RAMP2 only weakly induces amylin binding (2.3±0.3 pM/10^6 cells) (1,2). However, with elevated $G_{\alpha s}$ expression, an approximate 2-fold increase in ^{125}I-rAMY binding (6.8 ± 0.6 pM/10^6 cells) was evident, while $G_{\alpha i2}$, $G_{\alpha o}$ or $G_{\alpha q}$ had no effect. An effect of $G_{\alpha s}$ enrichment on the affinity for amylin was established by competitive inhibition of ^{125}I-rAMY by unlabelled amylin (IC_{50} reduced from 3.08 ± 0.70 nM for $hCTR_a$/RAMP1 transfectants, to 1.04 ± 0.10 nM for $hCTR_a$/RAMP1/$G_{\alpha s}$ and 0.55 ± 0.10 nM for $hCTR_a$/RAMP2/$G_{\alpha s}$). Enhanced $G_{\alpha i2}$, $G_{\alpha o}$ or $G_{\alpha q}$ expression led to a loss of ^{125}I-salmon CT (sCT) binding ($hCTR_a/G_{\alpha i2}$, 21.9 ± 2.6; $hCTR_a/G_{\alpha o}$, 22.6 ± 1.7; $hCTR_a/G_{\alpha q}$, 25.7 ± 0.6 pM/10^6 cells, compared with $hCTR_a$, 30.5 ± 1.5 and $hCTR_a/G_{\alpha s}$, 32.7 ± 0.6 pM), in the presence of RAMP1 or RAMP2. Similar loss in sCT binding also resulted when $G_{\alpha i2}$ or $G_{\alpha o}$ were transfected into CHO-K1 cells that express an endogenous CTR. These results suggest that the properties of both CTR and CTR/RAMP-derived receptor phenotypes are profoundly affected by the predominant G_α subtype and its expression level in cells.

1. Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, Fraser NJ, Main MJ, Foord SM Sexton PM (1999). *Mol. Pharmacol.* 56, 235-242.
2. Tilakaratne N, Christopoulos G, Zumpe E, Foord SM, Sexton PM (2000) *J. Pharmacol. Exp. Therap.* 294, 61-72.
3. Moore EM, Kuestner RE, Stroop SD, Grant FJ, Matthewes SL, Brady CL, Sexton PM, Findlay DM (1995). *Mol. Endocrinol.* 9, 959-968.

Deleted: 1,
Deleted: 2,
Deleted: is
Deleted:
Deleted: -
Deleted:) that
Deleted: the
Deleted: , calcitonin (CT), and helps to regulate
Deleted: The distinctive characteristic of classic CTR pharmacology is a high
Deleted: CT
Deleted: ,
Deleted:).
Deleted: up
Deleted: displays
Deleted:
Deleted: (h)
Deleted: ,
Deleted:)
Deleted: ,
Deleted: fact
Deleted: our
Deleted: transfected
Deleted: a RAMP and an individual
Deleted: subtype
Deleted: was increased from
Deleted: (mean ± s.e)
Deleted: enriched
Deleted:
Deleted: or
Deleted: 06
Deleted: 09
Deleted: 11
Deleted: A similar

Poster #18

Molecular characterisation of receptor activity modifying proteins (RAMPs)

Madhara Udawela[1], George Christopoulos[1], Kenji Kuwasako[2] and Patrick M Sexton[1]

[1]Howard Florey Institute, The University of Melbourne, Vic., Australia, 3010, [2]First Dept. Internal Medicine, Miyazaki Medical College, Miyazaki, Japan, 889-1692

RAMPs are recently discovered proteins, comprising three known members, RAMP1, 2 and 3 (1), that associate with the calcitonin receptor (CTR) and calcitonin receptor-like receptor (CLR) to determine their phenotype. Various chimeras and deletion mutants of RAMPS have been used previously to investigate the interactions of RAMPs and receptor (2,3). In this study, progressive N-terminal domain swap chimeras of RAMP1 and RAMP2 were used to examine the role of these regions in phenotypic induction with CTRs. These chimeras were transfected into COS-7 cells with CTRs, and levels of ^{125}I-amylin binding (n=4) were measured. The data suggested that the disulphide bonding pattern in the N-terminus may differ for RAMP1 and RAMP2 and that the domain responsible for induction of amylin receptor phenotype is not confined to one small region of the protein. Thus the interaction between RAMPs and the CTRs was complex and involved specific domains in the N-terminus as well as the transmembrane domain of the RAMP. RAMPs have a relatively small C-terminal intracellular tail (~10 amino acids). To investigate the role of this domain, deletion mutants were constructed for each RAMP. Deletion of the C-terminus markedly attenuated the induction of ^{125}I-amylin binding for RAMP1 and RAMP2 when expressed with either the human CTRa or CTRb isoforms. The RAMP3 deletion mutant also displayed reduced induction of binding but to a lesser extent than with RAMP1 and RAMP2. Co-transfection of Gαs with hCTR and mutant RAMPs caused limited recovery of binding, which was greater in hCTRb isoform co-transfected cells. These data contrast with that for RAMP/CLR heterodimers, where RAMP1 C-terminal deletion did not dramatically alter induction of CGRP phenotype (4,5). Together the data provides insights into the molecular basis for RAMP/receptor interaction and provides evidence for distinctive modes of interaction between RAMPs and CTRs and RAMPs and CLR.

1. McLatchie, L.M. et al. *Nature* **393**, 333-9. (1998)
2. Fraser, N.J. et al. *Mol Pharmacol* **55**, 1054-9. (1999)
3. Zumpe, E.T. et al. *Biochem Biophys Res Commun* **267**, 368-72. (2000)
4. Steiner, S. et al. *Biochemistry* **41**, 11398-11404. (2002)
5. Fitzsimmons, T.J. et al. *JBC* **278 (16)**, 14313-14320. (2003)

Poster #19

Coupling to Gα_q-mediated signaling downstream of 5HT$_{2C}$ receptors in Chinese hamster ovary cells is sufficient for ERK1/2 phosphorylation

Tim D Werry (1), Patrick M Sexton (1,2) & Arthur Christopoulos (1,2)

(1) Dept of Pharmacology, University of Melbourne, VIC 3010; (2) Howard Florey Institute, University of Melbourne, VIC 3010

The serotonin 5HT$_{2C}$ receptor (5HT$_{2C}$R) signals predominantly, although not exclusively, via coupling to the G$\alpha_{q/11}$ family of G proteins, and is well established as an important therapeutic target for a variety of CNS disorders. Despite previous demonstrations that the 5HT$_{2C}$R can induce cellular transformation and growth, the mechanisms that link 5HT$_{2C}$R activation to cellular growth pathways remain undetermined. The present study investigated the coupling of the 5HT$_{2C}$R to the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in a Chinese hamster ovary (CHO) cell line stably expressing the 5HT$_{2C}$R at levels approximating those found in the brain. We used selective pharmacological inhibitors to delineate the intracellular mechanisms leading to ERK1/2 phosphorylation downstream of 5HT$_{2C}$R activation. ERK1/2 phosphorylation mediated by 5HT (1μM) was abolished by pre-treatment of cells with ET-18-OCH$_3$ (100μM), staurosporine (1μM) and PD98059 (100μM), indicating the involvement of phosphatidylinositol-specific phospholipase C (PI-PLC), protein kinase C (PKC), and the ERK1/2 upstream kinase, MEK, respectively. Pretreatment of the cells with pertussis toxin (100ng/ml) was without effect, suggesting that G$_i$ proteins were unlikely to be involved. Inhibitors of phosphatidylcholine-specific PLC and Src, and the Ca^{2+} chelator, BAPTA/AM, also had no effect. Furthermore, responses following pre-treatment with inhibitors of PI 3-kinase, and of the EGF receptor (that is commonly transactivated by GPCRs to achieve ERK1/2 activation) were identical to those seen without pre-treatment. Taken together, these studies indicate that the traditional coupling of 5HT$_{2C}$R to Gα_q and PI-PLC is sufficient for ERK1/2 phosphorylation, and that the concept of stimulus trafficking to alternative signaling pathways need not be invoked in this instance.

---

[i] Nakamura, et al (1997) *Hum Genet* **99,** 38-41

[ii] Masi L, et al (1998) Biochem Biophys Res Commun **245,** 622-6.

[iii] Taboulet J, Frenkian M, Frendo JL, Feingold N, Jullienne A, de Vernejoul MC (1998) Hum Mol Genet **7**, 2129-33.