

## PROGRAM TIMETABLE

### Molecular Pharmacology of G Protein-Coupled Receptors

7:00 – 8:00 am      Registration

8:00 – 8:05 am      Introduction/Welcome      A. Christopoulos/P. Sexton

#### Session I: GPCR Signalling [8:05 – 10:20 am]

Chairs:                      *Arthur Christopoulos & Ian Dickerson*

8:05 – 8:50 am      **Graeme Milligan**      *Building signal transducosomes containing*  
**Plenary Speaker I**      *receptors, G proteins and Regulators of G protein*  
*Signalling*

8:50 – 9:35 am      **Macdonald Christie**      *Opioid agonists have different efficacy profiles for G-*  
**Plenary Speaker II**      *protein activation, rapid desensitization and*  
*endocytosis in a single  $\mu$ -opioid receptor expressing*  
*cell-line*

9:35 – 9:55 am      **Michelle Glass**      *G protein coupling of cannabinoid receptors*

9:55 – 10:20 am      **Michael Lew**      *Signal trafficking by angiotensin receptor agonists*

10:20 – 10:50 am                      MORNING TEA & POSTER VIEWING

#### Session II: Receptor Structure-Function [10:50 am – 1:00 pm]

Chairs:                      *Roger Summers & Andrew Tobin*

10:50 – 11:35 am      **Bob Graham**      *Towards accurate models of G protein-coupled*  
**Plenary Speaker III**      *receptors*

11:35 am – 12:20 pm      **Jean-Philippe Pin**      *Activation mechanism of family 3 GPCRs*  
**Plenary Speaker IV**

12:20 – 12:40 pm      **Roger Summers**      *Pleiotropic coupling of  $\beta$ 3-adrenoceptor splice*  
*variants*

12:40 – 1:00 pm      **Arthur Christopoulos**      *Allosteric modulation of G protein-coupled*  
*receptors*

1:00 – 2:00 pm

**LUNCH & POSTER VIEWING**

**Session III: Receptor – Protein Interactions [2:00 – 4:10 pm]**

Chairs: *Patrick Sexton & Jean-Philippe Pin*

2:00 – 2:45 pm **Michel Bouvier** *Protein-protein interactions in G protein coupled*  
**Plenary Speaker V** *receptor signalling*  
**(Sponsored by Perkin Elmer International)**

2:45 – 3:30 pm **Ian Dickerson** *CGRP-receptor component protein (RCP): A multi-*  
**Plenary Speaker VI** *protein complex required for G protein-coupled signal*  
*transduction*

3:30 – 3:50 pm **Karin Eidne** *GnRH and TRH receptors: Protein interactions in*  
**(Sponsored by** *living cells.*  
**Berthold, Australia)**

3:50 – 4:10 pm **Patrick Sexton** *Receptor activity modifying proteins*

4:10 – 4:40 pm

**AFTERNOON TEA & POSTER VIEWING**

**Session IV: Receptor Regulation [4:40 – 6:10 pm]**

Chairs: *Walter Thomas & Michel Bouvier*

4:40 – 5:25 pm **Andrew Tobin** *Novel mechanisms of receptor regulation*  
**Plenary Speaker VII**

5:25 – 5:45 pm **David Findlay** *Regulation of calcitonin receptors*

5:45 – 6:05 pm **Walter Thomas** *Regulation of angiotensin receptors*

6:05 – 6:10 pm Closing remarks Roger Summers

6:30 pm – 8:00 pm

**DRINKS / MIXER – University House**

## Session I:

### GPCR Signalling [8:05 – 10:20 am]

Chairs: *Arthur Christopoulos & Ian Dickerson*

8:05 – 8:50 am Plenary Speaker I

Graeme Milligan *Building signal transducosomes containing receptors, G proteins and Regulators of G protein Signalling*

8:50 – 9:35 am Plenary Speaker II

Macdonald Christie *Opioid agonists have different efficacy profiles for G-protein activation, rapid desensitization and endocytosis in a single  $\mu$ -opioid receptor expressing cell-line*

9:35 – 9:55 am

Michelle Glass *G protein coupling of cannabinoid receptors*

9:55 – 10:20 am

Michael Lew *Signal trafficking by angiotensin receptor agonists*

## **BUILDING SIGNAL TRANSDUCTOSOMES CONTAINING RECEPTORS, G PROTEINS AND REGULATORS OF G PROTEIN SIGNALLING.**

**Graeme Milligan.** Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, U.K.

Targeting of proteins to the appropriate cellular location and the formation of signal transductosomes is integral to effective signal transduction. G proteins are highly enriched in specialised membrane domains and this ensures that information transfer is more effective in these regions than in bulk membrane fractions. Furthermore, G proteins within such domains are regulated far more efficiently. Targeting of G protein  $\alpha$  subunits to such domains is dependent upon interplay between post-translational lipid acylation and interactions with G protein  $\beta/\gamma$  complexes and acylation deficient G proteins are generally mis-targeted and not activated by receptors. To explore the role of post-translational acylation and  $\beta/\gamma$  interactions in information transfer rather than cellular location we constructed fusion proteins between the  $\alpha_{1b}$ -adrenoceptor and forms of the  $\alpha$  subunit of  $G_{11}$  that were either resistant to acylation or bound  $\beta/\gamma$  with only low affinity. Although these forms of  $G_{11}$  were attached to the receptor, both displayed markedly lower capacity to exchange guanine nucleotide than the wild type G protein although for the  $\beta/\gamma$  binding defective form this was rescued by expression of high levels of  $\beta/\gamma$ . The kinetics of  $Ca^{2+}$  mobilisation by the  $\alpha_{1b}$ -adrenoceptor- $G\alpha_{11}$  fusion protein is slowed markedly in the presence of RGS4 or RGS16. Mutation of Cys residues 2 and 12, that are targets for post-translation acylation in RGS16, eliminated GAP activity but this was not the case when Cys98 was mutated, although this is within the G protein contact domain of the RGS. To explore these issues in a more quantitative fashion, a fusion protein was constructed between the  $\alpha_{2A}$ -adrenoceptor and wild type RGS4. Following co-expression with a pertussis toxin-resistant form of  $G_{o1}\alpha$  and membrane preparation detailed analysis of agonist-stimulated GTPase activity demonstrated that the fusion proteins functioned in a concerted fashion to first stimulate and then deactivate the G protein. Mutation of Asn<sup>128</sup> but not Asn<sup>88</sup> in the RGS element of the fusion protein obliterated GAP activity but not GTP loading by the receptor. In this context, now mutation of Cys 2 and Cys12 of RGS4 did not alter GAP activity but additional mutation of Cys95 did so. Building of signal transductosomes by generating open reading frames containing multiple interacting polypeptides can help to define the relative roles of residues in protein targeting and information processing.

# OPIOID AGONISTS HAVE DIFFERENT EFFICACY PROFILES FOR G-PROTEIN ACTIVATION, RAPID DESENSITIZATION AND ENDOCYTOSIS IN A SINGLE $\mu$ -OPIOID RECEPTOR EXPRESSING CELL-LINE

M.J. Christie, S.L. Borgland, M. Connor, P. Osborne<sup>1</sup>, J. B. Furness<sup>2</sup>.

Department of Pharmacology and The Medical Foundation, University of Sydney, Sydney NSW 2006, <sup>1</sup>Prince of Wales Medical Research Institute, Randwick, NSW, 2031, <sup>2</sup>Department of Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria, 3010.

The differential ability of various  $\mu$ -opioid (MOR) agonists to induce rapid desensitization and endocytosis could arise simply from differences in their efficacy to activate G-proteins or, alternatively, be due to differential capacity for activation of other signaling processes. Previous studies have been confounded because they generally did not resolve contributions of receptor activation within several seconds, rapid desensitization over several minutes and internalization over tens of minutes in a single system expressing a low density of receptors. We have used AtT-20 cells stably expressing a low density of FLAG-tagged MOR to compare the efficacies of a range of agonists to (1) activate G-proteins using inhibition of calcium channel currents ( $I_{Ca}$ ) as a reporter before and after inactivation of a fraction of receptors by  $\beta$ -chlornaltrexamine, (2) produce rapid, homologous desensitization of  $I_{Ca}$  inhibition and (3) internalize receptors. Using receptor inactivation, relative efficacies determined for G-protein coupling were DAMGO > methadone  $\geq$  morphine > pentazocine. The same rank order of efficacies for rapid desensitization was observed but greater concentrations of agonist were required than for G-protein activation. By contrast, relative efficacies for promoting endocytosis were DAMGO > methadone  $\gg$  morphine  $\geq$  pentazocine. Pertussis toxin completely abolished modulation of  $I_{Ca}$  by MOR as well as morphine induced endocytosis but only partially inhibited endocytosis produced by DAMGO. The actions of specific protein kinase inhibitors further resolved desensitization and endocytosis as distinct processes. Rapid desensitization was completely abolished by PI3 kinase inhibitors, wortmannin and LY294002 but inhibitors of other kinases heparin, staurosporine, PD98059, KN93 and PP2 had modest effects. By contrast a number of these kinase inhibitors disrupted internalization. These results indicate that the efficacy of opioids to produce activation of G-proteins and rapid desensitization is distinct from their capacity to internalize  $\mu$ -opioid receptors.

## G-PROTEIN COUPLING OF CANNABINOID RECEPTORS.

Michelle Glass<sup>1</sup> and John K Northup<sup>2</sup>

<sup>1</sup>Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand

<sup>2</sup>National Institute on Deafness and Other Communication Disorders, Rockville, Maryland, United States of America

Since the cloning of the cannabinoid CB1 and CB2 receptors in the early 1990's extensive research has focused on understanding their signal transduction pathways. While it has been known for sometime that both receptors can couple to intracellular signalling via pertussis toxin sensitive G-proteins (Gi/Go), the specificity and kinetics of these interactions have only recently been elucidated. We have developed an *in situ* reconstitution approach to investigating receptor-G-protein interactions. This approach involves chaotropic extraction of receptor containing membranes in order to inactivate or remove endogenous G-proteins. Recombinant or isolated brain G-proteins can then be added back to the receptors, and their activation monitored through the binding of [<sup>35</sup>S]-GTPγS. This technique has been utilised for an extensive study of cannabinoid receptor mediated activation of G-proteins, and also to establish the equivalence of myristoylated recombinant and native purified G-proteins. In these studies we have established that CB1 couples with high affinity to both Gi and Go type G-proteins. In contrast, CB2 couples strongly to Gi, but has a very low affinity for Go. We then examined the ability of a range of cannabinoid agonists to activate the Gi and Go via CB1. Conventional receptor theory suggests that a receptor is either active or inactive with regard to a G-protein and that the active receptor activates all relevant G-proteins equally. However, in this study we found that agonists could produce different degrees of activation, depending on which G-protein was present. Further studies have compared the ability of the two endocannabinoids to drive the activation of Gi or Go. These studies show that agonists can induce multiple forms of activated receptor that differ in their ability to catalyse the activation of Gi or Go. The ability of an agonist to drive a receptor into a conformation that can selectively target one pool of G-proteins over another suggests that agonists could be designed which show greater selectivity for one G-protein over another. If the receptor activates several G-protein classes, and one of these pathways leads to specific adverse effects, those adverse effects could be avoided by agonists that direct signalling in favour of more constructive pathways. Given the highly conserved nature of this receptor family, this phenomenon should hold for other GPCRs, therefore characterising this selectivity should advance drug discovery for the numerous disorders and diseases treated through the activation or inhibition of GPCRs.

## ANGIOTENSIN II AND ANGIOTENSIN III DIFFERENTIALLY ACTIVATE SIGNALLING PATHWAYS VIA THE AT<sub>1</sub> RECEPTOR.

MJ Lew<sup>1</sup>, AC Holloway<sup>1,2</sup> WG Thomas<sup>2</sup> & J Ziogas<sup>1</sup> Department of Pharmacology, University of Melbourne, Vic 3010 and Baker Heart Research Institute, Vic 8008.

Angiotensin AT<sub>1</sub> receptors signal via G<sub>q</sub>/11 and activation of phospholipase C, but have been shown to signal via G<sub>i/o</sub> and to stimulate a wide variety of other pathways. Physiological effects of AT<sub>1</sub> receptors include both immediate responses like vasoconstriction and more long-term, delayed responses like cellular growth. The commonly assumed natural ligand for AT<sub>1</sub> receptors is angiotensin II (AngII), but angiotensin III (des-Asp<sup>1</sup>-AngII, AngIII) is also likely to activate AT<sub>1</sub> receptors in physiological circumstances. Scant attention has been paid to AngIII as an activator of AT<sub>1</sub> receptors, so we compared the signalling properties of AT<sub>1</sub> receptors activated by AngII and AngIII with surprising results. In CHO-K1 cells stably transfected to express rat AT<sub>1A</sub> receptors, AngII and AngIII both elicited accumulation of inositol phosphates. AngII was about 60-fold more potent than AngIII (pEC<sub>50</sub>s 8.7 ± 0.3 and 6.9 ± 0.1) but their maximal responses were equivalent. The responses to AngII and AngIII were unaffected by peptidase inhibition with bestatin and amastatin. Both peptides elicited phosphorylation, and thus activation, of the MAPKs Erk 1 and Erk 2 with very high potency. AngII was nearly 10-fold more potent than AngIII, with a substantial response at 0.1 pM. Overall cellular metabolic responses to activation of AT<sub>1</sub> receptors, measured as extracellular acidification rate with a Cytosensor Microphysiometer, yielded the surprising result that AngII and AngIII were indistinguishable in time-course, maximum, and concentration-response curve location (pEC<sub>50</sub>s 9.3 ± 0.1 and 9.3 ± 0.1). There are large discrepancies in relative potencies of AngII and AngIII in these three different assays that cannot be explained in classical terms of agonist efficacy and are suggestive of ligand-directed trafficking of receptor signalling. The wide variation in absolute potency of the peptides between the assays is also notable and is suggestive of a low importance of PLC signalling in the responses to AT<sub>1</sub> activation in the CHO cells. AT<sub>1</sub> receptors are rapidly phosphorylated, uncoupled and internalised following activation, and those processes have capacity to influence the overall responses obtained. Therefore we determined the relative abilities of AngII and AngIII to elicit phosphorylation and internalisation of tagged AT<sub>1</sub> receptors in the CHO cells. AngII elicited phosphorylation of transiently transfected N-terminus-tagged AT<sub>1A</sub> receptors with a threshold of less than 0.1 nM. AngIII was at least 10-fold less potent and the response at 100 nM was only about 60% of that to AngII at the same concentration. Despite the marked difference in receptor phosphorylation elicited by AngII and AngIII, the peptides elicited internalisation of AT<sub>1A</sub>-GFP chimeras with equal potencies and time-courses. The internalisation results accord with the similarity of time-courses of ECAR responses to AngII and AngIII, but contrast with the receptor phosphorylation results. Perhaps only some of the phosphorylation sites on the AT<sub>1</sub> receptor are important determinants of internalisation. Overall this study has shown that the complex pattern of signalling by the AT<sub>1</sub> receptor is influenced by the nature of the ligand. This apparent ligand-directed trafficking of receptor signalling is important with respect to AT<sub>1</sub> receptor function, and also with respect to the physiological roles of the endogenous peptides AngII and AngIII.

## Session II:

### Receptor Structure-Function [10:50 am – 1:00 pm]

Chairs: *Roger Summers and Andrew Tobin*

10:50 – 11:35 am Plenary Speaker III

Bob Graham *Towards accurate models of G Protein-coupled receptors*

11:35 am – 12:20 pm Plenary Speaker IV

Jean-Philippe Pin *Activation mechanism of family 3 GPCRs*

12:20 – 12:40 pm

Roger Summers *Pleiotropic coupling of  $\beta$ 3-adrenoceptor splice variants*

12:40 – 1:00 pm

Arthur Christopoulos *Allosteric modulation of G protein-coupled receptors*

## TOWARDS ACCURATE MODELS OF G PROTEIN-COUPLED RECEPTORS

R. Peter Riek<sup>1</sup>, Isidore Rigoutsos<sup>2</sup>, Jiri Novotny<sup>1</sup> and **Robert M. Graham**<sup>1</sup>

<sup>1</sup>Victor Chang Cardiac Research Institute, Darlinghurst, NSW, Australia and <sup>2</sup>Bioinformatics and Pattern Discovery Research Group, IBM Research Division, Yorktown Heights, NY, USA

Despite the vast number of G protein-coupled receptors, a high-resolution structure has been determined for only a single member (rhodopsin) of this superfamily. Availability of accurate macromolecular models would greatly aid in understanding structure/function relationships for these receptors and for drug design. Development of such models requires an understanding of the relationship between protein amino acid sequence and its three dimensional structure; a relationship that remains very poorly defined. One of the promising methods of protein structure-prediction involves the use of amino acid sequence-derived patterns. Here we report the development of an algorithm for predicting sequence/structure relationships determined from an analysis of the high-resolution structures of all solved polytopic transmembrane proteins. We show that the transmembrane helices of these proteins contain frequent non- $\alpha$ -helical components, i.e.  $3_{10}$ -helices ("tight turns"),  $\pi$ -helices ("wide turns") and intrahelical kinks. In addition to their critical contribution to the overall architectural geometry of the receptor, failure to incorporate these elements into macromolecular receptor-models can cause marked deviations in side chain orientation and, thus, in key interhelical and ligand contacts. Sets of short peptides encoding these non-canonical motifs have been used as training sets to construct non-degenerate motif descriptors. A 'search engine' built of these motif descriptors correctly identifies, and discriminates amongst instances of the above "non-canonical" helical motifs contained in the SwissProt/TrEMBL database of protein primary structures. The results indicate that deviations from  $\alpha$ -helicity are encoded locally in sequence patterns only about 7- to 9-residues long. This search engine has now been applied to define the non- $\alpha$ -helical motifs in the  $\alpha_{1B}$ -adrenergic receptor structure, and this information used to refine a model of the receptor developed by amino acid replacement of the rhodopsin 3D crystal structure. This refined model shows the predicted orientation of a number of side chains, as defined previously from mutagenesis studies. These findings, therefore, auger well for the routine development of plausible models of G protein-coupled receptors. Moreover, the success of our approach to defining non-canonical elements in complex polytopic proteins foretells development of similar prediction tools capable of identifying other structural motifs from sequence alone.

## ACTIVATION MECHANISM OF FAMILY 3 GPCRS

**Pin JP**, Galvez T, Duthey B, Bessis AS, Kniazeff J, Acher F, Prézeau L

The family 3 G-protein coupled receptors is composed of receptors for physiologically important molecules such as  $\text{Ca}^{2+}$ , pheromones, sweet compounds and the main neurotransmitters GABA and glutamate. These family 3 receptors consist of two domains: a Venus Flytrap module (VFTM) responsible for agonist binding and a heptahelical domain (HD) responsible for G-protein activation. The VFTM is composed of two lobes separated by a large cleft where agonists bind. Upon binding, agonists likely stabilize a closed form of the VFTM, whereas antagonists prevent the full closure of this domain. In agreement with this proposal, we have identified the molecular determinants responsible for the antagonists action of two mGlu8 antagonists. Indeed, by mutating the identified residues we have been able to transform these two antagonists into full agonists (Bessis et al., 2002). We proposed that the closed state of the VFTM affects the equilibrium between the inactive and active forms of the HD. In agreement with this possibility, compounds interacting directly within the HD act as allosteric regulators of these receptors, being either inverse agonists or positive modulators (Parmentier, 2002). But how does the closure of the VFTM induce the necessary change in conformation within the HD to activate G-proteins? Recent data obtained with the  $\text{GABA}_B$  receptor highlight the importance of receptor dimerization in the activation process (Galvez et al., 2001). Indeed, the  $\text{GABA}_B$  receptor is composed of two subunits, the GB1 subunit responsible for agonist binding (Galvez et al., 2000; Kniazeff et al., 2002), and the GB2 subunit responsible for G-protein activation (Duthey et al., 2002). Our data are consistent with a model in which a large change in conformation of the dimer of VFTMs is transmitted to the dimer of HDs leading to the stabilization of the active form of the HD and G-protein activation.

- Bessis A-S, Rondard P, Gaven F, Brabet I, Triballeau N, Prézeau L, Acher F and Pin J-P (2002) Closure of the Venus Flytrap module of mGlu8 receptor and the activation process: insights from mutations converting antagonists into agonists. *Proc Natl Acad Sci (USA)* 17: 11097-11102.
- Duthey B, Caudron S, Perroy J, Bettler B, Fagni L, Pin J-P and Prézeau L (2002) A single subunit (GB2) is required for G-protein activation by the heterodimeric  $\text{GABA}_B$  receptor. *J Biol Chem* 277:3236-3241.
- Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prézeau L and Pin J-P (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal  $\text{GABA}_B$  receptor function. *EMBO J* 20:2152-2159.
- Galvez T, Prézeau L, Milioti G, Franek M, Joly C, Froestl W, Bettler B, Bertrand H-O, Blahos J and Pin J-P (2000) Mapping the agonist binding site of  $\text{GABA}_B$  type 1 subunit sheds light on the activation process of  $\text{GABA}_B$  receptors. *J Biol Chem* 275:41166-41174.
- Kniazeff J, Galvez T, Labesse G and Pin J-P (2002) No ligand binding in the GB2 subunit of the  $\text{GABA}_B$  receptor is required for activation and allosteric interaction between the subunits. *J Neurosci* 22:7352-7361.
- Parmentier M-L, Prézeau L, Bockaert J and Pin J-P (2002) A model for the functioning of family 3 GPCRs. *Trends Pharmacol Sci* 23:268-274.

## PLEIOTROPIC COUPLING OF $\beta_3$ -ADRENOCEPTOR SPLICE VARIANTS.

**R.J. Summers**

Molecular Pharmacology Laboratory, Department of Pharmacology, Monash University, PO Box 13 E, Melbourne, Victoria 3800, Australia.

Although not universal, alternative splicing of transcripts encoding G-protein coupled receptors has the potential to diversify the number of receptor subtypes beyond those encoded by distinct genes. Most splice variants show similar pharmacology but some show marked differences in signaling properties. The mouse  $\beta_3$ -adrenoceptor (AR) contains two introns both of which undergo alternate splicing (Evans *et al.*, 1999; Granneman *et al.*, 1995). Splicing of intron A 100bp upstream from the previously characterised start of exon 2 results in a mRNA coding for a splice variant termed the  $\beta_{3b}$ -AR which has a 17 amino acid C-terminus that differs from the 13 in the known  $\beta_{3a}$ -AR. Constructs carrying the coding region of the  $\beta_{3a}$ - and  $\beta_{3b}$ -AR were transfected into CHO-K1 cells and high (1200 fmol/mg), medium (500 fmol/mg) and low (100 fmol/mg protein) expressing clones selected. Receptor binding studies with [ $^{125}$ I]-cyanopindolol (ICYP) and a variety of agonist and antagonist competitors showed no significant differences in pharmacological properties between the splice variants. Functional responses were measured by increases in extracellular acidification rate (ECAR; cytosensor microphysiometer), by increases in cAMP accumulation or by examination of Erk 1/2 phosphorylation (Hutchinson *et al.*, 2002) in cells expressing either receptor. Exposure of CHO-K1 cells expressing high levels of the  $\beta_{3a}$ - or  $\beta_{3b}$ -AR to a variety of  $\beta$ -AR ligands resulted in a concentration-dependent increase in ECAR relative to baseline acidification rates. Maximum responses in cells expressing  $\beta_{3b}$ -AR were less for CL316243 ( $80.5 \pm 1.7$ , n=5-6); BRL37344 ( $80.0 \pm 4.2$ , n=4); L755507 ( $50.6 \pm 3.4$ , n=3-4) and (-)-isoprenaline ( $60.9 \pm 7.3$ , n=4) than in cells expressing  $\beta_{3a}$ -AR. Similar differences were observed for cAMP accumulation responses. PTX treatment ( $100\text{ng ml}^{-1}$  16h) increased maximum ECAR and cAMP responses to CL316243 in cells expressing the  $\beta_{3b}$ -AR but not in cells expressing the  $\beta_{3a}$ -AR at all levels of receptor expression indicating that  $\beta_{3b}$ - but not  $\beta_{3a}$ -AR couple to PTX sensitive G-proteins. Stimulation of high-expressing cells with CL316243 increased Erk 1/2 phosphorylation with pEC<sub>50</sub> values and maximum responses that were not significantly different in cells expressing either splice variant. Erk 1/2 phosphorylation was insensitive to PTX or H89 (PKA inhibitor) but was inhibited by LY294002 (PI3K $\gamma$  inhibitor), PP2 (c-Src inhibitor), genistein (tyrosine kinase inhibitor) and PD98059 (MEK inhibitor). However forskolin or cholera toxin failed to increase Erk 1/2 levels although both markedly increased cAMP accumulation in both  $\beta_{3a}$ - or  $\beta_{3b}$ -AR transfected cells. Comparison of concentration-response curves for CL316243 in high expressing cells for the 3 assays showed marked differences in agonist potency. pEC<sub>50</sub> values for  $\beta_{3a}$ - and  $\beta_{3b}$ -AR expressing cells were respectively for ECAR,  $10.79 \pm 0.07$ ;  $10.57 \pm 0.05$ , cAMP accumulation  $9.74 \pm 0.15$ ;  $9.87 \pm 0.10$  and Erk 1/2 phosphorylation  $7.28 \pm 0.26$ ;  $7.36 \pm 0.24$ . SR59230A, carvedilol and propranolol, usually regarded as  $\beta$ -AR antagonists, were full agonists for ECAR. At all 3 levels of receptor expression, compared to CL316243, SR59230A was a full agonist for ECAR but a partial agonist for cyclic AMP accumulation. This difference was more pronounced in 3T3-F442A cells that express mouse  $\beta_3$ -AR where SR59230A had higher efficacy than CL316243 for ECAR but acted as a competitive antagonist for cAMP accumulation. The studies demonstrate that  $\beta_3$ -AR can couple to ECAR, cAMP accumulation and Erk 1/2 phosphorylation with different efficacy. ECAR responses identify signaling pathways in addition to cAMP that can be selectively targeted by SR59230A. The findings emphasise the importance of measurement of drug efficacy in a variety of functional models.

- EVANS, B.A., PAPAIOANNOU, M., HAMILTON, S. & SUMMERS, R.J. (1999). Alternative splicing generates two isoforms of the  $\beta_3$ -adrenoceptor which are differentially expressed in mouse tissues. *Br. J. Pharmacol.*, **127**, 1525-442.
- GRANNEMAN, J.G. & LAHNERS, K.N. (1995). Regulation of mouse  $\beta_3$ -adrenergic receptor gene expression and mRNA splice variants in adipocytes. *American Journal of Physiology Cell Physiology*, **37**, C1040-C1044.
- HUTCHINSON, D.S., BENGTTSSON, T., EVANS, B.A. & SUMMERS, R.J. (2002). Mouse  $\beta_{3a}$ - and  $\beta_{3b}$ -adrenoceptors expressed in Chinese hamster ovary cells display identical pharmacology but utilize distinct signalling pathways. *British Journal of Pharmacology.*, **135**, 1903-14.

# ALLOSTERIC MODULATION OF G PROTEIN-COUPLED RECEPTORS

Arthur Christopoulos

Department of Pharmacology, University of Melbourne

G protein-coupled receptors (GPCRs) are natural allosteric proteins because agonist-mediated signaling by the receptor requires a conformational change in the protein that is transmitted between two topographically distinct binding domains, the orthosteric site recognized by the agonist, and an intracellular allosteric site recognized by the G protein. It is now becoming increasingly recognized, however, that the orthosteric ligand-bound GPCR can also form a ternary complex with other ligands or “accessory” proteins<sup>1, 2</sup>. Allosteric sites on GPCRs represent novel drug targets because allosteric modulators possess a number of theoretical advantages over classic orthosteric ligands, such as a ceiling level to the allosteric effect and a potential for greater GPCR subtype-selectivity. The simple ternary complex model (TCM) of allosteric interactions has proven useful in describing and quantifying the behavior of many allosteric modulators of GPCRs, and remains an important mechanistic starting point for studying GPCR allosterism. However, there are some instances where allosteric modulators appear to display more complex binding patterns than those predicted by the simple TCM. For example, we have found that the allosteric inhibitor, C<sub>7</sub>/3-phth, and the allosteric enhancer, alcuronium, can display biphasic or bell-shaped binding isotherms, respectively, when tested at the M<sub>2</sub> muscarinic acetylcholine receptor (mAChR), using [<sup>3</sup>H]N-methylscopolamine as the orthosteric radioligand. In the past, such behaviors of allosteric modulators have been explained in terms of complex mechanistic schemes, e.g., mixed orthosteric/allosteric binding modes, but we have found that both these behaviors are readily accommodated by the simple TCM if the effects of the modulators on orthosteric ligand binding kinetics are explicitly incorporated in the model. More recently, however, we have identified additional behaviors of some allosteric modulators of the mAChRs that do indeed require extensions of the simple TCM. For instance, although the modulator, gallamine, inhibits the binding of the agonist, acetylcholine, to the M<sub>2</sub> mAChR (negative binding cooperativity), it is sometimes able to weakly activate the receptor in its own right (positive activation cooperativity). Another example has been noted with alcuronium, which is able to enhance the binding affinity of the agonist, pilocarpine, while simultaneously decreasing its efficacy. Finally, although most studies of GPCR allosterism to date have primarily focused on acute GPCR binding and signaling properties, we have found that prolonged exposure of the M<sub>2</sub> mAChR to some allosteric modulators also leads to a change in the steady state cell-surface expression of this receptor. It is therefore evident that allosteric interactions can be manifested in a variety of ways. Nonetheless, the successful exploitation of allosteric phenomena can lead to novel therapeutic regimens that provide maximum benefit while causing minimal adverse effects. Importantly, the study of such phenomena will become of progressively greater import as the impact of newer and more sensitive GPCR screening technologies is absorbed and assimilated into the drug discovery process.

1. A. Christopoulos and T. Kenakin (2002) G protein-coupled receptor allosterism and complexing, *Pharmacol. Rev.* **54**, 323-374.
2. A. Christopoulos (2002) Allosteric binding sites on cell-surface receptors: Novel targets for drug discovery. *Nature Rev. Drug Discovery* **1**, 198-210.

### **Session III:**

#### **Receptor – Protein Interactions [2:00 – 4:10 pm]**

Chairs: *Patrick Sexton and Jean-Philippe Pin*

2:00 – 2:45 pm Plenary Speaker V

Michel Bouvier *Protein-protein interactions in G protein-coupled receptor signalling*

2:45 – 3:30 pm Plenary Speaker VI

Ian Dickerson *CGRP-receptor component protein (RCP): A multi-protein complex required for G protein-coupled signal transduction*

3:30 – 3:50 pm

Karin Eidne *GnRH and TRH receptors: Protein interactions in living cells*

3:50 – 4:10 pm

Patrick Sexton *Receptor activity modifying proteins*

# PROTEIN-PROTEIN INTERACTIONS IN G PROTEIN-COUPLED RECEPTOR SIGNALLING.

**Michel Bouvier**

Dept. de Biochimie, Local B-313-1, Université de Montréal, 2900 Édouard-Montpetit, C.P. 6128  
Succ. Centre-ville, Montréal, Québec, Canada H3C 3J7

The growing appreciation of the importance of protein-protein interactions in cell biology has led to the idea that, in addition to providing functional information about the protein itself, studying these interactions should lead to the identification of novel protein networks. This may be particularly useful when considering proteins involved in signal transduction. In fact, several *in vitro* assays have been designed towards this objective. However, characterization of the proteins and of their interactions in the environment where they are normally expressed remains a preferred goal. Conventional fluorescence microscopy techniques that are routinely used to visualize proteins in cells do not offer the resolution required to monitor molecular interactions. In contrast, spectroscopic approaches based on the non radiative transfer of energy provide such level of resolution. Thus, we exploited one of these approaches known as Bioluminescence Resonance Energy Transfer (BRET) to study protein-protein interactions in living mammalian cells. This technique is based on the non-radioactive transfer of energy between a luminescent donor (for example, *Renilla*-luciferase or *Rluc*) and a fluorescent acceptor (ex: a Green Fluorescent Protein; GFP). The strict dependence on molecular proximity between donor and acceptor molecules for energy transfer (<100 Å) makes BRET a system of choice for monitoring intermolecular interactions. Upon molecular proximity of the donor and acceptor molecules, the energy resulting from the catalytic degradation of coelenterazine, a cell-permeable substrate for *Rluc*, is transferred to GFP which, in turn, emits fluorescence at its characteristic wavelength. The fluorescence/luminescence ratio provides a measure of BRET and indicates the occurrence of protein-protein interaction. Recently, we applied this method to the study of G protein-coupled receptor (GPCR) signalling. Using the human  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) fused to *Rluc* as BRET donor ( $\beta_2$ AR-*Rluc*) and to GFP as BRET acceptor ( $\beta_2$ AR-GFP), we showed that this receptor exist as a constitutive homodimer under basal conditions. The generalization of this oligomerization assay has now been validated for a number of GPCR including the  $\beta_1$ AR, melatonin MT1 and MT2, chemokine CCR5 and CXCR4, vasopressin-V2 and V1a, oxytocin OT1 and GABA<sub>B</sub> receptors. Quantitative BRET analysis allowed determination of the stoichiometry of interaction, the relative affinity of the partners as well as the proportion of receptor engaged in oligomeric complexes. Also, the activation-dependent recruitment of the regulatory protein  $\beta$ -arrestin to the GPCR could be monitored by BRET. Taken together, these data demonstrate that BRET is a sensitive technique that permits monitoring of constitutive and dynamically regulated protein-protein interactions. In addition, it can provide useful bio-sensors to monitor multiple cellular activities in living cells.

## **CGRP-RECEPTOR COMPONENT PROTEIN (RCP): A MULTI-PROTEIN COMPLEX REQUIRED FOR G PROTEIN-COUPLED SIGNAL TRANSDUCTION.**

A.R. Davis, A.A. Tolun, M.A. Prado, B. Evans-Bain, and **I.M. Dickerson**

Departments of Physiology and Biophysics, Biochemistry and Molecular Biology, The University of Miami School of Medicine, P.O. Box 016430, Miami, FL, USA

Calcitonin gene-related peptide (CGRP) is a 37 amino acid, carboxyl-amidated neuropeptide. CGRP belongs to a family of homologous neuropeptides including calcitonin, adrenomedullin, and amylin characterized by an NH<sub>2</sub>-terminal disulfide ring structure. CGRP is expressed in neurons in a wide anatomical distribution in both central and peripheral nervous systems resulting in a wide range of physiologic functions. CGRP is best characterized for its potent vasodilator actions. CGRP is one of the most potent vasodilators known, and has the capacity to reduce blood pressure rapidly and effectively. CGRP binding results in increased intracellular cAMP, suggesting a G protein-coupled receptor. Our laboratory set out to clone the CGRP receptor, using an oocyte-based expression-cloning strategy. A single cDNA clone was identified, but upon sequencing this cDNA did not encode a characteristic G protein-coupled receptor. Instead, the activating cDNA encoded a 148 aa, hydrophilic protein with no homology to sequences in GenBank. We hypothesized that this protein was activating a CGRP receptor endogenous to *Xenopus* oocytes, and named this novel protein the CGRP-receptor component protein (RCP). RCP is broadly expressed in immortalized cell lines, making gain-of-function experiments difficult in cell culture. To circumvent this limitation we have expressed antisense constructs of RCP cDNA in NIH3T3 cells which are known to express CGRP receptors, and discovered that loss of RCP correlates with loss of CGRP-mediated signal transduction, but has no effect on CGRP receptor affinity or density. Therefore, RCP is not acting as a chaperone protein to route the CGRP receptor to the cell surface, but is instead coupling the receptor to the cellular signal transduction pathway. A stereotypic G protein-coupled receptor has recently been identified for CGRP, named the calcitonin receptor-like receptor (CRLR). CRLR requires a second protein named receptor activity modulating protein (RAMP1) for correct trafficking and pharmacology. We propose that a functional CGRP receptor is a complex of at least three proteins: the ligand binding protein (CRLR), a protein for trafficking and pharmacology (RAMP1), and a protein for signaling (RCP). CGRP-responsive cells express CRLR, RAMP1 and RCP, and RCP co-immunoprecipitates with CRLR and RAMP1, suggesting a protein complex. Preliminary data suggests that RCP may organize CRLR into functional complexes in lipid rafts in the cell membrane, although RCP may also work by directly coupling CRLR to signal transduction proteins such as G proteins. RCP appears to modulate CRLR function, as we found that RCP expression correlated with CGRP efficacy in mouse uterus during pregnancy. Thus, RCP may be a target for therapeutic modulation of CGRP peptidergic actions.

## **GNRH AND TRH RECEPTORS; PROTEIN INTERACTIONS IN LIVING CELLS.**

**Eidne KA**, Kroeger KM, Seeber RM, Dromey J and Hanyaloglu AC

Western Australian Institute for Medical Research (WAIMR), University of Western Australia, B Block, Sir Charles Gairdner Hospital, Perth, WA, Australia, 6009.

Genetically encoded luminescent and fluorescent fusion proteins in conjunction with biophysical methods such as bioluminescence resonance energy transfer (BRET) has allowed us to monitor dynamic protein-protein interactions involving G-protein coupled receptors (GPCRs). GPCRs have been reported to undergo oligomerization and the formation of receptor complexes could alter both receptor pharmacology and function to provide an additional level of regulation. Using BRET we have confirmed the existence of oligomeric GPCR complexes in living cells and have been able to quantitatively assess the functional interactions of GPCRs with adaptor proteins such as the  $\beta$ -arrestins as well as with several other partner molecules. We show that subtypes of the TRH receptor (TRHR1 & TRHR2) undergo oligomerization and that each subtype interacts differentially with  $\beta$ -arrestin 1 and 2 isoforms. TRHR2 does not utilize  $\beta$ -arrestin 1, however this interaction does occur when TRHR1 is also present, suggesting that formation of the hetero-oligomeric unit can alter receptor trafficking. Although another GPCR, the GnRH receptor (GnRHR) is also capable of forming oligomers, its unique features make it unable to utilize  $\beta$ -arrestins to promote agonist-dependent internalisation rates. We have investigated interactions between the GnRHR and other proteins and demonstrate a novel interaction between GnRHR and E2F4, a transcription factor involved in cell cycle arrest. By monitoring this interaction with BRET, we observed a rapid loss in binding between GnRHR and E2F4 after treatment with GnRH. This coincided with a GnRH-mediated change in E2F4 cellular distribution. GnRH mediates an antiproliferative effect in a range of cells expressing the GnRHR and our finding indicates the involvement of cell cycle arrest. By monitoring dynamic interactions of engineered BRET fusion protein partners involved in GPCR-mediated events, we can more fully understand the mechanisms of ligand-induced processes like receptor trafficking and cellular proliferation.

## RECEPTOR ACTIVITY MODIFYING PROTEINS

**Patrick M. Sexton**

Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, Victoria 3010.

The discovery of receptor activity modifying proteins (RAMPs) has led to a re-evaluation of what defines G protein-coupled receptor (GPCR) phenotypic behavior towards agonists and/or G proteins. The RAMP family comprises three accessory proteins (designated RAMP1, RAMP2 and RAMP3) that were originally identified during attempts to clone the receptor for calcitonin gene-related peptide (CGRP). Phenotypic receptor behavior corresponding to that of the native CGRP receptor could only be demonstrated in recombinant expression systems when another seven transmembrane receptor, the calcitonin receptor-like receptor (CL receptor) was co-expressed with RAMP1. Additional studies extended these observations to identify a general role of RAMPs in modifying the expression and the pharmacology of receptors related to the calcitonin family of peptides; CL receptor co expressed with RAMP2 or RAMP3 engendered adrenomedullin receptors, whereas calcitonin receptor co expression with individual RAMPs yielded amylin receptors with distinct phenotypes. Whether RAMPs interact with other receptors is less clear. The distribution of RAMP expression extends beyond that of both the CL receptor and the calcitonin receptor, suggesting they may have a wider role in receptor pharmacology. As the CL receptor and calcitonin receptors are members of the Class II subfamily of G protein-coupled receptors (GPCRs), we have recently investigated the potential for RAMP interaction with other Class II GPCRs. Using immunofluorescence confocal microscopy we have demonstrated, an interaction of RAMPs with 4 additional receptors, the VPAC1 vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor with all 3 RAMPs; the glucagon and PTH1 parathyroid hormone receptors with RAMP2 and the PTH2 receptor with RAMP3. No interaction was observed with the growth hormone releasing hormone, glucagon-like peptide (GLP) 1, GLP 2 or VPAC2 receptors. Unlike the interaction of RAMPs with the CL receptor or the CTR, in radioligand saturation and competition binding assays in whole cells and membranes, VPAC1 receptor/RAMP complexes did not show altered phenotypic behaviour when compared to the VPAC1 receptor alone in COS-7 cells. However, the VPAC1 receptor/RAMP2 heterodimer displays a significant enhancement of agonist-mediated phosphoinositide hydrolysis with no change in cAMP stimulation compared to the VPAC1 receptor alone. Our findings identify a new functional consequence of RAMP-receptor interaction, and suggest that RAMPs play a more general role in modulating cell-signaling through other GPCRs than is currently appreciated.

**Session IV:**

**Receptor Regulation [4:40 – 6:10 pm]**

Chairs: *Walter Thomas and Michel Bouvier*

4:40 – 5:25 pm Plenary Speaker VII

Andrew Tobin *Novel mechanisms of receptor regulation*

5:25 – 5:45 pm

David Findlay *Regulation of calcitonin receptors*

5:45 – 6:05 pm

Walter Thomas *Regulation of angiotensin receptors*

## NOVEL MECHANISMS OF RECEPTOR REGULATION

**Andrew B Tobin**

Department of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester. LE1 9HN UK.

Our laboratory has focused on agonist-mediated phosphorylation of GPCRs and the functional role played by this regulatory process. By using the human M<sub>3</sub>-muscarinic receptor as our model receptor we have determined that this receptor is phosphorylated by casein kinase CK1 $\alpha$  in an agonist-dependent manner. The significance of this finding is that it gives evidence for the hypothesis that agonist-dependent phosphorylation of GPCRs may be mediated by protein kinases distinct from the G-protein receptor kinase family. This is important in light of recent studies on a number of GPCRs demonstrating that agonist-phosphorylation is likely to be a complex process involving phosphorylation of the receptor on a number of sites and possibly in a hierarchal manner. This certainly appears to be the case of the M<sub>3</sub>-muscarinic receptor which is phosphorylated on serines which are arranged in clusters within the third intracellular loop. We have attempted to map these phosphorylation sites and assign a function to phosphorylation of particular clusters of serines. These studies have identified specific down-stream signalling pathways that are regulated by the phosphorylation status of the receptor and, interestingly, pathways which are regulated in a manner independent of receptor phosphorylation. In my presentation I will give details of these studies and give our thoughts on the role that multi-site phosphorylation plays in the regulation of GPCR signalling.

## REGULATION OF CALCITONIN RECEPTORS

**David Findlay**

Department of Orthopaedics and Trauma, University of Adelaide and Hanson Institute, Adelaide, S.A., Australia

Calcitonin (CT) is a 32 amino acid peptide hormone that has GPCR-mediated actions in a number of cell types and organs, the best recognised of which is a potent inhibition of osteoclastic bone resorption as a response to elevated serum calcium. Consistent with a role for CT in calcium metabolism, recent work suggests that renal CT receptors (CTR) are functionally linked to increased CYP24 mRNA levels in the kidney, CYP24 being the enzyme responsible for degradation of 1,25-dihydroxyvitamin D. Studies from our laboratory have indicated that CT can also modulate the growth and survival of cells expressing the CTR, and it has been found that the CTR is widely expressed in development and has a likely role in the early embryo. Indeed, it has recently been reported that CTR gene deletion is embryonic lethal in mice at about 10 days of gestation. CTR are also abundant in the brain, although we have a less developed notion of the actions of CT in the brain. Given the apparently diverse roles of CT in development, and postnatally, it is perhaps not surprising that regulation of the CTR is complex. Firstly, receptor function can be regulated at the protein level, by receptor activity modifying proteins (RAMPs) determining receptor 'phenotype', in terms of ligand specificity. This aspect of CTR function will be discussed by other speakers. Secondly, cell surface CTR levels are also determined by CT binding, the mechanisms for this 'homologous receptor regulation' being dependent on the cell type and the species. For example, CTR binding of CT in osteoclasts results in internalisation of CT-CTR complexes and rapid and profound down-regulation of CTR mRNA. CTR can activate a number of intracellular signalling pathways via different G proteins, which include the PKA, PKC and Erk 1/2 MAP kinase pathways. Down-regulation of CTR mRNA is mediated by PKA in mouse osteoclasts and PKC in the human. CT treatment of osteoclasts results in destabilisation of the CTR, an event that appears to involve AU-rich elements in the 3' UTR of the CTR mRNA. CTR binding of CT in non-osteoclastic cells also results in internalisation of CT-CTR complexes but with no observable effect on CTR mRNA. Finally, CTR expression is regulated transcriptionally, again in a cell-type dependent manner and as a function of cell differentiation and in response to agents such as glucocorticoids. The CTR gene 5'-flanking region has 3 recognised promoters, which operate in a tissue specific manner. The P3 promoter of the mouse CTR gene is osteoclast-specific and contains 8 putative NFAT/AP-1 sites, which are activated by calcium signalling. Thus, regulation of the CTR is multi-layered and complex, which reflects the apparently diverse roles of this intriguing molecule. Renewed interest in CT as an important physiological player and as a valuable therapy for bone disease will continue to stimulate research into the regulation of the CTR.

## **REGULATION OF ANGIOTENSIN RECEPTORS**

**Walter G. Thomas**

Molecular Endocrinology Laboratory, Baker Heart Research Institute, PO Box 6492, St Kilda Rd Central, Melbourne 8008, Australia

The type 1 angiotensin receptor (AT<sub>1</sub>R) mediates the homeostatic and pathological actions of the peptide hormone, angiotensin II. With regard to the processes that activate/deactivate seven transmembrane-spanning, G protein-coupled receptors (GPCRs), AT<sub>1</sub>Rs are among the most widely studied, serving as prototypes for GPCRs that bind and respond to peptide hormones. Arrestins are proteins that bind to activated and phosphorylated GPCRs and terminate initial signals emanating from these receptors. Arrestins can also act as scaffolds to recruit regulatory proteins, which mediate receptor internalization, and additional signalling molecules to increase the repertoire of receptor responses. My presentation will examine recent studies into the contribution of arrestins to the signalling, deactivation and trafficking of AT<sub>1</sub>Rs.

## FREE COMMUNICATIONS

**P1.** Michael F. Crouch, Francis Willard & Leise Berven. *Thrombin stimulates the clustering and sensitization of EGF receptors of Swiss 3T3 cells without transactivation*

**P2.** Michael F. Crouch, Andrea J. Brown, Georgina L. Harvey & Anthony R. Dyer. *High throughput cell-based screening of receptor signal transduction pathways*

**P3.** Angela M. Finch, Fang Lin, Valerie Sarramegna, Songhai Chen, Anne Cunningham & Robert M. Graham. *Structural determinants of  $\alpha_1$ -adrenergic receptor subtype-selective phosphorylation and internalisation*

**P4.** D. M. Findlay, S. Hay, L. J. Raggatt, S. Bouralexis & A. Evdokiou. *Calcitonin decreases the adherence and survival of HEK-293 cells by a caspase-independent mechanism*

**P5.** Ross A. D. Bathgate, Daniel J. Scott, Ping Fu, Tania Ferrara, Sharon Layfield, John D. Wade & Geoffrey W. Tregear. *The novel G protein-coupled receptors LGR7 and LGR8 are the receptors for relaxin and insulin 3*

**P6.** Daniel J. Scott, Laura J. Parry, Geoffrey W. Tregear & Ross A. D. Bathgate. *Cloning and expression of mouse and rat homologues of novel G protein-coupled receptors, LGR7 and LGR8: The receptors for relaxin and insulin 3*

**P7.** Dana S. Hutchinson, Bronwyn A. Evans, Arthur Christopoulos & Roger J. Summers. *The  $\beta_3$ -adrenoceptor (AR) antagonist SR59230A displays agonist properties at the mouse  $\beta_3$ -AR*

**P8.** J. Nevzorova, B. A. Evans & R. J. Summers. *The role of cyclic AMP in  $\beta_2$ -adrenergic receptor mediated glucose transport in the rat skeletal muscle cell line L6*

**P9.** Lauren May, Patrick M. Sexton & Arthur Christopoulos. *Acute and chronic effects of allosteric modulators on  $M_2$  acetylcholine receptors (mAChRs)*

**P10.** Anne E. Luebke & Ian M. Dickerson. *CGRP-receptor component protein (RCP) in cochlear CGRP-mediated signal transduction*

**P11.** Maria Morfis, George Christopoulos, Arthur Christopoulos, Madhara Udawela, Marc Laburthe, Alain Couvineau, Kenji Kuwasako & Patrick M. Sexton. *Novel receptor partners and function of receptor activity modifying proteins*

**P12.** Nicola J. Smith, Patrick M. Sexton & Arthur Christopoulos. *Novel effects of prolonged ligand exposure and cell confluence on human  $5HT_{2C}$  receptor-mediated signalling in Chinese hamster ovary (CHO) cells.*

**P13.** N. Tilakaratne, K. Smyth, B. Popp, R. J. Summers & P. M. Sexton. *Modulation of beta-adrenergic receptor properties by the receptor activity modifying proteins (RAMPs)*

**P14.** Vi Pham, John Wade, Sharon G. McDowall, Maribel Quiza & Patrick M. Sexton. *Photoaffinity scanning in the mapping of the biomolecular interface between residue 19 of calcitonin and its receptor*

**P15.** Ping Fu, Pei-Juan Shen, Ross A. D. Bathgate, Andrew L. Gundlach & Geoffrey W. Tregear. *Novel G protein-coupled receptor for insulin-like peptide 3 (INSL3) – LGR8: Distribution of LGR8 mRNA in rat brain and peripheral tissues*

- P16. Andrew L. Siebel, Helen M. Gehring & Laura J. Parry.** *What is regulating uterine mesotocin receptors in the Tammar wallaby?*
- P17. Bridget R. Southwell.** *Location and kinetics of tachykinin receptors in rat and guinea pig small intestine*
- P18. Madhara Udawela, Maria Morfis, Kenji Kuwasako & Patrick M. Sexton.** *Trafficking of receptor activity modifying proteins (RAMPs) to the cell surface*
- P19. N. M. Broxton, B. A. Evans & R. J. Summers.** *Ligand directed signalling of  $\alpha_{1A}$ -adrenoceptors*

## **THROMBIN STIMULATES THE CLUSTERING AND SENSITIZATION OF EGF RECEPTORS OF SWISS 3T3 CELLS WITHOUT TRANSACTIVATION**

*Michael F. Crouch\**, *Francis S. Willard<sup>^</sup>* and *Leise A. Berven<sup>#</sup>*

*\*TGR BioSciences, Thebarton, SA, <sup>^</sup>Univ. of North Carolina, Chapel Hill, USA and <sup>#</sup> CMRI Westmead, NSW*

### **ABSTRACT**

The G-protein-coupled thrombin receptor can induce cellular responses in some systems by transactivating the EGF receptor. This is in part due to the stimulation of ectoproteases that generate EGF receptor ligands. We show here that this cannot account for the stimulation of proliferation or migration by thrombin of Swiss 3T3 cells. Thrombin has no direct effect on the activation state of the EGF receptor or of its downstream effectors. However, thrombin induces the subcellular clustering of the EGF receptor at F-actin-containing structures at the leading edge and actin arcs of migrating cells in association with other signalling molecules, including Shc and PLC $\gamma$ 1. In these thrombin-primed cells, the subsequent migratory response to EGF is potentiated. Thrombin did not potentiate the EGF stimulated EGF receptor phosphorylation. Thus, in Swiss 3T3 cells the G-protein coupled thrombin receptor can potentiate the EGF tyrosine kinase receptor response when activated by EGF, and this appears to be due to the subcellular concentration of the receptor with downstream effectors and not only to the overall ability of EGF to induce receptor transphosphorylation. Thus, the EGF receptor subcellular localization which is altered by thrombin appears to be an important determinant of the efficacy of downstream EGF receptor signalling in cell migration.

Poster # 2.

## **HIGH THROUGHPUT CELL-BASED SCREENING OF RECEPTOR SIGNAL TRANSDUCTION PATHWAYS**

Michael F. Crouch, Andrea J. Brown, Georgina L. Harvey and Anthony R. Dyer  
TGR BioSciences Pty Ltd, and Australian Proteome Analysis Facility (APAF), Adelaide Node, 31 Dalglish St, Thebarton, 5031 SA.

TGR BioSciences is part of the Proteomic Major National Research Facility, with Macquarie, Sydney and NSW Universities. TGR forms the Adelaide Node of this Australian Proteomic Analysis Facility (APAF), and will provide 'Functional Proteomic' screening of bioactive molecules. This will involve detection of bioactive proteins and (non-protein molecules) in cell based assays. Detection of bioactive molecules, such as growth factors and other hormones, is best achieved in cell-based assays. The parameters measured in such assays have changed over recent years with the elucidation of many of the intracellular receptor signal transduction pathways. Measurement of such events has largely replaced the use of less sensitive and more time-consuming assays such as of cell growth and differentiation. The methodology usually employed to assess receptor signaling is the western blot, with proteins from cellular extracts being probed with activation-specific antibodies (eg phospho-specific) or with extracts being immunoprecipitated and then the presence of a protein in the immunoprecipitate being assessed by standard or epitope-specific antibody probing of a western blot. This procedure is, however, also laborious and time consuming, and is limited in the numbers of samples that can be handled at one time. To overcome this bottleneck, we have developed a high throughput screening platform that largely replaces western blotting as the method of choice for studying signal transduction pathway activation. These methods are homogeneous (ie no washing steps), are as sensitive as western blotting, use minimal sample, and can be completed in a matter of a few hours. This methodology is presently being expanded in the number of target signaling systems that can be measured, and is being automated with robotic liquid handling to increase throughput to handle large sample numbers anticipated in the APAF Facility.

## **STRUCTURAL DETERMINANTS OF $\alpha_1$ -ADRENERGIC RECEPTOR SUBTYPE-SELECTIVE PHOSPHORYLATION AND INTERNALISATION**

Angela M Finch<sup>1</sup>, Fang Lin<sup>1</sup>, Valerie Sarramegna<sup>1</sup>, Songhai Chen<sup>1</sup>, Anne Cunningham<sup>2</sup> and Robert M Graham<sup>1,3</sup>

<sup>1</sup>Molecular Cardiology Unit, Victor Chang Cardiac Research Institute, St Vincent's Hospital, Darlinghurst, NSW 2010, Sydney and Faculty of Medicine, University of New South Wales, Kensington, NSW 2033, Australia; and <sup>2</sup>School of Women's and Children's Health, Faculty of Medicine, University of New South Wales, Kensington, NSW 2033, Australia.

$\alpha_1$ -Adrenergic receptors ( $\alpha_1$ -ARs) are members of the G protein-coupled receptor (GPCR) superfamily. Molecular cloning and pharmacological studies have identified three  $\alpha_1$ -AR subtypes,  $\alpha_{1A}$ ,  $\alpha_{1B}$  and  $\alpha_{1D}$ . Like other GPCRs, signalling by  $\alpha_1$ -ARs is regulated by a series of cellular processes, including phosphorylation and internalisation. Recent evidence suggests that the three subtypes may have distinct cellular distributions at steady state, which may contribute to their distinct pharmacological characteristics. We have investigated the structural basis for differences in agonist-stimulated cellular trafficking and phosphorylation of the  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs. At steady state, predominant cell-surface expression was evident for both subtypes. However, agonist-stimulated internalisation and phosphorylation were observed only for the  $\alpha_{1B}$ -AR.

To study the mechanisms and structural basis for these differences in agonist-stimulated internalisation and phosphorylation of the  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs, we constructed C-terminal truncated receptors and chimeric receptors in which the C-terminal tail, the 3<sup>rd</sup> intracellular loop or both were exchanged for that of the other subtype. The chimeric receptors were expressed using stable transfection in HEK293 cells. Agonist-stimulated internalisation was investigated using immunofluorescent staining, confocal microscopy and assessed by ligand binding. Agonist-stimulated phosphorylation was examined via <sup>32</sup>P<sub>i</sub>-labelling.

Phosphorylation and internalisation of the  $\alpha_{1B}$ -AR were abolished with truncation of its C-terminal tail. However, constitutive phosphorylation and internalisation, which could be further enhanced with agonist-stimulation, were evident with a chimeric receptor consisting of the  $\alpha_{1A}$ -AR with the  $\alpha_{1B}$ -AR C-tail. Additional substitution of the  $\alpha_{1A}$ -AR third intracellular loop with that of the  $\alpha_{1B}$ -AR resulted in cell surface receptors which could be phosphorylated and internalised with agonist stimulation. A plausible interpretation of these findings is that in the ground state negative endocytic signals in the third loop of  $\alpha_1$ -ARs ensure their plasma membrane retention. With agonist-stimulation, however, positive endocytic signals in the C-tail are disinhibited, allowing phosphorylation and internalisation of those subtypes able to interact productively with the endocytic machinery.

To confirm our hypothesis, the reciprocal chimeric receptors were constructed. Phosphorylation of these chimerae is consistent with our hypothesis. Agonist stimulated internalisation of the  $\alpha_{1B}$ -AR with the  $\alpha_{1B}$ -AR C-tail and/or  $\alpha_{1A}$ -AR third intracellular loop is currently under investigation.

## **CALCITONIN DECREASES THE ADHERENCE AND SURVIVAL OF HEK-293 CELLS BY A CASPASE-INDEPENDENT MECHANISM**

DM Findlay, S Hay, LJ Raggatt, S Bouralexis, and A Evdokiou. Department of Orthopaedics and Trauma, University of Adelaide, Adelaide, S.A. 5000

We recently reported<sup>1</sup> that CT can profoundly inhibit the growth of HEK-293 cells transfected with the hCTR. We also obtained preliminary evidence that suggested a role for CT in cell survival, which appeared to be dependent on the cell growth conditions. In the present study we have investigated the pro-apoptotic action of CT in conditions of low serum concentration.

We have found that CT treatment of HEK-293 cells stably transfected with the insert-negative form of the human CTR (HR12 cells) caused a time-dependent decrease in cell number associated with loss of cellular attachment. Loss of cellular adherence in CT-treated cultures caused programmed cell death (PCD) in serum deplete, but not serum replete, conditions. PCD was shown by condensation and cleavage of nuclear DNA, failure of cells to exclude trypan blue dye, annexin V staining, and appearance of hypodiploid cells and cell fragments in FACS analysis. The accumulation of non-adherent cells and cell death was concomitant with increased intracellular activity of caspase-3. However, inhibition of caspase activation in HR12 cells did not prevent CT-mediated loss of attachment and did not maintain the viability of non-adherent cells, indicating that caspase activation accompanied, but was probably not causal, of the loss of cell viability. The Erk1/2 inhibitor, PD 98059, inhibited the loss of cellular adherence and the consequent reduction in cell numbers.

These results demonstrate that CT can negatively affect cell survival and identify roles for cell adherence and MAPK activation in this process.

1. Evdokiou, A, Raggatt, L-J, Atkins, GJ, Findlay, DM. Calcitonin receptor-mediated growth suppression of HEK-293 cells is accompanied by induction of p21<sup>waf1/cip1</sup> and G2/M arrest. *Mol. Endocrinol.* 13: 1738-1750, 1999.

## THE NOVEL G PROTEIN-COUPLED RECEPTORS LGR7 AND LGR8 ARE THE RECEPTORS FOR RELAXIN AND INSULIN 3

Ross AD Bathgate, Daniel J Scott, Ping Fu, Tania Ferraro, Sharon Layfield, John D Wade and Geoffrey W Tregear.

*Howard Florey Institute, University of Melbourne, Victoria, Australia.*

Insulin 3 (INSL3, relaxin-like factor, RLF) and relaxin are both members of the insulin/IGF superfamily of peptide hormones. Both peptides 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. Problems with testis descent, leading to cryptorchidism, are very common, with 2-5% of boys requiring corrective surgery and having an increased risk of testicular cancer and infertility. INSL3 is essential for the development of a ligament called the gubernaculum, however the mechanisms by which it exerts its effects are not known. Relaxin is a peptide hormone which is primarily a product of pregnancy where it is essential for the development of the reproductive tract. More recent data suggests that it is also essential for normal tissue collagen turnover, hence relaxin knockout mice show progressive fibrosis in various tissues including the heart and lung as they age. Relaxin therefore has great clinical potential as an anti-fibrotic. Research into these two peptide hormones has been limited by the inability of researchers to identify specific receptors.

Recently relaxin was shown to activate two orphan leucine-rich repeat-containing G protein-coupled receptors (LGR), LGR7 and LGR8, resulting in cAMP release [1]. The LGR7 is the relaxin receptor, however evidence suggested that the LGR8 should be the receptor for INSL3 [1]. Using the human LGR7 and LGR8 expressed in 293T cells together with relaxin and INSL3 peptides we have studied the ligand binding and activation specificity of both LGR7 and LGR8. Ligand binding was measured using <sup>33</sup>P-labelled human gene 2 (H2) relaxin and activation via measurement of cAMP accumulation. Cells transfected with human LGR7 bound H2 relaxin with high affinity ( $EC_{50} = 0.28$  nM) but only bound INSL3 with very low affinity ( $EC_{50} = 3.85$   $\mu$ M). In contrast although cells transfected with human LGR8 bound H2 relaxin with high affinity ( $EC_{50} = 1.3$  nM) they had a ten-fold higher affinity for INSL3 ( $EC_{50} = 0.16$  nM). Interestingly INSL3 ( $EC_{50} = 0.036$  nM) had a much higher potency in stimulating cAMP release from LGR8 transfected cells than H2 relaxin ( $EC_{50} = 9.9$  nM). In contrast the ability of both H2 relaxin ( $EC_{50} = 0.018$  nM) and INSL3 ( $EC_{50} = 0.926$   $\mu$ M) to stimulate cAMP release from LGR7 receptor transfected cells reflected their ligand binding affinities. The data indicate that relaxin may be interacting with the LGR8 receptor at multiple sites, only one of which results in cAMP production. This hypothesis is further strengthened by the ability of rat relaxin to partially bind to the LGR8 receptor ( $EC_{50} = 17.8$  nM; Max displacement 70% of <sup>33</sup>P-relaxin binding), and its inability to stimulate cAMP release at concentrations up to 10  $\mu$ M. This inability of rat relaxin to stimulate cAMP production is probably due to the absence of a critical Tryptophan residue in the B-chain of the peptide which has been shown to be essential for INSL3 activity and is also present in the H2 relaxin peptide. In fact other relaxin peptides which contain this Trp residue have similar activities on the LGR8 receptor. Therefore although the LGR8 receptor fulfills all the criteria of being the INSL3 receptor, the biology of INSL3 action and hence testis descent may be complicated in species other than rodents by the actions of relaxin. 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.

1. S.Y. Hsu et al, *Science* **295**, 671 (2002)
2. Kubota, Temelcos, Bathgate et al., *Molecular Human Reproduction* **8**: 900-905 (2002).

## **CLONING AND EXPRESSION OF MOUSE AND RAT HOMOLOGUES OF NOVEL G PROTEIN-COUPLED RECEPTORS, LGR7 AND LGR8; THE RECEPTORS FOR RELAXIN AND INSULIN 3**

Daniel J Scott, Laura J Parry, Geoffrey W Tregear and Ross AD Bathgate  
*Howard Florey Institute, University of Melbourne, Victoria*

The peptide hormones Relaxin and Insulin 3 (INSL3) are both members of the insulin/IGF superfamily. Relaxin, classically known as a hormone of pregnancy, acts on multiple tissues to induce varying effects primarily via collagen remodelling. INSL3 is expressed in the testicular Leydig cells and mediates testicular descent by inducing development of a specific ligament, the gubernaculum. Recently relaxin was shown to bind two orphan leucine-rich repeat-containing G protein-coupled receptors (LGR), LGR7 and LGR8, causing cAMP activation [1]. Subsequently, LGR8 was shown to be activated by INSL3 (see abstract by Bathgate et al). The LGR family comprises LH, FSH and TSH receptors, 3 orphan receptors, and LGR7 and LGR8. All contain a characteristic extracellular ectodomain with leucine rich repeats. Human LGR7 and LGR8 [1], were used to search the Celera and NCBI genome and EST databases for novel homologues. Particular emphasis was placed on identifying mouse and rat homologues.

We identified a full mouse LGR7 sequence using the Celera genome database and almost complete rat LGR7 and LGR8 sequences in the NCBI high throughput genome database. Furthermore, ESTs corresponding to chicken, pig and zebrafish LGR7 and bovine LGR8 were also discovered. Rat, mouse, pig and bovine sequences were confirmed using RT-PCR and sequencing. Mouse and Rat LGR7 expression was confined to tissues known to possess functional relaxin receptors, such as cervix, cerebral cortex and myometrium, while LGR8 was expressed predominantly in the fetal gubernaculum, the site of INSL3 action.

Human LGR7 exists in two alternately spliced isoforms [1]. We identified mouse and rat LGR7 splice variants of a similar nature, with the spliced region shifted 100 nucleotides in the C terminal direction. Unlike the human splice variant which results in a full length receptor which is expressed at the cell surface the rodent variant results in a short protein sequence comprising only the LDL-receptor domain from the N-terminus of the receptor. Interestingly using quantitative real-time PCR we have shown that the transcript encoding this short protein sequence makes up 50% of the LGR7 transcripts in late pregnant mouse reproductive tract. In contrast the brain and other non-reproductive tissues express only very small amount of the splice variant. This variant therefore has the potential to have an important function during late pregnancy. Recent work has demonstrated that the LDL-receptor domain is important for activation of the LGR7 receptor (Bathgate & Hsueh, unpublished) therefore the protein product of the splice variant has the potential to block relaxin signalling in late pregnancy.

By aligning LGR7 and LGR8 family members we have identified regions of high homology that could also be involved in ligand binding and activation. Overall, the identification of novel homologues of LGR7 and LGR8 will significantly aid in further characterisation of the precise roles and downstream processes involved in relaxin and INSL3's actions.

1. S.Y. Hsu et al, *Science* **295**, 671 (2002)

## THE $\beta_3$ -ADRENOCEPTOR (AR) ANTAGONIST SR59230A DISPLAYS AGONIST PROPERTIES AT THE MOUSE $\beta_3$ -AR.

Dana S. Hutchinson<sup>1,2</sup>, Bronwyn A. Evans<sup>1</sup>, Arthur Christopoulos<sup>3</sup> & Roger J. Summers<sup>1</sup>.

<sup>1</sup>*Department of Pharmacology, Monash University, Melbourne, Victoria 3800, Australia.*

<sup>2</sup>*Department of Physiology, The Wenner-Gren Institute, Stockholm University, SE10691 Stockholm, Sweden.*

<sup>3</sup>*Department of Pharmacology, University of Melbourne, Parkville, Victoria 3100, Australia*

**Introduction:** This study examines the action of the  $\beta_3$ -AR antagonist SR59230A<sup>(1)</sup> at the cloned mouse  $\beta_3$ -AR expressed in CHO-K1 cells, and the endogenously expressed  $\beta_3$ -AR in mouse 3T3-F442A adipocytes.

**Methods:** Functional responses were measured by increases in cAMP accumulation or increases in extracellular acidification rate (EAR; cytosensor microphysiometer)<sup>(2)</sup> to determine the agonist and antagonist properties of SR59230A.

**Results:** In high expressing cells (1200fmol/mg protein), SR59230A blocked CL316243 ( $\beta_3$ -AR agonist) mediated increases of cAMP accumulation at low concentrations but at high concentrations, stimulated cAMP production even in the absence of CL316243. In medium (500 fmol/mg protein) and low (100 fmol/mg protein) expressing cells, SR59230A alone produced small increases in cAMP, and blocked CL316243 mediated increases of cAMP. In the cytosensor, SR59230A acted as a full agonist for EAR at all levels of receptor expression and blocked CL316243 responses only in medium and low expressing cells. In 3T3-F442A cells (150 fmol/mg protein), SR59230A had no agonist actions on cAMP levels and antagonised CL316243 mediated increases of cAMP; however, in the cytosensor, SR59230A acted as an agonist and increased EAR.

**Conclusion:** Therefore SR59230A displays both agonist and antagonist actions at the mouse  $\beta_3$ -AR and may increase EAR by signalling pathways distinct from cAMP accumulation.

(1) Manara L, Badone D, Baroni M, Boccardi G, Cecchi R, Croci T, Giudice A, Guzzi U, Landi M, Le Fur G. *Br J Pharmacol.* 1996; 117:435-442

(2) Hutchinson DS, Bengtsson T, Evans BA, Summers RJ. *Br J Pharmacol.* 2002;135:1903-1914

**THE ROLE OF CYCLIC AMP IN  $\beta_2$ -ADRENERGIC RECEPTOR MEDIATED GLUCOSE TRANSPORT IN THE RAT SKELETAL MUSCLE CELL LINE L6.**

J Nevzorova, BA Evans. & RJ Summers.

Department of Pharmacology, Monash University, VIC 3800

$\beta_2$ -Adrenergic receptors (AR) mediate an increase in glucose transport (GT) in the rat skeletal muscle cell line L6 via a phosphatidylinositol-3 kinase (PI3K) dependent mechanism (Nevzorova *et al.*, 2002). The  $\beta_2$ -AR is a Gs coupled receptor that links to adenylate cyclase, and stimulation of L6 cells with the  $\beta_2$ -AR selective agonist zinterol results in a significant increase in cyclic AMP (cAMP) accumulation. In this study we investigated the relationship between accumulation of cAMP and the stimulation of GT in L6 cells. We found a significant increase in GT in response to zinterol ( $10^{-7}$ M), which was maintained with stimulation for 3 hours. In contrast, cAMP accumulation was initially high but then progressively diminished over 3-hour stimulation, suggesting that the  $\beta_2$ -AR signalling pathway was desensitised. This most likely occurs due to phosphorylation of the  $\beta_2$ -AR by protein kinase A (PKA). There is evidence that phosphorylation of the  $\beta_2$ -AR by PKA results in its uncoupling from Gs and coupling to Gi, which activates other signalling pathways including MAP kinase (Daaka *et al.*, 1997) and PI3K (Zhu *et al.*, 2001). Since PI3K is known to be involved in GT this may provide an explanation for the ability of  $\beta_2$ -AR to facilitate GT in L6 cells. To investigate the coupling of the  $\beta_2$ -AR to different signalling pathways in L6 cells we measured agonist-stimulated changes in extracellular acidification rate (ECAR) in the cytosensor microphysiometer. This may prove to be a convenient approach for examining cellular activation associated with glucose uptake and metabolism, as these processes are thought to be major contributors to extracellular acidification. We investigated the coupling of the  $\beta_2$ -AR to Gi by testing the effect of pertussis toxin (PTX) on zinterol, isoprenaline (non-selective  $\beta$ -AR agonist) and BRL37344 ( $\beta_3$ -AR) ECAR responses. In the short term, these responses were not sensitive to PTX. Further studies are now in progress to examine the effects of PTX on ECAR responses in L6 cells desensitised by longer exposure to high concentrations of  $\beta$ -AR agonists. In conclusion, our results suggest that maintenance of high cAMP levels is not required for an increase in GT after stimulation of the  $\beta_2$ -AR. We are currently testing the hypothesis that phosphorylation of the  $\beta_2$ -AR by PKA is necessary for the coupling of the receptor to PI3K that leads to stimulation of GT in L6 cells.

J. Nevzorova, T. Bengtsson, B.A. Evans & R.J. Summers (2002) *Brit. J. Pharmacol.*, **137**: 9-18

W.-Z. Zhu, M. Zheng, W.J. Koch, R.J. Lefkowitz, B.K. Kobilka & R.-P. Xiao (2001) *PNAS*, **98**: 1607-1612

Y. Daaka, L.M. Luttrell, & R.J. Lefkowitz (1997) *Nature*, **390**: 88-91

## ACUTE AND CHRONIC EFFECTS OF ALLOSTERIC MODULATORS ON M<sub>2</sub> MUSCARINIC ACETYLCHOLINE RECEPTORS (MACHRS)

Lauren May, Patrick M. Sexton and Arthur Christopoulos

Department of Pharmacology and Howard Florey Institute of Experimental Medicine and Physiology, University of Melbourne

The ternary complex model (TCM) describes the effects of allosteric modulators on the binding properties of G protein-coupled receptors (GPCRs) in terms of the modulator's affinity for the GPCR ( $pK_B$ ) and the co-operativity ( $\alpha$ ) between the agonist-binding (orthosteric) and allosteric sites<sup>1</sup>. The aim of the present study was to evaluate the acute and chronic effects of three allosteric modulators, heptane-1,7-bis-[dimethyl-3'-phthalimidopropyl] (*C*<sub>7</sub>/3-phth), gallamine and alcuronium, on the binding of the hydrophilic antagonist, [<sup>3</sup>H]N-methylscopolamine (<sup>3</sup>H-NMS), in intact CHO cells expressing the human M<sub>2</sub> mAChR. Using standard equilibrium binding methods, we found that the binding of gallamine was well-fitted by the simple TCM, but that the binding of the inhibitor, *C*<sub>7</sub>/3-phth, and the enhancer, alcuronium, was more complex than predicted by the model; *C*<sub>7</sub>/3-phth yielded a biphasic curve, while alcuronium, yielded a bell-shaped curve. Subsequent experiments revealed that the modulators could slow the dissociation of the radioligand to such an extent that the system never attains a true equilibrium state. Application of a kinetic, rather than an equilibrium, TCM to the data yielded the following parameters; *C*<sub>7</sub>/3-phth,  $pK_B=5.7\pm 0.2$   $\log\alpha = -1.00\pm 0.05$ ,  $n = 4$ ; alcuronium,  $pK_B = 6.1\pm 0.1$   $\log\alpha = 0.22\pm 0.07$ ,  $n = 4$ ; gallamine,  $pK_B = 5.7\pm 0.1$   $\log\alpha = -1.30\pm 0.11$ ,  $n = 7$ . The chronic effects of the modulators or the orthosteric antagonists, atropine or NMS, were then investigated by treating the cells with a  $10 \times K_B$  concentration of each agent for 24 hours, followed by extensive washing and <sup>3</sup>H-NMS saturation binding. Although pretreatment with atropine and gallamine significantly enhanced cell surface M<sub>2</sub> mAChR expression, this was not observed with either NMS, *C*<sub>7</sub>/3-phth or alcuronium. In conclusion, our findings support the use of the TCM to quantify the acute effects of allosteric modulators in radioligand binding assays, but suggest that chronic drug effects on receptor expression vary from ligand to ligand.

1. A. Christopoulos (2002) Allosteric binding sites on cell-surface receptors: Novel targets for drug discovery. *Nature Rev. Drug Discovery* **1**, 198-210.

## **CGRP-RECEPTOR COMPONENT PROTEIN (RCP) IN COCHLEAR CGRP-MEDIATED SIGNAL TRANSDUCTION.**

Anne E. Luebke<sup>1,3</sup> and Ian M. Dickerson<sup>2,3</sup> Departments of Otolaryngology<sup>1</sup>, Physiology and Biophysics<sup>2</sup>, and the Neuroscience Program<sup>3</sup>, University of Miami School of Medicine, Miami, FL 33136 USA.

Calcitonin Gene-Related Peptide (CGRP) has been implicated in neurotransmission between olivocochlear (OC) efferent terminals and their cochlear targets. Throughout the mammalian cochlea, OC terminals in the inner hair cell (IHC) area are immunopositive for CGRP; in guinea pig, mouse, and rat, outer hair cell (OHC) terminals are CGRP-positive, as well [Cabanillas & Luebke 2002, *Hear Res* 3845:1-4; Maison et al., 2001, *Assoc Res Otolaryn Abstr.* #307]. In the lateral line (hair cell-containing organ), bath application of CGRP increases spontaneous discharge rates in afferent fibers [Bailey & Sewell 2000, *J Neurosci* 20:5163-9]. We have discovered a novel protein required for signal transduction at receptors for calcitonin gene-related peptide (CGRP). This protein, named the CGRP-receptor component protein (RCP) is an intracellular peripheral membrane protein that coimmunoprecipitates with the calcitonin receptor-like receptor (CRLR) from cell lysates, and is required for CGRP-mediated signal transduction [Evans et al., 2000, *J Biol Chem.* 275(40):31438-43; Prado et al., 2001, *Peptides* 22(11):1773-81]. CRLR functions as a CGRP receptor when coexpressed with an accessory protein named receptor activity modifying protein-1 (RAMP1), which affects intracellular trafficking and the pharmacologic specificity of CRLR [McLatchie et al., 1998, *Nature* 393(6683):333-9]. RCP was originally discovered in a screen to clone the receptor for CGRP from the cochlea, and has no homology to other proteins in GeneBank beside itself [Luebke et al., 1996, *Proc Natl Acad Sci U S A* 93(8):3455-60]. We hypothesize that RCP is part of a complex of proteins that together constitute a functional CGRP receptor. We have performed immunoprecipitations for CRLR, RAMP1, and RCP and show that these molecules can be co-immunoprecipitated from cochlear extracts. In addition, we have generated antibodies to CGRP-RCP and show that CGRP-RCP protein is present near CGRP-positive nerve endings in the inner spiral region (near inner hair cells) of the cochlea. Future studies are designed to determine if the excitatory effect of CGRP release by the olivocochlear system on auditory nerve responses is mediated by the CGRP-RCP signalling.

Supported by grants from the Public Health Service (R01 DC03086, R01 DK52328) and the Chiles Endowment Biomedical Research Program of the Florida Department of Health BM028.

## **NOVEL RECEPTOR PARTNERS AND FUNCTION OF RECEPTOR ACTIVITY MODIFYING PROTEINS**

Maria Morfis<sup>1</sup>, George Christopoulos<sup>1</sup>, Arthur Christopoulos<sup>1,2</sup>, Madhara Udawela<sup>1</sup>, Marc Laburthe<sup>3</sup>, Alain Couvineau<sup>3</sup>, Kenji Kuwasako<sup>4</sup> and Patrick M. Sexton<sup>1</sup>

<sup>1</sup>Howard Florey Institute, The University of Melbourne, Vic, <sup>2</sup>Dept. of Pharmacology, The University of Melbourne, Vic, <sup>3</sup>INSERM U410, Faculte de Medecine Xavier Bichat, Paris, France and <sup>4</sup>First Department of Internal Medicine, Miyazaki Medical College, Miyazaki, Japan.

The receptor activity modifying proteins (RAMPs) comprise a family of three accessory proteins that heterodimerize with the calcitonin receptor-like receptor (CL receptor) or with the calcitonin receptor (CTR) to generate different receptor phenotypes. However, RAMPs are more widely distributed across cell and tissue types than the CTR and CL receptor, suggesting additional roles for RAMPs in cellular processes. We have investigated the potential for RAMP interaction with a number of Class II G protein-coupled receptors (GPCRs) in addition to the CL receptor and the CTR. Using immunofluorescence confocal microscopy we demonstrate, for the first time, that N-terminal epitope-tagged RAMPs interact with at least 4 additional receptors, the VPAC1 vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor with all 3 RAMPs; the glucagon and PTH1 parathyroid hormone receptors with RAMP2 and the PTH2 receptor with RAMP3. Unlike the interaction of RAMPs with the CL receptor or the CTR, in radioligand saturation and competition binding assays in whole cells and membranes, VPAC1 receptor/RAMP complexes did not show altered phenotypic behaviour when compared to the VPAC1 receptor alone in COS-7 cells. Furthermore, the cell surface expression of VPAC1 receptor was also unaffected by the co-expression of RAMPs; quantitatively determined by specific binding of <sup>125</sup>I labelled IgG antibody to N-terminally FLAG-tagged-VPAC1 receptors. However, the VPAC1 receptor/RAMP2 heterodimer displays a significant enhancement of agonist-mediated phosphoinositide hydrolysis with no change in cAMP stimulation compared to the VPAC1 receptor alone. Our findings identify a new functional consequence of RAMP-receptor interaction, and suggest that RAMPs play a more general role in modulating cell-signaling through other GPCRs than is currently appreciated.

**NOVEL EFFECTS OF PROLONGED LIGAND EXPOSURE AND CELL CONFLUENCE ON HUMAN 5HT<sub>2C</sub> RECEPTOR-MEDIATED SIGNALING IN CHINESE HAMSTER OVARY (CHO) CELLS**

Nicola J. Smith, Patrick M. Sexton and Arthur Christopoulos

Department of Pharmacology and Howard Florey Institute of Experimental Medicine and Physiology, University of Melbourne

In contrast to many other G protein-coupled receptors, 5HT<sub>2C</sub> receptors downregulate after prolonged *in vivo* exposure to both agonists and antagonists. The molecular determinants of this phenomenon are currently unknown. Previously, we investigated the effects of prolonged ligand exposure on constitutively active, human 5HT<sub>2C</sub> receptors stably overexpressed (120 pmol/mg protein) in CHO cells. Paradoxically, we found that pretreatment with inverse agonists significantly enhanced agonist-mediated signaling whereas pretreatment with neutral antagonists enhanced agonist-independent receptor signaling<sup>1</sup>. In order to ascertain whether these differential effects were due to pleiotropic receptor coupling to different signaling pathways as a consequence of overexpression, the present study was undertaken using a CHO cell line that expressed the 5HT<sub>2C</sub> receptor at much lower levels (50 – 200 fmol/mg protein). Cells were pre-treated for 24 h with 10 × K<sub>i</sub> concentrations of the partial agonist, DOI, the neutral antagonist, 5-methoxygramine (5-MXG), or the inverse agonist mianserin (MSN), before extensive washout and determination of 5-HT-mediated [<sup>3</sup>H]phosphatidyl inositol (PI) accumulation. In agreement with our previous finding using overexpressed receptors, pretreatment with MSN caused a significant enhancement of agonist-mediated signaling with no change in the basal response but, interestingly, this effect was lost when the pretreatment time was extended to 72 h. In contrast, pretreatment with either DOI or 5-MXG had no effect on any system response parameters compared to vehicle-pretreated cells. Unexpectedly, we also found that the acute manifestation of agonistic behavior by each of the ligands tested was highly dependent on the level of CHO cell confluence. At 70-80% cell confluence, 5-HT and DOI behaved as agonists in PI signaling, whereas 5-MXG and MSN behaved as neutral antagonists. At higher levels of confluence, signaling by 5HT and DOI was markedly diminished whereas MSN and 5-MXG revealed significant inverse agonist behavior. Our findings suggest that 5HT<sub>2C</sub> receptor signaling is highly sensitive to acute and chronic regulation by ligands and cellular background.

1 A. Christopoulos and M.G. Devlin. *Proc. Aust. Clin. Exp. Pharmacol. Toxicol.* 2001; **9**, 47.

## MODULATION OF BETA-ADRENERGIC RECEPTOR PROPERTIES BY THE RECEPTOR ACTIVITY MODIFYING PROTEINS (RAMPS)

N Tilakaratne<sup>1</sup>, K Smyth<sup>1</sup>, B Popp<sup>2</sup>, RJ Summers<sup>2</sup> and PM Sexton<sup>1</sup>

<sup>1</sup> Howard Florey Institute of Experimental Physiology & Medicine, Parkville, VIC,

<sup>2</sup> Department of Pharmacology, Monash University, Clayton, VIC.

Pharmacological diversity in hormone receptors, resulting from their interaction with the receptor activity modifying proteins (RAMPs), has been demonstrated for the calcitonin receptor (CTR), calcitonin-like receptor (CLR) and the VPAC receptor (VPAC-R), all of which are members of the class II G protein coupled receptor (GPCR) subfamily (1-3). A frequently raised question, in relevance to RAMPs, is whether these proteins interact with members of the other GPCR subfamilies as well. To address this issue, we examined the effects of enhanced RAMP expression on the native beta-adrenergic receptors (class I GPCR) in the human neuroblastoma cells - SK-N-MC. A mixed population of  $\beta_1$  and  $\beta_3$ -adrenergic receptors ( $\beta_1$ -AR and  $\beta_3$ -AR) has been previously demonstrated in these cells (4). Reverse transcription-polymerase chain reaction (RT-PCR) analysis on RNA extracted from SK-N-MC showed that there is endogenous expression of RAMPs, at varying levels (R2>R1>R3). RAMP1 enrichment by transient-transfection resulted in a 25-50% loss of <sup>125</sup>I-cyanopindolol ([<sup>125</sup>I]-CYP) binding to whole cells. RAMP2, -3 or empty vector transfection did not alter the [<sup>125</sup>I]-CYP binding. Competitive displacement of [<sup>125</sup>I]-CYP by the non-selective antagonist propranolol or the non-selective agonist isoproterenol (ISO), varied among vector (V), R1 or R3-transfectants (the IC<sub>50</sub> values for propranolol were; V = 0.72, R1 = 2.79 and R3 = 6.72 nM, and for ISO; V = 2492, R1 = 1921 and R3 = 20 nM). RAMP3-enriched cells, not R1, R2 or vector-transfectants, showed increased basal cAMP levels, which may be attributed to any of the multitude of receptors expressed in SK-N-MC, including the CLR which is a known RAMP-interacting receptor. However, a RAMP-effect specific to the  $\beta$ -ARs was evident from the enhanced potency of ISO in stimulating cAMP production in all RAMP-transfectants, when compared with the vector-transfected controls. Comparative studies using compounds with greater selectivity-R0363 (for  $\beta_1$ -AR) or L755507 (for  $\beta_3$ -AR), revealed that the enhanced cAMP response was selective to the  $\beta_3$ -AR in R3-enriched cells. These results suggest that the  $\beta$ -ARs in the SK-N-MC cells are prone to differential modulation by the expression level of each of the RAMPs.

### References:

1. McLatchie LM et al (1998) *Nature* 393, 333-339
2. Christopoulos, G. et al (1999) *Mol Pharmacol* 56, 235-242
3. Christopoulos A. et al (2002) unpublished
4. Esbenshade TA et al. (1992) *Mol Pharmacol* 42, 753-759

Poster # 14.

**PHOTOAFFINITY SCANNING IN THE MAPPING OF THE BIMOLECULAR INTERFACE BETWEEN RESIDUE 19 OF CALCITONIN AND ITS RECEPTOR.**

Vi Pham, John Wade, Sharon G. McDowall, Maribel Quiza & Patrick M. Sexton

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

Understanding receptor function, particularly in terms of ligand recognition by calcitonin receptors, may aid in the rational design of calcitonin analogues with increased potency and improved selectivity for therapeutic purposes. To directly identify interacting sites between calcitonin (CT) and its receptor (CTR), we carried out photoaffinity labeling studies followed by protein digestion and mapping of the radiolabeled photoconjugated receptor. A photoactive sCT analogue [Arg<sup>11,18</sup>, Bpa<sup>19</sup>]sCT incorporating a photolabile Bpa (p-benzoyl-L-phenylalanine) into position 19 of the ligand has been used to demonstrate spatial proximity between residue 19 and the amino-terminal extracellular domain of receptor. Cyanogen bromide (CNBr) digestion indicated that binding was predominately to the region delimited by receptor residues M<sup>59</sup>-M<sup>133</sup>, with an additional interaction site elsewhere in the N-terminus. Mutation of Leu 90 to Met led to a decrease in the CNBr - generated fragment consistent with interaction occurring within residues 90 and 133. Binding within this 42-amino-acid fragment was further refined by digestion with the endoproteases Asp-N and Lys-C to the distal 12 residues corresponding to K<sup>120</sup>-M<sup>133</sup>. Within this region, residue S<sup>129</sup> may form an important contact with residue 19 of sCT because mutant S<sup>129</sup>M expressed highly on the cell surface, but showed a significant decrease in specific binding. Further analysis of CNBr, Asp-N and Lys-C digestions suggested that the second interaction site was delimited by the residues D<sup>57</sup>-M<sup>59</sup>. These results provide the first direct demonstration of a contact domain between calcitonin and its receptor, and will contribute towards the modelling of the CT-CTR interface.

## **NOVEL G-PROTEIN-COUPLED RECEPTOR FOR INSULIN-LIKE PEPTIDE 3 (*INSL3*) - LGR8: DISTRIBUTION OF LGR8 MRNA IN RAT BRAIN AND PERIPHERAL TISSUES**

Ping Fu, Pei-Juan Shen, Ross AD Bathgate, Andrew L Gundlach and Geoffrey W Tregear

*Howard Florey Institute of Experimental Physiology and Medicine  
The University of Melbourne, Parkville, 3010, Victoria*

Cryptorchidism is a common developmental disorder in humans, affecting 1 to 3% of newborn males. It can result in defects in spermatogenesis, infertility and is associated with an increased risk for testicular malignancy. LGR8 is a member of the leucine-rich repeat containing G protein-coupled receptor family (LGR4-8) and shows considerable structural similarity to the recently identified relaxin receptor, LGR7 [1]. Mice homozygous for a deletion of the LGR8 or 'Great' gene exhibit cryptorchidism [2,3], which is primarily caused by abnormal gubernaculum development. This phenotype is very similar to that of *INSL3*-deficient mice, suggesting that LGR8 may be a receptor for *INSL3*. *INSL3*, also known as relaxin-like factor, belongs to the insulin/relaxin superfamily and the peptide is essential for testicular descent. Recently, we have shown that *INSL3* is capable of binding with high affinity to human LGR8 transfected 293T cells, and of mediating the activation of LGR8 through a cAMP-dependent pathway ([4]; 4Bathgate *et al.*, this meeting), further suggesting that LGR8 is an *INSL3* receptor.

LGR8 mRNA has been detected by RT-PCR in a number of tissues including brain, testis, kidney, thyroid gland, bone marrow, uterus and muscle [1]. However, the regional and cellular distribution of LGR8 gene expression in these tissues is unknown. In this study, [<sup>35</sup>S]-labeled antisense oligonucleotides and *in situ* hybridization were used to localize LGR8 mRNA in various rat tissues. In brain, expression of LGR8 mRNA was principally restricted to the thalamus, with the highest level detected in the parafascicular thalamic nucleus and more moderate levels observed in the medial habenular nucleus, several dorsolateral and ventrolateral nuclei and in anterior pretectal areas. In this respect, the distribution of LGR8 mRNA is quite distinct from that of LGR7 mRNA, which, while found within the thalamus, is present in different nuclei. In kidney, LGR8 gene expression was confined to the renal cortex and was particularly enriched in glomeruli. In testis, LGR8 mRNA was localized within the seminiferous tubules. However, *in situ* hybridization analysis and RT-PCR indicate that only truncated transcripts are present, suggesting that any LGR8 protein produced in testis may not function as an *INSL3* receptor. In gubernaculum, LGR8 expression was very high at 16 days of gestation, and then decreased sharply just before parturition. The expression pattern of the receptor correlates well with the expression profile of *INSL3* mRNA in testis and the timing of gubernaculum development [5].

Overall, the presence of LGR8 mRNA in the brain, kidney and testis suggests that LGR8 not only plays an essential role in gubernaculum growth during fetal development, but also has additional potential functions in other tissues in postnatal life.

[1] Hsu, S.Y. *et al.*, *Science* 295 (2002) 671-674.

[2] Overbeek, P.A. *et al.*, *Genesis* 30 (2001) 25-35.

[3] Gorlov, I.P. *et al.*, *Hum. Mol. Gen.* 11 (2002) 2309-2318.

[4] Kumagai, J. *et al.*, *J Biol Chem* 277 (2002) 31283-31286.

[5] Kubota, Y. *et al.*, *Mol. Hum. Reprod.* 8 (2002) 900-905.

## WHAT IS REGULATING UTERINE MESOTOCIN RECEPTORS IN THE TAMMAR WALLABY?

Andrew L. Siebel, Helen M. Gehring, Laura J. Parry.

*Department of Zoology & Howard Florey Institute, University of Melbourne, Victoria.*

The oxytocin receptor (OTR) belongs to the class-1 G protein-coupled, seven transmembrane domain receptor superfamily and is primarily coupled via G<sub>q</sub> proteins to phospholipase C-β. The cDNA encoding the human OTR was first isolated by Kimura *et al.*<sup>1</sup>. In all species to date, the OTR gene is a single-copy gene and encodes a protein of between 388 and 392 amino acids. The OTR sequence is highly conserved among species in the transmembrane domain regions and extracellular loops which are important for ligand binding and selectivity. Uterine smooth muscle contractions are stimulated by the nonapeptide hormone oxytocin (OT) in most eutherians or mesotocin (MT) in Australian marsupials. Like OT, MT is synthesized in the magnocellular nuclei of the hypothalamus and stored in the posterior pituitary. Upon stimulation of the pituitary, MT is released into the blood and binds to its cognate receptor in the myometrium. We have sequenced 850bp of the tammar wallaby MT receptor (MTR) and shown it to have between 82-86% homology compared with human, ovine and bovine sequences. The ligand-receptor interaction and subsequent activation of second messenger systems is well described in the literature. However, the factors that regulate OTRs have not been established.

The tammar wallaby has two anatomically separate uteri with a single conceptus contained in one uterus (gravid), with the contralateral uterus remaining empty. This unique reproductive tract anatomy enables local embryo-derived factors that regulate contractile associated proteins to be differentiated from systemic factors. Previous studies showed a marked increase in MTR expression in the myometrium of the gravid uterus between Days 22 and 24 of the 26-day gestation<sup>2,3</sup>. The objective of this study was to determine what factors are regulating uterine MTRs in the tammar wallaby. Our first experiment involved surgical removal of the embryo from the gravid uterus on either Day 17 or Day 20 of the 26-day gestation. Animals were euthanized on Day 23 and MTR concentrations were measured in the myometrium of both uteri, using quantitative real-time PCR and a radioreceptor binding assay (<sup>125</sup>I-OTA) respectively. In a second group, the embryo was removed on Day 17 and dental rubber (≈ 3ml) was inserted into the gravid uterus to artificially stretch the uterus (n=6). In sham-operated controls (n=5), there was a significant increase in MTR mRNA and receptor concentrations in the gravid uterus compared with the nongravid on Day 23 of gestation. Surgical removal of the embryo at both stages effectively eliminated uterine distension and resulted in significantly lower MTR mRNA and receptor concentrations compared with the gravid uterus of sham-operated controls. However, artificial distension of the uterus on Day 17, in the absence of the embryo, did not cause an increase in MTRs on Day 23. There was no significant difference in MTR mRNA concentrations between the artificially distended uterus and non-distended gravid uterus lacking the embryo. In a related study, we examined myometrial MTRs in animals that appeared to be pregnant with a distended uterus. However these uteri contained an abnormally developed fetus and avascular placenta. In these animals, MTRs were significantly higher in the distended uterus compared with the nondistended uterus, and did not differ from controls. These data demonstrate that whilst uterine occupancy may be important, local embryo-derived factors appear to regulate MTRs in the gravid uterus. Furthermore, uterine distension on Day 17 does not appear to be essential for the increase in MTRs observed on Day 23 of gestation.

1. Kimura *et al.* 1992, *J Steroid Biochem Mol Biol*, 42, 253-258.
2. Parry *et al.* 1997, *Biol Reprod*, 56, 200-7.
3. Siebel 2002, *et al. Biol Reprod* 66:1237-43.

## LOCATION AND KINETICS OF TACHYKININ RECEPTORS IN RAT AND GUINEA PIG SMALL INTESTINE

Bridget R Southwell

Murdoch Childrens Research Institute, Royal Childrens Hospital, Parkville, 3052.

In mammalian intestine, contraction and relaxation of muscle is coordinated by intrinsic neurons. Tachykinins (TK) are cotransmitters in cholinergic excitatory and sensory neurons and TKs applied to isolated intestine induce contraction. 3 TK receptors (neurokinin 1 (NK1), NK2 and NK3) have been cloned and sequenced. They are 7TM- GPCR and undergo ligand-induced endocytosis. Physiological and pharmacological studies suggested all 3 receptors were present in the muscle and ganglia in small intestine, but the precise locations of receptors was difficult to determine. I have used receptor kinetics and confocal microscopy to unravel the location of TK receptor subtypes in intact intestine. The precise location of NK1, NK2 and NK3 receptors in rat and guinea pig small intestine was determined using fluorescent antibodies, fluorescent ligands, specific agonists, antagonists and confocal microscopy. Intact tubes of gut were incubated with agonists and antagonists, then fixed and processed for fluorescence immunohistochemistry. Ligand-induced receptor endocytosis was quantified using confocal microscopy<sup>1</sup>. The identity of receptors was determined by the ability of specific agonists (Sar<sup>9</sup>Met<sup>11</sup>-SP and senktide ) or antagonists (CP-9994, SR- 142801) to induce or inhibit endocytosis. NK2r were present on the muscle throughout the intestine<sup>2</sup>.

In rat myenteric ganglia, NK1r and NK3r were present on the same neurons. These contain calbindin, ChAT and SP and are primary afferent (sensory) neurons<sup>3,4</sup>. Both receptors underwent endocytosis that was concentration dependent, in response to exogenous SP and NKA ( $K_{max}$  10<sup>-5</sup>M), but showed specific endocytosis in response to Sar<sup>9</sup>Met<sup>11</sup>-SP and senktide<sup>5</sup>. NK1r underwent endocytosis and recycling in response to endogenous release of ligand, while NK3r did not, showing there is independent activation of the receptors due to local concentrations of native ligands. NK1 and NK3r were also present on neurons containing VIP and NOS (secretomotor neurons) in the submucosal ganglia.

In contrast, in guinea pig myenteric ganglia, NK1r and NK3r were present on different neurons. NK1r was predominantly present on inhibitory neurons containing nitric oxide synthase (NOS)<sup>6</sup>, while NK3r was predominantly present on sensory and excitatory neurons containing calbindin and ChAT<sup>7</sup>. In addition, NK1r was present on muscle cells, interstitial cells of Cajal in the myenteric and deep muscular plexuses and on epithelial cells in the mucosa<sup>8,9</sup>. Locations of receptors was confirmed by internalisation of fluorescent ligand and receptors. Interstitial cells of Cajal in the myenteric plexus layer and in the deep muscular plexus are closely associated with nerve fibres containing TK and the presence of NK1r on these cells suggests a role in mediating excitatory transmission to the muscle and mediation of pacemaker activity by nerve fibres.

These results show that while TKs are present in the same nerve fibres, TK receptors have different cellular distributions in rat and guinea pig small intestine. This leads to different effects for TK in the two species and highlights the need to determine the location of receptors for each species. The combination of receptor kinetics and confocal microscopy allows the localisation of receptor subtypes on individual cells dispersed in a complex tissue. Combined with double labelling for cell specific markers, this technique provides a powerful tool for distinguishing the complex distribution of multiple members of a receptor family.

1. Southwell, BR et al 1998 *Neuroscience* 87, 925-31.
2. Portbury, AL et al. 1996 *Cell Tissue Res* 286, 281-92.
3. Mann, PT et al 1999 *Neuroscience* 91, 353-62.
4. Mann, PT et al 1999 *Cell Tissue Res* 297, 241-8.
5. Jenkinson, KM et al 2000 *Neuroscience* 100, 191-9.
6. Southwell, BR et al 1996 *Histochem Cell Biol* 106, 563-71.
7. Jenkinson, KM et al 1999 *Histochem Cell Biol* 112, 233-46.
8. Southwell, BR et al 2001 *Gastroenterology* 120, 1140-51.
9. Lavin, ST et al 1998 *Histochem Cell Biol* 110, 263-71.

## **TRAFFICKING OF RECEPTOR ACTIVITY MODIFYING PROTEINS (RAMPS) TO THE CELL SURFACE.**

Madhara Udawela<sup>1</sup>, Maria Morfis<sup>1</sup>, Kenji Kuwasako<sup>2</sup> and Patrick M. Sexton<sup>1</sup>

<sup>1</sup>*Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, Vic. 3010 and* <sup>2</sup>*First Dept. Internal Medicine, Miyazaki Medical College, Miyazaki, Japan.*

Receptor activity modifying proteins (RAMPs) are recently discovered proteins, comprising three known members, RAMP1, -2 and -3, that associate with the calcitonin receptor (CTR) and calcitonin receptor-like receptor (CRLR) to determine their phenotype. To examine the distribution of RAMPs a number of groups have utilised N-terminal epitope tags. Characterisation of tagged RAMP1 indicated that it was only expressed at the cell surface when heterodimerised with either CTR or CRLR<sup>(1,2)</sup>. Recent work with FLAG tagged RAMP's 2 or 3 has suggested that they may be cell surface expressed in the absence of receptor, an action that was dependent on glycosylation of these RAMPs<sup>(3)</sup>. In this study we have used confocal microscopy to compare the trafficking of RAMP's 2 or 3 using N-terminal epitope tagged constructs in either COS-7, HEK-293 or BHK cells. The HA-tagged constructs contained the tag following the natural predicted signal sequence, while the c-myc tagged constructs had the natural leader sequence replaced by a well-characterised signal peptide, the influenza haemagglutinin signal sequence, followed by c-myc tag. Additional experiments were also performed with RAMP3 containing the V5 epitope at the C-terminus. As seen by Flahaut and colleagues<sup>(3)</sup>, a high level of cell surface expression, in the absence of receptor co-expression, occurred for both c-myc tagged RAMP2 and RAMP3 containing the artificial signal sequence, which could be further increased with receptor co-expression. In contrast, the HA-tagged RAMPs containing the natural signal sequence showed very little cell surface expression in the absence of receptor. High level expression, however, was seen upon receptor co-expression. Similar data was obtained using the C-terminal V5-tagged RAMP3, which was principally retained inside the cell in the absence of receptor. These data suggest that either the nature of the tags, or the use of artificial signal sequence may effect the normal distribution of proteins within the cell.

### References:

- (1) McLatchie LM, et al, *Nature* 1998; 393:333-339.
- (2) Christopoulos G, et al, *Mol. Pharmacol.* 1999; 56:235-242.
- (3) Flahaut M, et al. *J Biol Chem.* 2002;277:14731-14737.

## LIGAND DIRECTED SIGNALLING OF $\alpha_{1A}$ - ADRENOCEPTORS

NM Broxton, BA Evans, RJ Summers

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

The  $\alpha_1$ -adrenoceptor ( $\alpha_1$ -AR) family consists of the  $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1D}$  and the putative  $\alpha_{1L}$  subtypes (Weinberg *et al.*, 1994). The  $\alpha_{1A}$ -AR has four functional and five truncated non-functional isoforms. The functional isoforms have variant lengths and sequences within the C-terminal tail while the truncated ones lack transmembrane domain VII (Chang *et al.*, 1998; Coge *et al.*, 1999). All of the functional isoforms of the  $\alpha_{1A}$ -AR couple to Gq and signal through the IP<sub>3</sub>/DAG pathway (Chang *et al.*, 1998). Coupling to additional G-proteins has been reported for the hamster  $\alpha_{1B}$ -AR where Gs can be activated to produce cAMP (Horie *et al.*, 1995). Noradrenaline also stimulates cAMP accumulation in cells transfected with functional  $\alpha_{1A}$ -AR isoforms (Chang *et al.*, 1998). Functional responses of  $\alpha_{1A}$ -AR isoforms stably expressed in CHO-K1 cells have been studied using a cAMP radio-immunoassay and extracellular acidification rate (ECAR) responses in the cytosensor microphysiometer. The relative potency (pEC<sub>50</sub>) of agonists for cAMP and ECAR were respectively noradrenaline: 5.0±0.1, 7.4±0.1; phenylephrine 5.4±0.03, 7.8±0.1; methoxamine 4.7±0.1, 7.1±0.2; cirazoline 6.5±0.1, 9.5±0.6; and A61603 7.5±0.1, 8.7±0.2 activated cAMP accumulation and stimulated an ECAR response in the cytosensor (n=3-5). However, oxymetazoline and clonidine failed to stimulate cAMP accumulation although they both potently stimulated ECAR with pEC<sub>50</sub> values respectively of 8.4±0.3 and 7.6, n=2-4). The rank order of potency of the agonists for cAMP accumulation were A61603 > cirazoline > phenylephrine > noradrenaline, methoxamine >> oxymetazoline, clonidine; and for ECAR: cirazoline > A61603 > oxymetazoline > phenylephrine > clonidine > noradrenaline > methoxamine. The results suggest that ligand directed signalling of the  $\alpha_{1A}$ -AR occurs and that ligands can display different potency for stimulation of particular signalling pathways.

Weinberg DH *et al.* (1994) *Biochem. Biophys. Res. Commun.* **201**:1296-1304.

Chang DJ *et al.* (1998) *FEBS Letters.* **422**: 279-283.

Coge F *et al.* (1999) *Biochem.J.* **343**: 231-239.

Horie K *et al.* (1995) *Mol. Pharmacol.* **48**: 392-400.