Accepted Manuscript

The effects of RAMPs upon cell signalling

Sarah J. Routledge, Graham Ladds, David R. Poyner

PII: S0303-7207(17)30198-3

DOI: 10.1016/j.mce.2017.03.033

Reference: MCE 9907

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 29 July 2016

Revised Date: 1 February 2017

Accepted Date: 24 March 2017

Please cite this article as: Routledge, S.J., Ladds, G., Poyner, D.R., The effects of RAMPs upon cell signalling, *Molecular and Cellular Endocrinology* (2017), doi: 10.1016/j.mce.2017.03.033.

The effects of RAMPs upon cell signalling

Sarah J Routledge[1]*, Graham Ladds[1] and David R Poyner[2]

[1]Department of Pharmacology, University of Cambridge, Cambridge CB2 1PD, United Kingdom

[2]School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom

*Corresponding author; Sarah J Routledge, Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1PD, United Kingdom; +44 1223 334028; sr760@cam.ac.uk

Abstract

G protein-coupled receptors (GPCRs) play a vital role in signal transduction. It is now clear that numerous other molecules within the cell and at the cell surface interact with GPCRs to modulate their signalling properties. Receptor activity modifying proteins (RAMPs) are a group of single transmembrane domain proteins which have been predominantly demonstrated to interact with Family B GPCRs, but interactions with Family A and C receptors have recently begun to emerge. These interactions can influence cell surface expression, ligand binding preferences and G protein-coupling, thus modulating GPCR signal transduction. There is still a great deal of research to be conducted into the effects of RAMPs on GPCR signalling; their effects upon Family B GPCRs are still not fully documented, in addition to their potential interactions with Family A and C GPCRs. New interactions could have a significant impact on the development of therapeutics

Keywords

Receptor activity modifying protein, G protein-coupled receptor, signalling, trafficking, coupling.

Abbreviations

AM, adrenomedullin; AMY, amylin; CaSR, calcium-sensing receptor, CGRP, calcitonin gene-related peptide; CHO, chinese hamster ovary; CLR, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; CRF, corticotrophin releasing factor; ECD, extracellular domain; ECL, extracellular loops; GCGR, glucagon receptor; GLP, glucagon-like peptide; GLP, GLP2R, glucagon-like peptide receptor 2; GPCR, G protein-coupled receptor; GPR30, G protein coupled estrogen receptor 1; GRKs, G protein-coupled receptor kinases; h, human; HEK, human embryonic kidney; m, mouse; NHERF-1, Na+/H+ exchanger regulatory factor-1; PTX, pertussis toxin; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; PTHrP, parathyroid hormone related peptide; r, rat; RAMP, receptor activity modifying protein; s, salmon; VPAC, vasoactive intestinal peptide.

1. Introduction

In order to communicate and respond to their surrounding environment, cells utilise a vast array of signalling molecules ranging from neurotransmitters,

photons of light, lipids and hormones. Signals from many of these molecules are transduced by G protein-coupled receptors (GPCRs) which comprise the largest family of membrane proteins, with more than 800 of these seven transmembrane domain receptors now identified in the human genome[1]. As such, these receptors play a crucial role in mediating most physiological responses and are implicated in many disease states, making them valuable targets for drug development.

In the classical model, upon receptor activation, GPCRs undergo a conformational change and activate an associated heterotrimeric G protein. GDP is exchanged for GTP on the Gα subunit, which dissociates from the βγ subunit. These liberated subunits then activate downstream effector molecules such as adenylyl cyclase and phospholipase C, resulting in stimulation or inhibition of an intricate web of signalling pathways within the cell to control processes including transcription, translation and metabolism [2, 3](Fig. 1). There are 16 known Gα subunits, 5β and 12γ in humans, with the potential of hundreds of combinations[4]. In addition, there are thought to be G protein-independent signalling pathways activated by GPCRs[2] such as through β arrestins[5] and G protein-coupled receptor kinases (GRKs)[6].

GPCRs are much more complex than first envisioned; they were initially thought to behave like switches, with an inactive state and no signalling, or an active state initiating a signalling cascade. It is now clear that GPCRs occupy numerous conformations, which are associated with the activation of a range of signalling pathways. These conformations are stabilised by ligands, therefore certain agonists bias the receptor for a particular pathway or combination of pathways in comparison to another[3]. Complicating this system further, many GPCRs have been shown to interact with additional components[7]. Allosteric modulators bind to the receptors at a different location to the orthosteric ligand binding site. This further influences the pharmacology by altering orthosteric ligand affinity or efficacy, and in some cases may themselves act as allosteric agonists or antagonists[8, 9].

One such group of proteins that can have a significant impact upon GPCR location, ligand binding and signalling are the receptor activity modifying proteins (RAMPs), which were first identified through research into possible CGRP (calcitonin gene-related peptide) receptors. One of the candidates, the then orphan Family B GPCR calcitonin receptor-like receptor (CLR), was difficult to study and responses to CGRP only appeared to occur in HEK293T cells and not others such as COS7 cells lines[10]. This information suggested the requirement of another component for a functional receptor, which was present in HEKs. The elusive component was discovered 1998, when McLatchie *et al* injected Xenopus oocytes with the cDNA of SK-N-MC cells, which contain endogenous CGRP receptors. They identified a population of cells with larger responses to CGRP and isolated the cDNA of a 148 amino acid single-pass membrane protein, which they named RAMP1[11]. Upon co-expression of CLR with RAMP1 in cells that did not contain endogenous CGRP receptors, a response to CGRP was observed

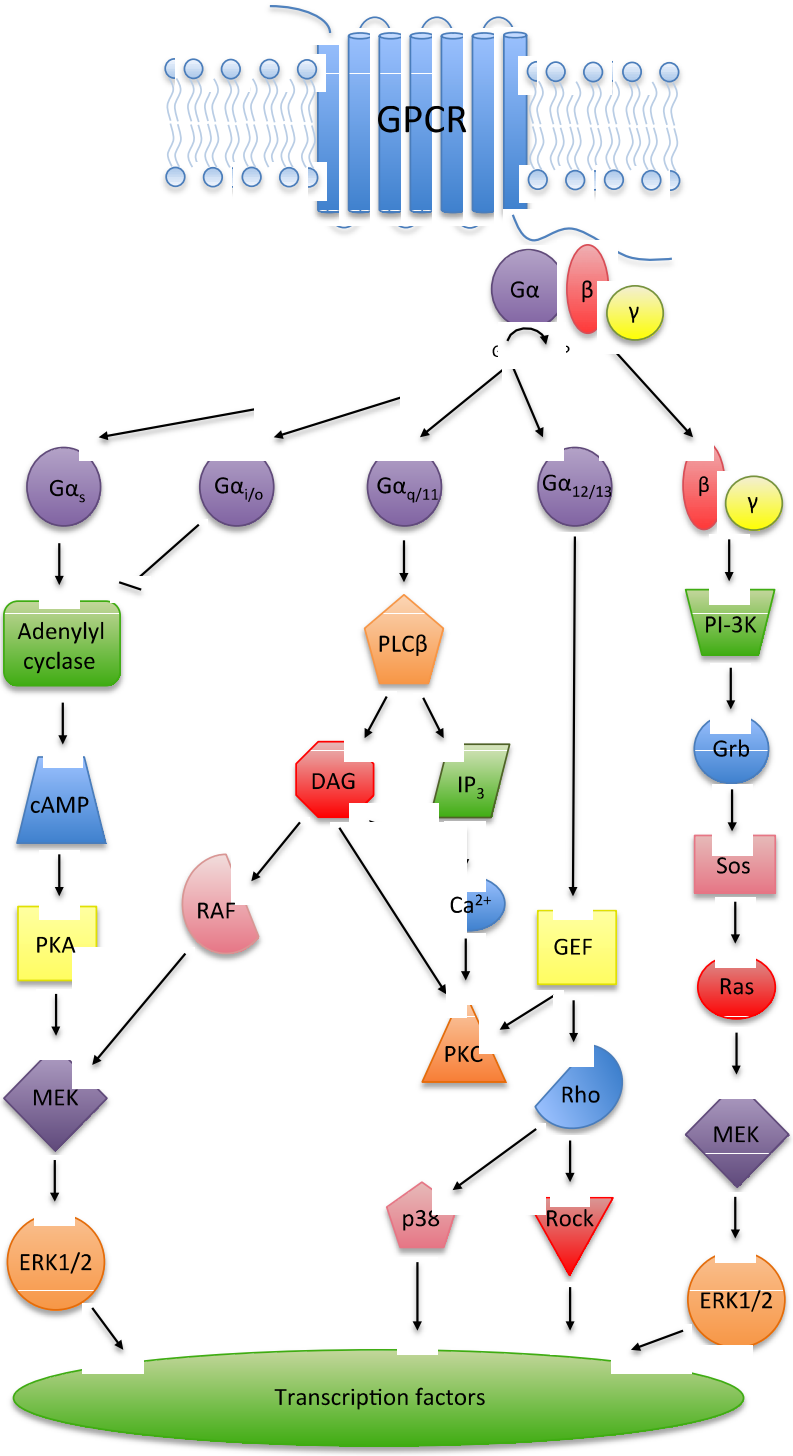

Figure 1. Signalling pathways of GPCRs.

equivalent to that seen in SK-N-MCs. Further investigation demonstrated that RAMP1 was required to transport CLR to the cell surface in order to form a functional receptor able to become bound and activated by CGRP[11]. Database searches identified two RAMP-like proteins named RAMP2 and RAMP3 with 31% homology to one another. RAMP2 and RAMP3 were found to form the adrenomedullin 1 and 2 receptors (AM1R, AM2R) together with CLR[11, 12]. The RAMPs by themselves, like CLR, show only poor cell surface expression; however the RAMP/CLR heterodimers are efficiently trafficked to the outside of the cell.

The interactions of the RAMPs with CLR and calcitonin receptor (CTR) are now well studied, providing us with better insight into the role of these accessory proteins[13]. It is now known that RAMPs can interact with some GPCRs to alter the pharmacology of the receptors by allosterically affecting the structure, altering ligand specificity and pharmacology, and in trafficking certain receptors. Several Family B receptors have now been shown to interact with the RAMPs, in addition to emerging interactions with GPCRs from Family A and C (summarised in Table 1). The consequences of these interactions in many cases are still unclear. Here we discuss research that has been conducted to investigate the role of RAMPs upon GPCR signalling; these findings are highlighted in Table 2. Other aspects of RAMPs have been recently reviewed elsewhere[14].

2. RAMP interactions with Family B GPCRs

2.1 CLR

The role of RAMPs in translocating CLR to the cell surface have been described above; it should further be noted that CLR by itself appears to be unable to bind with appreciable affinity any of the endogenous peptide ligands within the CGRP/calcitonin family. Two recent studies have cast some light on how RAMPs can influence peptide binding to CLR. Crystal structures of the extracellular domain (ECD) of CLR in combination with either the ECD of RAMP1 and a CGRP analogue or RAMP2 and an adrenomedullin (AM) fragment show that the RAMPs interact with the C-terminal residue of the peptide (F37-amide for CGRP, Y52-amide for AM). For CGRP, F37 contacts W84 of RAMP1. (Fig. 2a). In RAMP2, the equivalent residue, F111 cannot make the necessary contact but instead there is an interaction with E101and Y52 of AM (Fig. 2b). In RAMP1, the equivalent of E101, W74, fails to contact CGRP.There are no further direct contacts between either peptide and the RAMPs. Instead the peptides have turn structures, not seen in other peptide ligands for family B GPCRs which contact CLR. There is evidence for some small but potentially significant RAMP-dependant shifts in the conformation of the contact residues on CLR, suggesting that the RAMPs act in part by allostery[15].

The RAMPs also seem to exert an effect on the extracellular loops (ECLs) of CLR. This has been investigated by mutagenesis; for each RAMP a different set of residues within the ECLs appear to be important. On the basis of molecular modelling, it has been suggested that RAMP-induced conformational changes in ECL3 may be particularly important[16].

Table 1: Summary of known RAMP interactions with GPCRs

GPCR	RAMP interaction partner	Required for/ enhances receptor trafficking			Influences peptide binding affinity			Modulates signalling		
		RAMP1	RAMP2	RAMP3	RAMP1	RAMP2	RAMP3	RAMP1	RAMP2	RAMP3
CaSR	RAMPS 1 and 3	X		X				X		
CLR	RAMPS □, □ and □	X	X	X	X	X	X	X	X	X
CTR	RAMPS □, □ and □				X	X	X	X		X
CRF1R	RAMP 2		X						X	
Glucagon	RAMP 2					X			X	
GPR30	RAMP 3			X						
GLP2R	RAMPS □, □ and □							X	X	X
PTHR1	RAMP 2									
PTHR2	RAMP 3									
Secretin	RAMP 3			X						
VPAC1	RAMPS □, □ and □								X	
VPAC2	RAMPS □, □ and □							X	X	

Table 2: RAMP modulation of GPCR signalling

GPCR	Family	Agonists investigated	G-proteins modulated by RAMPs	RAMP interaction	Effect upon signalling	Other effects	Refs
GPR30 or GPER	A	Estrogen Tamoxifen G-1		RAMP3	Unknown	Trafficking to cell surface	[17, 18]
CLR	B	CGRP AM1 AM2	Gαs, Gαi/o	RAMP1 RAMP2 RAMP3	RAMP1 promotes Gαs coupling of CGRP and Gαi coupling of AM and AM2. RAMP2 promotes Gαs coupling of AM.	Confer ability to bind endogenous peptide ligands. Traffic receptor to the cell surface. RAMP3 decreases CLR internalisation.	[19-21]
CTR	B	Calcitonin Amylin CGRP	Gαs, Gαq	RAMP1 RAMP2 RAMP3	RAMPs 1 and 3 increase Gαs coupling relative to Gαq and erk activation	Enhance affinity for amylin and CGRP	[22-26]
CRF1R	B	CRF Urocortin Sauvagine	Gαi/o, Gαq, Gα12/13	RAMP2	No effect with Gαs. Enhanced basal and E_{max} with Gαi/o. Enhanced Emax with Gq. Enhanced agonist potency with Gα12/13 Enhanced Ca^{2+} signalling with CRF and urocortin but not sauvagine.	Enhanced trafficking to cell surface	[27]
Glucagon	B	Glucagon GLP1 Oxyntomodulin	Gαs, Gαi	RAMP2	Reduced coupling to Gαi when activated by glucagon but not	Binding of GLP-1 abolished	[28-30]

					oxyntomodulin. Enhanced Gαs coupling with oxyntomodulin		
GLP2R	B	GLP2	Gαs	RAMP1 RAMP2 RAMP3	RAMPs may alter basal signalling.		Unpublished data
PTH1	B			RAMP2	Not determined		[31]
PTH2	B			RAMP3	Not determined		[31]
Secretin	B	Secretin		RAMP3	No effect on cAMP, ERK1/2 phosphorylation, intracellular Ca^{2+} or internalization	Trafficking	[32]
VPAC1	B	VIP	Possible Gαq coupling	RAMP1 RAMP2 RAMP3	RAMP2 enhances E_{max} for inositol phosphate production		[31]
VPAC2	B	VIP	Gαi/o	RAMP1 RAMP2 RAMP3	RAMP 1and 2 enhanced basal coupling to Gαi/o and VIP potency		[27]
Calcium sensing	C	Cinacelcet, neomycin	Possible Gαq coupling	RAMP1 RAMP3	RAMP1 may enhance Ca^{2+} signalling.	Enhanced trafficking to cell surface.	[33, 34]

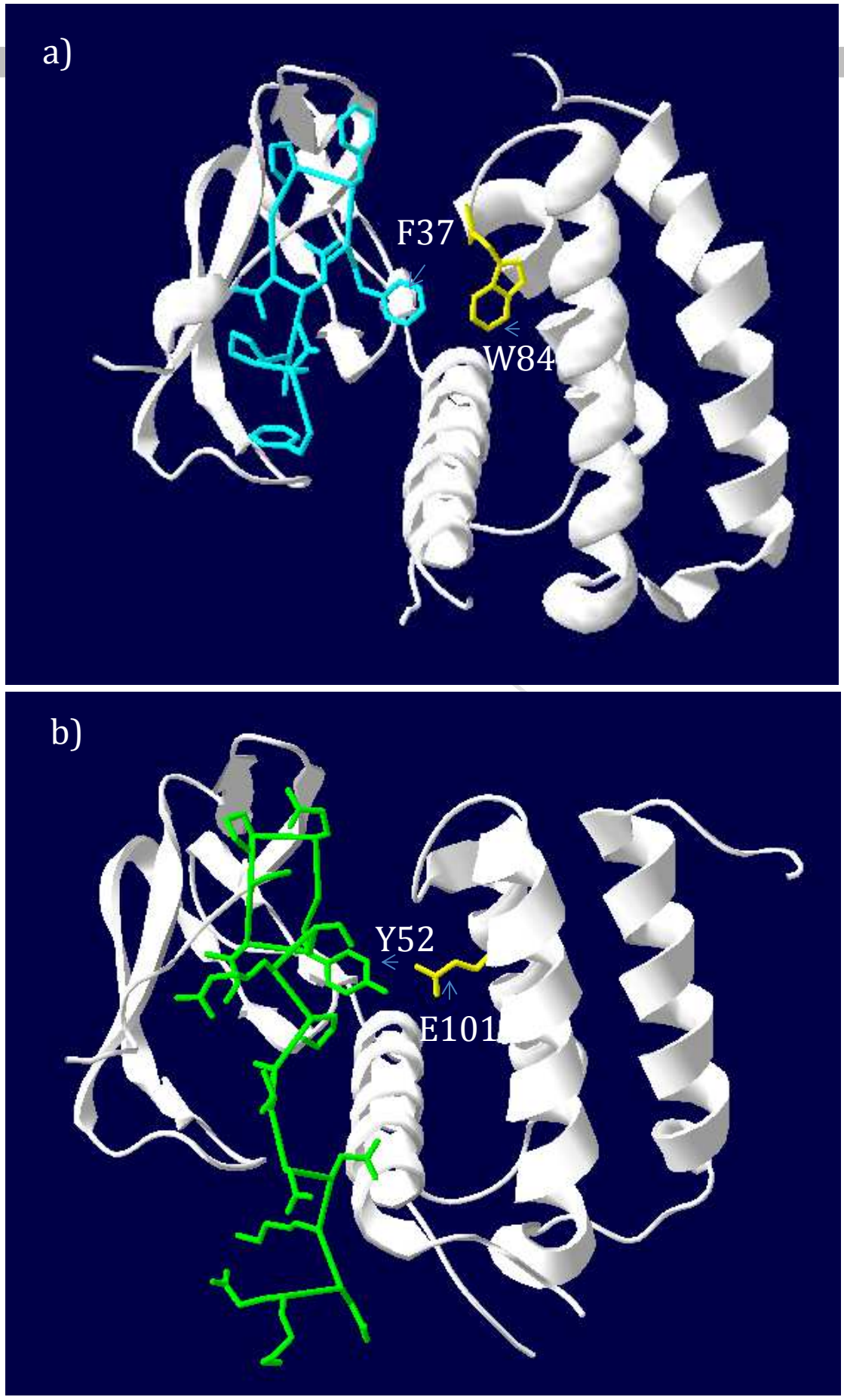

Figure 2. Structure of ligand-bound RAMP-CLR complexes. (a) RAMP1/CLR (white) with $CGRP_{27-37}$ [D^{31},P^{34},F^{35}] (blue) bound (4RWG); (b) RAMP2/CLR (white) with AM_{22-52} (green) (4RWF). The key residues involved in RAMP/ligand interactions are shown.

Little work has been done to investigate if RAMPs influence G protein selectivity of CLR. However, in both HEK293 cells and a model yeast system, they alter Gαs/Gαi/ Gαq coupling in a ligand-dependent manner[21]. It has been demonstrated that RAMP3 can interact with Na+/H+ exchanger regulatory factor-1 (NHERF-1) and this prevents receptor internalisation. This remains the only detailed study to investigate the effect of RAMPs on receptor internalisation[20].

2.2 CTR

The CTR was first cloned in 1991[35], and is known to have several isoforms with distinct pharmacology and signalling properties [24, 36, 37]. The most commonly expressed splice variant has a 16 amino acid insert in the first intracellular loop either present (denoted CTb receptor) or absent (denoted the CTa receptor)[24]. Differences in these two isoforms include reduced internalization in addition to reduced Gs and Gq signalling of the CTb isoform[24]. Activation of CTRs leads to effects in the bone, CNS, gastrointestinal and reproductive systems[36].

Amylin is a peptide with substantial homology to CT, CGRP and adrenomedullin. Levels of amylin in circulation increase upon eating and physiological effects include the inhibition of glucagon secretion, gastric emptying and food consumption[22]. Two groups discovered that the CTR interacts with the RAMPs to form a receptor for amylin[22, 38]. In the most comprehensive study, it was found that COS-7 cells expressing the CTa receptor isoform in association with RAMP1 or RAMP3 led to formation receptors for amylin with differing affinities[22], and later found that RAMP2 together with CT also resulted in an amylin receptor distinct from the RAMP1 and RAMP3 phenotypes, although these findings were influenced by the cell line and also the isoform of CTR expressed[23]. All three RAMPs couple to CTa and CTb, however creation of an AMY receptor with RAMP2 appears to favour the CTb variant[23, 24]. Unlike CLR, CTR does not require association with RAMPs for cell surface expression[22].

It has now been demonstrated that the AMY1 receptor has highest affinity for salmon CT (sCT), followed by amylin and CGRP and low affinity for mammalian CT. The AMY2 and 3 receptors parallel this pharmacology with lower affinities for CGRP[39]. Since the CTR signals through Gs and Gq, it is assumed that the AMY receptors also couple to these G proteins, although coupling of G proteins with the CTb isoforms may be reduced[39].

Several studies have provided mechanistic insight into how RAMPs alter ligand binding to CTR. An extensive mutagenesis screen of the ECD of CTR[40] suggested that the RAMPs had allosteric actions; on the basis of molecular modelling, it was suggested that the RAMPs might influence the dynamics of loop 5 and residues immediately C-terminal of the CTR. Similarly, based on the structure of the ECD of the CTR in complex with a salmon calcitonin analogue, it has been suggested that the RAMPs change the orientation of R126 in loop 5 of CTR to enhance the affinity of the receptor for amylin[41]. The structure suggests that the C-terminus of calcitonin is unlikely to be able to interact with

the RAMPs. In view of the key role of the C-terminal residues of CGRP and AM, it is surprising that the equivalent residue of amylin, Y37, is of little importance for binding[42]. This raises questions as to the importance of direct RAMP contacts with amylin. It is also interesting that RAMPs enhance the affinity of a CTR/CLR orthologue from *Branchiostoma floridae* to bind its calcitonin/CGRP orthologues[43]. Whilst the authors of this study interpret their results in terms of the RAMPs enhancing cell surface expression of the CTR/CLR orthologue, the peptides which it binds appear much closer to calcitonin than CGRP at their C-termini and so it is not clear that they make direct contact with the RAMPs. If this is correct, it would further strengthen the case for an allosteric role of RAMPs.

A study into the role of the C-terminus of the RAMPs upon interaction of the CTRa isoform was conducted by Sexton *et al* in 2006, and was the first study to illustrate that the C-terminus is involved in signalling. Chimeric RAMPs with C-terminal domains swapped were created; RAMP1 with the C-terminus of RAMP2, and RAMP2 with the C-terminus of RAMP1. CTRa co-expressed with chimeras containing a RAMP1 C-terminus exhibited similar cAMP signalling profiles to RAMP1 and CTRa with high affinity for hCT, hCGRP and rAMY (rat amylin), despite the RAMP2-CTRa receptor having lower affinities for CGRP[44].

The RAMP2 C-terminus-containing chimeras also had similar signalling profiles to RAMP2 and CTRa [44]. These findings suggested that while the N-terminus contributes to the peptide binding site and the TMD to receptor-RAMP stability, the C-terminus, although relatively short at 10 amino acids, is involved in determining the signalling profile of amylin receptors generated from the CTR. Deletion of a large proportion of the C-terminus results in a loss of high affinity amylin receptors[25]. Following on from this, RAMP1 and RAMP3 were found to significantly increase the potency of AMY at AMY1 and AMY3 receptors via Gs mediated cAMP production, but only slight increases in Ca^{2+} and ERK1/2 activation were observed when compared to CTRa without RAMPs. This implies that RAMPs affect G protein-coupling efficiency of the AMY receptors, and induce more efficient coupling to Gαs than other G proteins[26].

2.3 CRF1R

There are two subtypes of corticotrophin releasing factor (CRF) receptor in humans. When activated, these receptors predominantly signal through Gs and are involved in the synthesis and release of adrenocorticotropic hormone (ACTH) and β-endorphins from pituitary glands. They have been implicated in stress and anxiety-related endocrine responses[45]. A RAMP2 interaction has been demonstrated for the type 1 receptor (CFR1). A study by Wootten *et al* demonstrated that this interaction leads to the improved trafficking of the receptor to the cell surface, as well as affecting signalling[27]. There was no effect of RAMP2 to coupling of the receptor to Gαs upon challenge with the agonists CRF, urocortin 1 and sauvigne. However, GTPγS binding revealed improved coupling of CRF1 to Gαi/o/t/z, Gαq/11 and Gα12/13 in the presence of RAMP2. Improvements in G protein coupling were not found to be a result of enhanced trafficking of the receptor to the cell surface. RAMP2 interactions resulted in greater basal coupling of CRF1 to Gi/o/t/z and a higher maximum

response when stimulated by CRF. Improved Gq/11 coupling led to an increase in maximum Ca^{2+} with both CRF and urocortin 1, but not sauvagine. This demonstrates that the signalling effects of RAMP-receptor interactions may be ligand dependent. Investigations with inhibitors suggested that the elevated Ca^{2+} in the presence of RAMP2 came from extracellular sources in addition to intracellular pools, whereas the CRF1 alone mobilized intracellular stores[27].

2.4 Glucagon receptor

Glucagon is a peptide involved in blood glucose regulation and generally opposes the effects of insulin. Activation of its receptor leads to conversion of glycogen to glucose in the liver where it is released into the blood to maintain glucose levels[46]. The glucagon receptor (GCGR) couples to Gαs, Gαi/o and Gαq/11[28-30], and has been shown to traffic RAMP2 to the cell surface[28, 31]. Further studies demonstrated that upon co-expression of its receptor with RAMP2, glucagon was more potent and E_{max} was increased[28]. This was not due to enhanced cell surface expression of GCGR, or an effect upon ligand affinity. A yeast system developed to allow coupling of human GPCRs and chimeric G proteins to activate an endogenous yeast-mating pathway[47] was used to investigate coupling of GCGR to Gαs and Gαi. Activation of individual pathways can be determined in relation to yeast cell growth, and findings suggested that co-expression of RAMP2 with the GCGR in the GPA1/Gαs containing strain resulted in an increase in the maximum response and potency of glucagon. When expressed in the GPA1/Gαi expressing strain, RAMP2 led to a reduction in response. In HEK293 cells, there was no significant change was observed in Gαs activation, however Gi coupling was significantly decreased, thereby elevating the cAMP response. PTX treatment of cells to prevent activation of Gαi resulted in an increase in Gαs coupling with GCGR alone, but did not affect the maximum cAMP produced when coexpressed with RAMP2. These results suggest that RAMP2 reduced coupling of GCGR to Gαi, and uncovers an important role for the RAMPs in modulating G protein coupling and cell signalling[28].

Another significant finding by this study is that this is effect ligand specific. Oxyntomodulin is a less potent agonist at the GCGR than its cognate ligand, and RAMP2 co-expression also led to increased potency on cAMP production without affecting the binding affinity. Here, studies with pertussis toxin (PTX) and in yeast suggest the effects are due to augmented coupling of Gs, rather than reduced coupling to Gαi[28].

In addition, RAMP2 was capable of abolishing binding of GLP-1, which is a partial agonist at the GCGR. Liraglutide, a GLP-1 analogue and weak GCGR agonist used in diabetes treatment, was also unable to bind the receptor in the presence of RAMP2. This effect is not seen at the GLP-1 receptor, where GLP-1 binding and activation is not affected by RAMPs, and is therefore receptor-specific[28, 29].

2.5 GLP2R

Glucagon-like peptide 2 (GLP-2) is a peptide derived from proglucagon and secreted from intestinal enteroendocrine L cells and has a 40% similarity to

other proglucagon-derived peptides, GLP-1 and glucagon[48]. Activation of the GLP-2 receptor (GLP-2R) by its 33 amino acid peptide causes signalling through Gαs, leading to crypt cell proliferation in the small intestine, and has been found to improve nutrient absorption in patients with short bowel syndrome and to regulate blood glucose[48, 49].

The GLP-2R had previously been investigated for RAMP interaction, but none had been detected[31]. We have recently investigated the GLP2R for interaction with the RAMPs by cell surface ELISA in HEK293Ts and our preliminary data suggests each of the RAMPs were detected at the cell surface upon coexpression with GLP2-R. The data also suggest that the RAMPs change either the basal or maximum stimulation of cAMP.

2.6 PTHR1 and PTHR2

Parathyroid hormone (PTH) regulates blood calcium levels as well as mineral ions and is secreted from the parathyroid cells in response to low extracellular Ca^{2+} and elevated extracellular phosphate[50, 51]. It has two receptors, PTHR1 and PTHR2. Another similar peptide, parathyroid hormone related peptide (PTHrP) is also able to activate PTHR1, but not PTHR2[51]. PTHrP is normally involved in lactation where it promotes calcium mobilisation from bone, and in long bone development. Secretion is increased in tumours causing a rise in serum calcium, resulting in development of humoral hypercalcemia malignancy syndrome[51].

The study by Christopolous *et al* investigated cell surface expression of the RAMPs upon coexpression with the PTHRs for the first time. An interaction was observed with PTHR1 and RAMP2 and with PTHR2 and RAMP3[31], however the consequences of these interactions are currently unknown.

2.7 Secretin

The secretin receptor was the first member of the Family B GPCRs to be cloned, and as such represents the model receptor for the family[52]. It was first cloned by Ishihara *et al* in 1991[53], and its biological roles include bile stimulation, gastric pepsin secretion and release of insulin from the pancreas upon intake of glucose[53, 54]. Secretin receptors are expressed in the brain, stomach, pancreas, kidneys and the liver, and are thought to couple to Gαs and Gαq[54, 55].

Harikumar *et al* demonstrated an interaction of the secretin receptor and RAMP3 for the first time in 2009[32]. The receptor is normally able to traffic to the cell surface alone, however RAMP3 restored this ability to a mutant receptor (G241C) unable to traffic, suggesting a role for RAMP3 as a chaperone whether required for normal expression or not[32]. Investigation into possible effects of RAMP3 upon signalling of the secretin receptor were conducted, however no changes were observed to cAMP, ERK1/2 phosphorylation, intracellular Ca^{2+} or internalization of the receptor. In addition, RAMP3 appeared to have no effect upon binding of secretin to its receptor, and unsurprisingly, no interaction was

observed with the N-terminus of the receptor with RAMP3. Instead, interaction sites were found to be with TM6 and TM7. Interestingly, the study discovered that RAMP3 interacted with a homodimer of the secretin receptor, and that the receptor competed for RAMP3 with CLR, thus reducing functional CLR-RAMP3 generated adrenomedullin receptors at the cell surface[32].

2.8 VPAC1R

Vasoactive Intestinal Peptide activation of VPAC1 leads to growth and development and is involved in immune response[56]. The successful cloning of VPAC1R from rat lung cDNA library was published in 1992[57]. VPAC1R has been reported to couple to Gαs, Gαi/o and Gαq[58] as well as numerous other second messengers such as tyrosine kinases, calcium channels, MAPK and RhoA GTPases[59]. Stimulation of VPAC1R with VIP predominantly stimulates cAMP production, with lower levels of phosphoinositide (PI) hydrolysis, an indication of PLC activation and Gαq coupling[27, 31, 59]. Increases in calcium levels have also been observed [58]. Christopoulos *et al* observed trafficking to the cell surface of all three RAMPs upon coexpression with VPAC1R [31]. Following upon these findings, they discovered that the RAMPs did not affect ligand binding, nor did they alter expression levels of the VPAC1R at the cell surface[31]. Upon further investigation into possible effects upon cell signalling, it was found that the RAMPs did not affect cAMP production, but RAMP2 significantly enhanced the hydrolysis of PI[31]. They suggested that RAMP2 may improve the signalling efficiency of the receptor.

2.9 VPAC2R

The VPAC2R was first cloned in 1993 by Lutz *et a*l from a rat pituitary cDNA library[60]. Initial investigations for interaction of the VPAC2R in HEK293 cells did not reveal any interactions[31], but a later study by Wootten *et al* demonstrated trafficking of all three RAMPs to the cell surface when co-transfected with VCAP2R in HEK293S and CHO-K1 cells, with larger effects seen in the former[27]. These findings highlight the variations between cell lines and the authors noted that the expression levels of RAMPs in each cell type should be considered when interpreting data.

While the study did not find any significant changes to binding of VPAC2 agonists VIP, BAY55-9837, PCAP-27 and PHM-27 in the presence of the RAMPs, G protein coupling, however, was affected. GTPγS binding assays demonstrated that although there were no RAMP-mediated changes to Gαs coupling when stimulated with VIP, there were significant increases in basal coupling to Gαi/o in HEK293S and CHO-K1 cells with RAMP1 and RAMP2 co-transfection[27]. In addition, VIP appeared to increase the potency of this coupling with RAMP1. No change to coupling with Gαq/11 or Gα12/13 was observed for VPAC2R alone or with any of the RAMPs[27].

2.10 GPR30

G protein coupled estrogen receptor 1, or GPR30, is expressed in the human and rodent heart and activation by estradiol, a form of estrogen, mediates pleiotropic function in the cardiovascular system in addition to the endocrine, immune and CNS and may be involved in cardioprotection[17]. Lenhart *et al* recently described for the first time the interaction of RAMP3 with GPR30[17], which they theorized could interact due to evidence that estrogen regulates *Ramp3* gene expression. They found that RAMP3 increases GPR30 expression at the cell surface[61].

At present, there is currently no known effect of RAMPs upon the signalling profile of this receptor.

2.11 Calcium-sensing receptor

The calcium-sensing receptor (CaSR) is a Family C GPCR that is able to bind Ca^{2+} and is therefore involved in calcium homeostasis. It is also capable of binding Mg^{2+}, Zn^{2+} and Ni^{2+} in addition to antibiotics like neomycin[34]. This receptor is also involved in PTH and CT secretion[33, 34]. It is capable of coupling to several G proteins including Gαs, Gαi, Gαq, and Gα12/13[34].

It was first shown by Bouschet *et al* to interact with RAMPs 1 and 3 but not RAMP2, making this the first known interaction between a RAMP and a Family C GPCR[62]. This has now been demonstrated in both transfected cell lines and endogenously expressing cells[34, 62, 63]. RAMP1 and 3 interactions are a requirement in order to traffic the receptor to the cell surface[34, 62, 63]. In addition, RAMP3 association has been shown to lead to further glycosylation of CaSR[62].

Expanding upon this research, Desai *et al* demonstrated that RAMP1 also played a role in the signalling of the receptor. Knockdown of RAMP1 expression by siRNA in medullary thyroid carcinoma TT cells, endogenously expressing RAMP1 only, resulted in a 50% reduction in Ca^{2+} signalling by Cinacelcet (a CaSR allosteric modulator) and neomycin (a CaSR agonist)[34]. Stoichiometric analysis revealed there to be approximately 1.6 times more RAMP3 associated with CaSR than with RAMP1; the authors suggested that receptors may interact with more than one molecule of RAMP[34], however, this has yet to be fully explored.

2.12 The role of RAMPs in pathophysiology

The upregulation of RAMPs and the modulation of receptor response to ligands, in particular to AM, are involved in numerous disease states and several studies have investigated knockout mice to better understand their role. In an investigation into skin wound healing, RAMP1 (-/-) mice displayed reduced wound-induced angiogenesis and lymphangiogenesis and their ability to heal wounds was decreased compared to WT mice [64]. RAMP2 (-/-) mice have been demonstrated to die *in utero* as a result of improper vascular development and edema, an outcome that is also observed in AM(-/-) mice [65]. Heterozygous RAMP2 (+/-) knockout acute and chronic cerebral ischemia models to

investigate cerebrovascular disease demonstrated greater upregulation of AM gene expression compared to WT mice after induction of infarction. This was thought to compensate for reduced RAMP2 expression[65]. The findings suggested a protective role for RAMP2 with the AM receptor complex by reducing oxidative stress, inflammation and restoring blood flow, thereby protecting against brain injury.

RenTgMK mice, used to model cardiac hypertrophy and chronic hypertension where male mice have increased cardiac hypertrophy and reduced survival compared to females, were investigated in addition to RAMP3 (-/-) knockout[61]. An increase in AKT activation (a regulator of cardiomyocyte cell survival and apoptosis) was observed in male RenTgMK; RAMP 3 (-/-) mice when compared to female RenTgMK and RenTgMK; RAMP 3 (-/-) mice, with an associated increase in cardiac apoptosis. The males also exhibited significant depressed systosolic function and renal damage when compared to the females. In addition, female mouse hearts displayed increased *Ramp3* gene expression during cardiovascular stress[61]. These findings suggest that there is a sex-dependent role for RAMP3 as a cardioprotectant, linked to oestrogen-regulated *Ramp3* gene expression [61]. A study on RAMP-receptor trafficking found that the interaction of the RAMP3 PDZ type 1 motif with NSFs (*N*-ethylmaleimide-sensitive factor) promoted targeting of the CLR-RAMP3 complex for recycling after internalization upon agonist stimulation. The authors suggest that since RAMP3 expression is increased the myocardium of rats with chronic heart failure, this may then allow for improved recycling of AM receptors and therefore extend exposure to the protective effect of AM in this condition and others such as type 1 and 2 diabetes and chronic glomerulonephritis where AM is elevated[20].

3. Conclusions

RAMPs modulate GPCRs in numerous ways. The simplest of these is by acting as molecular chaperones and this may have been the first function to appear in evolution[43] and is seen across Families A, B and C of GPCRs. However, beyond this, they can also modulate ligand binding and cell signalling. Although first characterised for their effects on conferring the ability of CLR to bind to its native peptide agonists, the most common effect across GPCRs seems to be modulation of cell signalling. These effects can manifest themselves as changes in agonist potency (without any change in affinity), the size of the maximum response and basal activity. Furthermore, the effects are frequently agonist-specific. These suggest that the RAMPs work by altering the conformation of the transmembrane domain of the GPCRs (Figure 3). The ECD of the RAMPs may influence the ECD of the GPCR and, through this, the transmembrane domain; for some Family B GPCRs, the ECD is an allosteric regulator of signalling[66]. There are also likely to be direct interactions of the RAMPs with the ECLs of the GPCRs, which will change alter the conformation of the transmembrane helices. The transmembrane domain of the RAMP must pack against the transmembrane helices of the GPCRs and this may alter either their conformation or their movements during receptor activation. Finally the C-termini of the RAMPs can interact with the intracellular loops of the GPCRs and possibly the G proteins

themselves. All these provide potential mechanisms allowing RAMPs to tune GPCR signalling.

For the future, there is still significant work to document the full range of RAMP interactions with all of the Family B GPCRs. The recent work showing that RAMP1 can influence calcium signalling at the CaSR suggests that the effects on signal transduction may extend at least to Family C GPCRs. Very little work has been done to investigate the influence of RAMPs on β-arrestins or the interaction of other proteins with GPCRs, but the influence of RAMP3 on CLR internalisation illustrates that this may be significant[20]. The effects of RAMPs depend heavily on the cell line in which the receptor is expressed; the molecular basis for this is not clear but it implies that the physiological consequences of RAMP expression are crucially dependant on the cells in which they are expressed. If this is understood, then there is considerable potential to develop drugs that are targeted against specific RAMP/GPCR complexes.

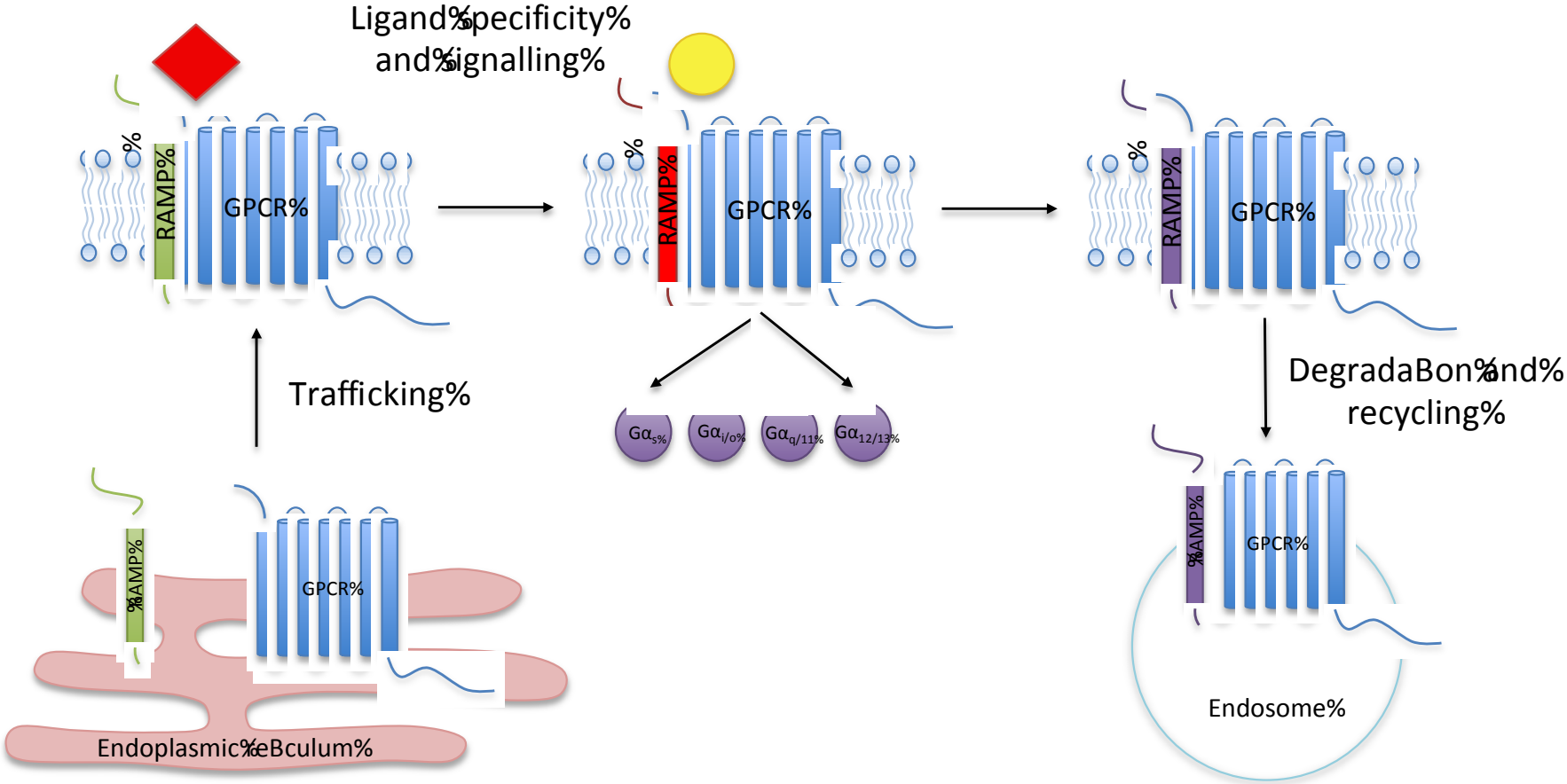

Figure 3. Mechanisms of RAMP interactions

Figure Legends

Figure 1. Signalling pathways of GPCRs.

Figure 2. Structure of ligand-bound RAMP-CLR complexes. (a) RAMP1/CLR (white) with $CGRP_{27-37}$ [D^{31},P^{34},F^{35}] (blue) bound (4RWG); (b) RAMP2/CLR (white) with AM_{22-52} (green) (4RWF). The key residues involved in RAMP/ligand interactions are shown.

Figure 3. Mechanisms of RAMP interactions

Competing interests
The authors declare that they have no competing interests.

Funding
This work was supported by BBSRC grants BB/M00015X/1 and BB/M000176/1.

References

1. Kobilka B: G Protein Coupled Receptor Structure and Activation*. Biochim Biophys Acta* 1768(4), 794–807 (2007).
2. Marinissen MJ, Gutkind JS: G-protein-coupled receptors and signaling networks: emerging paradigms*. Trends Pharmacol Sci* 22(7), 368-376 (2001).
3. Violin JD, Crombie AL, Soergel DG, Lark MW: Biased ligands at G-protein-coupled receptors: promise and progress*.* 35(7), p308–316 (2014).
4. Hillenbrand M, Schori C, Schöppe J, Plückthun A: Comprehensive analysis of heterotrimeric G-protein complex diversity and their interactions with GPCRs in solution*. PNAS* 112(11), 1181–1190 (2015).
5. Shenoy SK, Drake MT, Nelson CD *et al.*: β-Arrestin-dependent, G Protein-independent ERK1/2 Activation by the β2 Adrenergic Receptor*. The Journal of Biological Chemistry* 281, 1261-1273 (2006).
6. Watari K, Nakaya M, Kurose H: Multiple functions of G protein-coupled receptor kinases *Journal of Molecular Signaling* 9(1), (2014).
7. Magalhaes AC, Dunn H, Ferguson SSG: Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins *Br J Pharmacol* 165(6), 1717–1736 (2012).
8. Langmead CJ, Christopoulos A: Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox *Trends Pharmacol Sci* 27(9), 475–481 (2006).
9. Kenakin T, Miller LJ: Seven Transmembrane Receptors as Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery*. Pharmacol Rev* 62(2), 265–304 (2010).
10. Han ZQ, Coppock HA, Smith DM *et al.*: The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium*. J Mol Endocrinol* 18(3), 267-272 (1997).

11. Mclatchie LM, Fraser NJ, Main MJ *et al.*: RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor *Nature* 393, 333-339 (1997).
12. Hilairet S, Foord SM, Marshall FH, Bouvier M: Protein-protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. *. J Mol Biochem* 276(31), 29575-29581 (2001).
13. Sexton PM, Albiston A, Morfis M, Tilakaratne N: Receptor activity modifying proteins*. Cellular Signalling* 12(2), 73–83 (2001).
14. Hay DL, Pioszak AA: Receptor Activity-Modifying Proteins (RAMPs): New Insights and Roles*. Annu Rev Pharmacol Toxicol* 56, 469-487 (2016).
15. Booe JM, Walker CS, Barwell J *et al.*: Structural Basis for Receptor Activity-Modifying Protein-Dependent Selective Peptide Recognition by a G Protein-Coupled Receptor*. Mol Cell* 58(6), 1040-1052 (2015).
16. Watkins HA, Chakravarthy M, Abhayawardana RS *et al.*: Receptor Activity-modifying Proteins 2 and 3 Generate Adrenomedullin Receptor Subtypes with Distinct Molecular Properties*. J Biol Chem* 291(22), 11657-11675 (2016).
17. Lenhart PM, Broselid S, Barrick CJ, Leeb-Lundberg LMF, Caron KM: G-protein Coupled Receptor 30 Interacts with Receptor Activity Modifying Protein 3 and Confers Sex-Dependent Cardioprotection*. J Mol Endocrinol* 51(1), 191–202 (2013).
18. Broselid S, Berg KA, Chavera TA *et al.*: G protein-coupled Receptor 30 (GPR30) Forms a Plasma Membrane Complex with Membrane-associated Guanylate Kinases (MAGUKs) and Protein Kinase A-anchoring Protein 5 (AKAP5) That Constitutively Inhibits cAMP Production*. The Journal of Biological Chemistry* 289, 22117-22127 (2014).
19. Weston C, Dowell SJ, Poyner DR, Ladds G: *Exploring The Effect Of The G Protein Subunit On RAMP-Mediated Signal Modulation Of Calcitonin-related-like Receptor*. In: *Proceedings of the British Pharmacological Society*. 2013.
20. Bomberger JM, Spielman WS, Hall CS, Weinman EJ, Parameswaran NJ: Receptor activity-modifying protein (RAMP) isoform-specific regulation of adrenomedullin receptor trafficking by NHERF-1*. J Biol Chem* 280(25), 23926-23935 (2005).
21. Weston C, Winfield I, Harris M *et al.*: Receptor Activity-modifying Protein-directed G Protein Signaling Specificity for the Calcitonin Gene-related Peptide Family of Receptors*. J Biol Chem* 291(42), 21925-21944 (2016).
22. Christopoulos G, Perry KJ, Morfis M *et al.*: Multiple Amylin Receptors Arise from Receptor Activity-Modifying Protein Interaction with the Calcitonin Receptor Gene Product *Molecular Pharmacology* 56(1), 235-242 (1999).
23. Tilakaratne N, Christopoulos G, Zumpe E, Foord SM, Sexton PM: Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment*. J Pharmacol Exp Ther* 294(1), 61–72 (2000).
24. Poyner DR, Sexton PM, Marshall I *et al.*: International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin

receptors. *Pharmacol Rev* 54(2), 233-246 (2002).
25. Udawela M, Christopoulos G, Morfis M *et al.*: A critical role for the short intracellular C terminus in receptor activity-modifying protein function. *Mol Pharmacol* 70, 1750–1760 (2006).
26. Morfis M, Tilakaratne N, Furness SGB *et al.*: Receptor Activity-Modifying Proteins Differentially Modulate the G Protein-Coupling Efficiency of Amylin Receptors *Endocrinology* 149(11), 5423-5431 (2008).
27. Wootten D, Lindmark H, Kadmiel M *et al.*: Receptor activity modifying proteins (RAMPs) interact with the VPAC2 receptor and CRF1 receptors and modulate their function. *Br J Pharmacol* 168(4), 822–834 (2013).
28. Weston C, Lu J, Li N *et al.*: Modulation of Glucagon Receptor Pharmacology by Receptor Activity-modifying Protein-2 (RAMP2) *J Biol Chem* 290(38), 23009–23022 (2015).
29. Weston C, Poyner DR, Patel V, Dowell SJ, Ladds G: Investigating G protein signaling bias at the glucagon-like peptide-1 receptor in yeast. *Br J Pharmacol* 171, 3651–3665 (2014).
30. Xu Y, Xie X: Glucagon receptor mediates calcium signaling by coupling to Gαq/11 and Gαi/o in HEK293 cells. *J Recept Signal Transduct Res* 29, 318–325 (2009).
31. Christopoulos A, Christopoulos G, Morfis M *et al.*: Novel receptor partners and function of receptor activity-modifying proteins. *J Biol Chem* 278(5), 3293-3297 (2003).
32. Harikumar KG, Simms J, Christopoulos G, Sexton PM, Miller LJ: Molecularbasisofassociationof receptor activity-modifying protein 3 with the family B G protein–coupled secretin receptor. *Biochemistry* 48, 11773–11785 (2009).
33. Huang C, Miller RT: The calcium-sensing receptor and its interacting proteins. *J Cell Mol Med* 11(5), 923–934 (2007).
34. Desai AJ, Roberts DJ, Richards GO, Skerry TM: Role of Receptor Activity Modifying Protein 1 in Function of the Calcium Sensing Receptor in the Human TT Thyroid Carcinoma Cell Line. *PLOS ONE* 9(1), (2014).
35. Lin HY, Harris TL, Flanery MS *et al.*: Expression cloning and characterization of a porcine renal calcitonin receptor. *Science* 254(5034), 1022-1024 (1991).
36. Masi L, Brandi ML: Calcitonin and calcitonin receptors. *Clin Cases Miner Bone Metab* 4(2), 117–122 (2007).
37. Sexton PM, Houssami S, Hilton JM *et al.*: Identification of brain isoforms of the rat calcitonin receptor. *Mol Endocrinol* 7(6), 815-821 (1993).
38. Muff R, Bühlmann N, Fischer JA, Born W: An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. *Endocrinology* 140(6), 2924-2927 (1999).
39. Hay DL, Christopoulos G, Christopoulos A, Sexton PM: Amylin receptors: molecular composition and pharmacology. *Biochemical Society Transactions* 32(5), 865-867 (2004).
40. Gingell JJ, Simms J, Barwell J *et al.*: An allosteric role for receptor activity-modifying proteins in defining GPCR Pharmacology. *Cell Discovery*, (In Press).
41. Johansson E, Hansen JL, Hansen AM *et al.*: Type II Turn of Receptor-bound Salmon Calcitonin Revealed by X-ray Crystallography. *J Biol Chem*, (2016).

42. Lee SM, Hay DL, Pioszak AA: Calcitonin and Amylin Receptor Peptide Interaction Mechanisms: INSIGHTS INTO PEPTIDE-BINDING MODES AND ALLOSTERIC MODULATION OF THE CALCITONIN RECEPTOR BY RECEPTOR ACTIVITY-MODIFYING PROTEINS. *J Biol Chem* 291(16), 8686-8700 (2016).
43. Sekiguchi T, Kuwasako K, Ogasawara M *et al.*: Evidence for Conservation of the Calcitonin Superfamily and Activity-regulating Mechanisms in the Basal Chordate Branchiostoma floridae: INSIGHTS INTO THE MOLECULAR AND FUNCTIONAL EVOLUTION IN CHORDATES. *J Biol Chem* 291(5), 2345-2356 (2016).
44. Udawela M, Christopoulos G, Tilakaratne N, Christopoulos A, Albiston A, Sexton PM: Distinct Receptor Activity-Modifying Protein Domains Differentially Modulate Interaction with Calcitonin Receptors. *Mol Pharmacol* 69, 1984–1989 (2006).
45. Taché Y, Martinez V, Wang L, Million M: CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. *British Journal of Pharmacology* 141, 1321–1330 (2004).
46. Unson CG: Molecular determinants of glucagon receptor signaling *Biopolymers (Peptide Science)* 66, 218–235 (2002).
47. Dowell SJ, Brown AJ: Yeast assays for G-protein-coupled receptors. *Receptors Channels* 8, 343-352 (2002).
48. Walsh NA, Yusta B, Dacambra MP, Anini Y, Drucker DJ, Brubaker PL: Glucagon-like peptide-2 receptor activation in the rat intestinal mucosa. *Endocrinology* 144(10), 4385 – 4392 (2003).
49. Guan X, Karpen HE, Stephens J *et al.*: GLP-2 Receptor Localizes to Enteric Neurons and Endocrine Cells Expressin Vasoactive Peptides and Mediates Increased Blood Flow. *Gastroenterology* 130(1), 150-164 (2006).
50. Pioszak AA, Xu HE: Molecular recognition of parathyroid hormone by its G protein-coupled receptor. *PNAS* 105(13), 5034-5039 (2008).
51. Gensure RC, Gardella TJ, Juppner H: Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. *Biochem Biophys Commum* 328(3), 666-678 (2005).
52. Miller LJ, Dong M, Harikumar KG: Ligand binding and activation of the secretin receptor, a prototypic family B G protein-coupled receptor. *Br J Pharmacol* 166(1), 18–26 (2012).
53. Ishihara T, Nakamura S, Kaziro Y, Takahashi T, Takahashi K, Nagata S: Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J* 10, 1635–1641 (1991).
54. Afroze S, Meng F, Jensen K *et al.*: The physiological roles of secretin and its receptor. *Annals of translational medicine* 1(3), (2013).
55. Garcia GL, Dong M, Miller LJ: Differential determinants for coupling of distinct G proteins with the class B secretin receptor *Am J Physiol Cell Physiol* 302, 1202–1212 (2012).
56. Couvineau A, Ceraudo E, Tan Y-V, Nicole P, Laburthe M: The VPAC1 receptor: structure and function of a class B GPCR prototype *Front Endocrinol* 3(139), (2012).

57. Ishihara T, Shigemoto R, Mori K, Takahashi K, Nagata S: Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* 8, 811–819 (1992).
58. Dickson L, Aramori I, Mcculloch J, Sharkey J, Finlayson K: A systematic comparison of intracellular cyclic AMP and calcium signalling highlights complexities in human VPAC/PAC receptor pharmacology. *Neuropharmacology* 51, 1086–1098 (2006).
59. Dickson L, Finlayson K: VPAC and PAC receptors: From ligands to function *Pharmacology & Therapeutics* 121(3), 294–316 (2009).
60. Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ: The VIP2 receptor: Molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett* 334(1), 3–8 (1993).
61. Barrick CJ, Lenhart PM, Dackor RT, Nagle E, Caron KM: Loss of receptor activity-modifying protein 3 exacerbates cardiac hypertrophy and transition to heart failure in a sex-dependent manner *J Mol Cell Cardiol* 52(1), 165–174 (2012).
62. Bouschet T, Martin S, Henley JM: Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane. . *Journal of Cell Science* 118, 4709–4720 (2005).
63. Bouschet T, Martin S, Henley JM: Regulation of calcium sensing receptor trafficking by RAMPs. *Advances in experimental medicine and biology* 744, 39–48 (2012).
64. Kurashige C, Hosono K, Matsuda H, Tsujikawa K, Okamoto H, Majima M: Roles of receptor activity-modifying protein 1 in angiogenesis and lymphangiogenesis during skin wound healin in mice. *The FASEB Journal* 28(3), 1237-1247 (2014).
65. Igarashi K, Sakurai T, Kamiyoshi A *et al.*: Pathophysiological roles of adrenomedullin-RAMP2 system in acute and chronic cerebral ischemia. *Peptides* 62, 21-31 (2014).
66. Zhao LH, Yin Y, Yang D *et al.*: Differential Requirement of the Extracellular Domain in Activation of Class B G Protein-Coupled Receptors. *J Biol Chem*, (In press).

Highlights

- RAMPs have been shown to interact predominantly with Family B GPCRs, and recently with Family A and Family C GPCRs.
- RAMPs enhance trafficking to the cell surface of several GPCRs
- RAMPs can alter GPCR signalling including enhancing coupling to certain G-proteins.