BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 1059–1068

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: MOLECULAR PHARMACOLOGY OF G PROTEIN-COUPLED RECEPTORS

REVIEW

Structure–function relationships of the N-terminus of receptor activity-modifying proteins

Tao Qi and Debbie L Hay

School of Biological Sciences, University of Auckland, Auckland, New Zealand

The receptor activity-modifying proteins (RAMPs) are a family of three single transmembrane proteins that have been identified as accessory proteins to some G-protein-coupled receptors (GPCRs). They can regulate their pharmacology, forward trafficking and recycling, depending on the GPCR. The best characterized receptor complexes formed by RAMPs and GPCRs are the calcitonin peptide family receptors. The association of RAMP1 with the calcitonin receptor-like receptor (CL) constitutes the calcitonin gene-related peptide receptor, whereas RAMP2 or 3 with CL generates adrenomedullin receptors. In this case, the RAMPs substantially alter the pharmacology and trafficking properties of this GPCR. Amylin receptor subtypes are formed from calcitonin receptor (CTR) interactions with RAMPs. Although the RAMPs themselves are not responsive to calcitonin peptide family ligands, there is clear evidence that they participate in ligand binding, although it is still unclear whether this is by directly participating in binding or through allosteric modulation of CL or CTR. A considerable amount of mutagenesis data have now been generated on RAMPs to try and identify the residues that play a role in ligand interactions, and to also identify which residues in RAMPs interact with CL and CTR. This review will focus on RAMP mutagenesis studies with CL, summarizing and discussing the available data in association with current RAMP models and structures. The data reveal key regions in RAMPs that are important for ligand binding and receptor interactions.
British Journal of Pharmacology (2010) **159,** 1059–1068; doi:10.1111/j.1476-5381.2009.00541.x; published online 10 December 2009

This article is part of a themed section on Molecular Pharmacology of GPCR. To view the editorial for this themed section visit http://dx.doi.org/10.1111/j.1476-5381.2010.00695.x

Keywords: receptor activity-modifying proteins; calcitonin receptor; calcitonin receptor-like receptor; calcitonin; calcitonin gene-related peptide; adrenomedullin; amylin; mutagenesis

Abbreviations: AM, adrenomedullin; AM2, intermedin; Amy, amylin; AMY, amylin receptor phenotype; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5amino-l-[[4-(4-pyridinyl)-l-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl); CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; GPCR, G-protein-coupled receptor; h, human; r, rat; RAMP, receptor activity-modifying protein; WT, wild type

Introduction

The calcitonin family of peptides includes calcitonin, calcitonin gene-related peptide (CGRP), adrenomedullin (AM), AM2 (also known as intermedin) and amylin (Amy). Genetic models, combined with traditional pharmacological techniques, have revealed roles for these peptides in a wide range of processes including bone formation, nutrient intake, vasodilation, cardioprotection, pain perception, neurogenic inflammation, angiogenesis and lymphangiogenesis. Thus, the family is of considerable clinical potential, and the receptors for these peptides are important drug targets.

The receptors for these peptides belong to family B (secretin-like) G-protein-coupled receptors (GPCRs), namely the calcitonin receptor (CTR) and calcitonin receptor-like receptor (CL). However, for most members of the calcitonin peptide family, the presence of another protein is required to form a functional receptor with CL or CTR at the cell surface. This protein is known as a receptor activity-modifying protein

Correspondence: Debbie Hay, School of Biological Sciences, University of Auckland, 3A Symonds Street, Private Bag 92019, Auckland, New Zealand. E-mail: dl.hay@auckland.ac.nz
Received 30 May 2009; revised 13 August 2009; accepted 16 September 2009

(RAMP). In 1998, McLatchie and colleagues isolated a single cDNA that encoded a 148-amino-acid protein during attempts to expression-clone the gene encoding the human CGRP receptor from cells derived from a human neuroblastoma (SK-N-MC cells) (McLatchie *et al.*, 1998). Xenopus oocytes expressing this protein gave rise to a large concentration-dependent response to CGRP, compared with the endogenous response. This protein was named RAMP1, which together with CL reconstituted a functional CGRP receptor (McLatchie *et al.*, 1998). Co-expression of these two components is obligatory for the full functionality of a CGRP receptor, as neither of them induce a significant response to CGRP when transfected alone (McLatchie *et al.*, 1998). Besides human RAMP1, another two proteins belonging to the RAMP family were also cloned, which were named as RAMP2 (cloned from SK-N-MC cells) and RAMP3 (cloned from human spleen) (McLatchie *et al.*, 1998). CL expressed with RAMP2 or 3 generates receptors for AM (AM_1 and AM_2, respectively), a relative of CGRP. Thus, the interaction between RAMPs and receptors confers ligand specificity. Among the calcitonin family peptides, calcitonin is the only known ligand that does not require RAMP to bind to its receptor. Co-expression of a RAMP molecule with CTR, however, generates AMY receptors. Note that the abbreviation Amy denotes the peptide, but AMY denotes the receptor for this peptide. The pharmacology of these receptors has been investigated in different cell types, which has been extensively reviewed (Sexton *et al.*, 2001; Poyner *et al.*, 2002; Kuwasako *et al.*, 2004; Hay *et al.*, 2006b). Table 1 summarizes the molecular composition of the calcitonin peptide family receptors and their pharmacological profiles.

Interestingly, the modulating role of RAMPs is not limited to calcitonin family peptide-binding events. The fact that RAMPs are more widely distributed across cell and tissue types than CTR or CL suggests additional roles for RAMPs (McLatchie *et al.*, 1998). There is now evidence that at least four other receptors couple with RAMPs. They are the VPAC1 receptor (vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide, with all three RAMPs), PTH-1 receptor (parathyroid hormone, with RAMP2) and PTH-2 receptor (with RAMP3) (Christopoulos *et al.*, 2003). In addition, RAMP1 and RAMP3 have also been reported to complex with a family C GPCR, the calcium-sensing receptor. This association is required for receptor trafficking (Bouschet *et al.*, 2005). Indeed, being ubiquitously expressed in many cells, it will not be surprising to identify RAMPs coupling with not only more GPCRs, but also other proteins. For example, an interaction between RAMP1 and β-tubulin has been reported, suggesting broader biological roles for RAMPs than as GPCR accessory proteins (Kunz *et al.*, 2007).

All three human RAMPs share a common structure: a short intracellular C-terminus (~9 amino acids), a single transmembrane (TM) domain (~22 amino acids) and a long extracellular N-terminus (~91 amino acids for RAMP1 and RAMP3, ~102 amino acids for human RAMP2). In addition, there is a predicted signal sequence of ~26 to ~44 amino acids at the N-terminus. While the N-terminus is the major determinant of receptor pharmacology (Fraser *et al.*, 1999; Zumpe *et al.*, 2000), the TM and C-terminus have been suggested to be important in RAMP–receptor interactions (Zumpe *et al.*, 2000; Udawela *et al.*, 2006b) and intracellular signalling for AMY receptors (Udawela *et al.*, 2006a; Morfis *et al.*, 2008) respectively. Furthermore, the C-terminus of RAMP also possesses some specific functional features including an endoplasmic reticulum retention signal in human RAMP1 and type-1 PDZ1 recognition sequence in human RAMP3, which directs CL/RAMP3 complexes to recycling (Kuwasako *et al.*, 2000; Hilairet *et al.*, 2001a; Steiner *et al.*, 2002; Bomberger *et al.*, 2005). Studies on the TM region and C-terminus of RAMPs have been reviewed elsewhere (Hay *et al.*, 2006b; Parameswaran and Spielman, 2006; Sexton *et al.*, 2009). This review will therefore focus on the N-terminus of RAMPs.

Early experimental evidence indicated that the N-terminus of RAMPs contributes to the peptide-binding pocket together with the N-terminus of CL, as cross-linking using ^{125}I-AM and ^{125}I-CGRP showed that the RAMPs located close to the peptide-binding pocket in the CL/RAMP complexes (Hilairet *et al.*, 2001b). However, it is still an open question as to whether RAMPs modulate receptor pharmacology either by directly providing points of contact or allosterically altering the receptor structure to allow selective ligand binding. Therefore, any effect observed in mutagenesis studies for RAMPs is likely to reflect an impact on the overall receptor complex. Therefore, functional data for RAMP mutants should not be considered in isolation, but as part of a receptor complex.

So far, the molecular analysis of RAMPs has mostly been limited to their ability to form CGRP and AM receptors. There is also some information on how AMY receptors are affected by residue substitutions, but the amount of information available is too sparse to draw any meaningful conclusions. In the interests of brevity, only CGRP and AM receptors (i.e. CL/RAMP complexes) will therefore be discussed in this review. A pattern is emerging of key regions involved in conferring pharmacological specificity and receptor interactions.

Table 1 Molecular composition and pharmacological profiles of human CGRP, AM, calcitonin and AMY receptors (Hay *et al.*, 2006b)

Receptor type	*Molecular composition*	*Pharmacological profiles*
CGRP receptor (CGRP)	CL + RAMP1	αCGRP > AM > rAmy
Adrenomedullin receptor (AM_1)	CL + RAMP2	AM > αCGRP > rAmy
Adrenomedullin receptor (AM_2)	CL + RAMP3	AM > αCGRP > rAmy
Calcitonin receptor (CTR)	CTR alone	hCT > rAmy, αCGRP > AM
Amylin receptor (AMY_1)	CTR + RAMP1	rAmy > αCGRP > hCT > AM
Amylin receptor (AMY_2)	CTR + RAMP2	Poorly defined
Amylin receptor (AMY_3)	CTR + RAMP3	rAmy > αCGRP > AM

The nomenclature conforms to Alexander *et al.* (2008). CT, calcitonin.

The structure of RAMPs

The primary amino acid sequences of the three human RAMPs reveal about 31% identity and about 56% similarity to each other (Figure 1). Across species, the TM regions show high conservation, while N-termini are less conserved. This high degree of variability in the N-terminus suggests that this region is most likely to contribute to the pharmacological specificity that these proteins engender. However, within each RAMP, the sequence homology in the N-terminus is considerably greater across species. Despite low N-terminal sequence homology, all RAMPs across species contain four conserved cysteine residues, which presumably form disulphide bonds, suggesting a common secondary structure.

Before any RAMP structure was available, attempts were made to predict the structural characteristics of RAMPs. The first human RAMP1 structure was modelled using an *ab initio* method, where an *ab initio* modelling protocol was developed for the structural prediction of RAMP family proteins (Simms *et al.*, 2006). The secondary structure was obtained by a consensus from two prediction routines. These were selected based on their ability to predict the secondary structure of 16 diverse, small, helical, disulphide bond-containing peptides of known structure. The structure predicted from this *ab initio* model suggested that the human RAMP1 N-terminus comprised three α-helices (α-helix 1, α-helix 2 and α-helix 3), and that residues 118–139 form a small TM domain. The disulphide bonding pattern predicted by this model was tested using site-directed mutagenesis at cysteine residues where the arrangement of disulphide bonds was determined using double mutants of every cysteine pair (Simms *et al.*, 2006).

Another RAMP structural model was later built by Benítez-Páez from a primary sequence alignment of 38 RAMPs (1–3) across different species that were retrieved from a PSI blast search with a human RAMP1 sequence lacking the signal peptide (Benítez-Páez, 2006). The model predicted in this study had a similar organization as the *ab initio* model generated by Simms and colleagues, but with some differences including the disulphide bonding pattern and the orientation of α-helix 3. The author later published a follow-up study where phylogenetic and statistical analyses were carried out on the RAMPs across different species (Benítez-Páez and Cárdenas-Brito, 2008). The study suggested a collection of residues that could potentially be functionally important, many of which were found in the extracellular domains of RAMPs. The TM helix of RAMP3 was also modelled in this study. Interestingly, some of the residues that were predicted to be important for receptor function from their analyses are localized on the TM helix (Benítez-Páez and Cárdenas-Brito, 2008).

The tri-helical organization stabilized by three disulphide bonds for the human RAMP1 N-terminus [residues 27–107 ($RAMP1_{27–107}$)] was confirmed by a crystal structure (Kusano *et al.*, 2008) (Figures 1 and 2A). This indicates that α-helix 1 is formed by residues E29 to V51, and is kinked at residue L39. It is followed by a small helical structure consisting of E53 to L55, which sits within the loop connecting α-helices 1 and 2. α-helix 2 is anti-parallel to the other two helices, starting at W59 and ending at L80, whereas α-helix 3 is formed by residues from A87 to Y100. The interacting residues forming the three disulphide bonds predicted by Simms *et al.* were confirmed in the crystal structure as C27–C82, C40–C72 and C57–C104. The structure is maintained by multiple hydrophobic interaction sites, which mostly locate between α-helices 1 and 2.

Based on the electrostatic potential distribution on the solvent accessible surface of the crystal structure of the human RAMP1 N-terminus, a hydrophobic patch located on α-helices 2 and 3 was identified (Kusano *et al.*, 2008). Residues that are likely to constitute the receptor (CL) and ligand (CGRP) binding interfaces were proposed from this area. F93, H97 and F101 were proposed to form the CL binding interface, whereas residues R67, D71, W74, E78 and W84 were predicted to constitute part of the ligand (CGRP) binding pocket. The residues which are thought to form the CL binding interface as identified in the crystal structure are highly conserved across RAMPs, supporting the notion that these residues have an important role in RAMP function. The contribution of some of these residues to either receptor or ligand interaction has been tested experimentally; these studies are discussed below. Nevertheless, further experimental evidence is needed to confirm these assignations.

Despite the low sequence identities between three RAMPs, they are believed to adopt a similar fold. This prediction is supported in the sequence comparison that shows that the

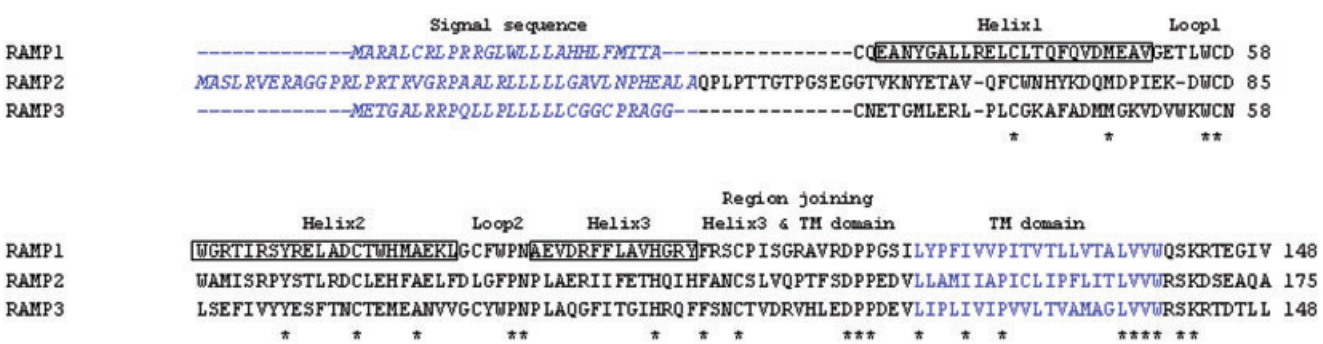

Figure 1 Amino acid sequence alignment of the three human RAMPs. The predicted signal sequence (italic font) and TM region are shown in blue. The boxes indicate residues comprising the three α-helices with loop regions in between based on the crystal structure of RAMP1 N-terminus (Kusano *et al.*, 2008). The asterisk symbols indicate those residues that are conserved among the three RAMPs beyond the signal sequence.

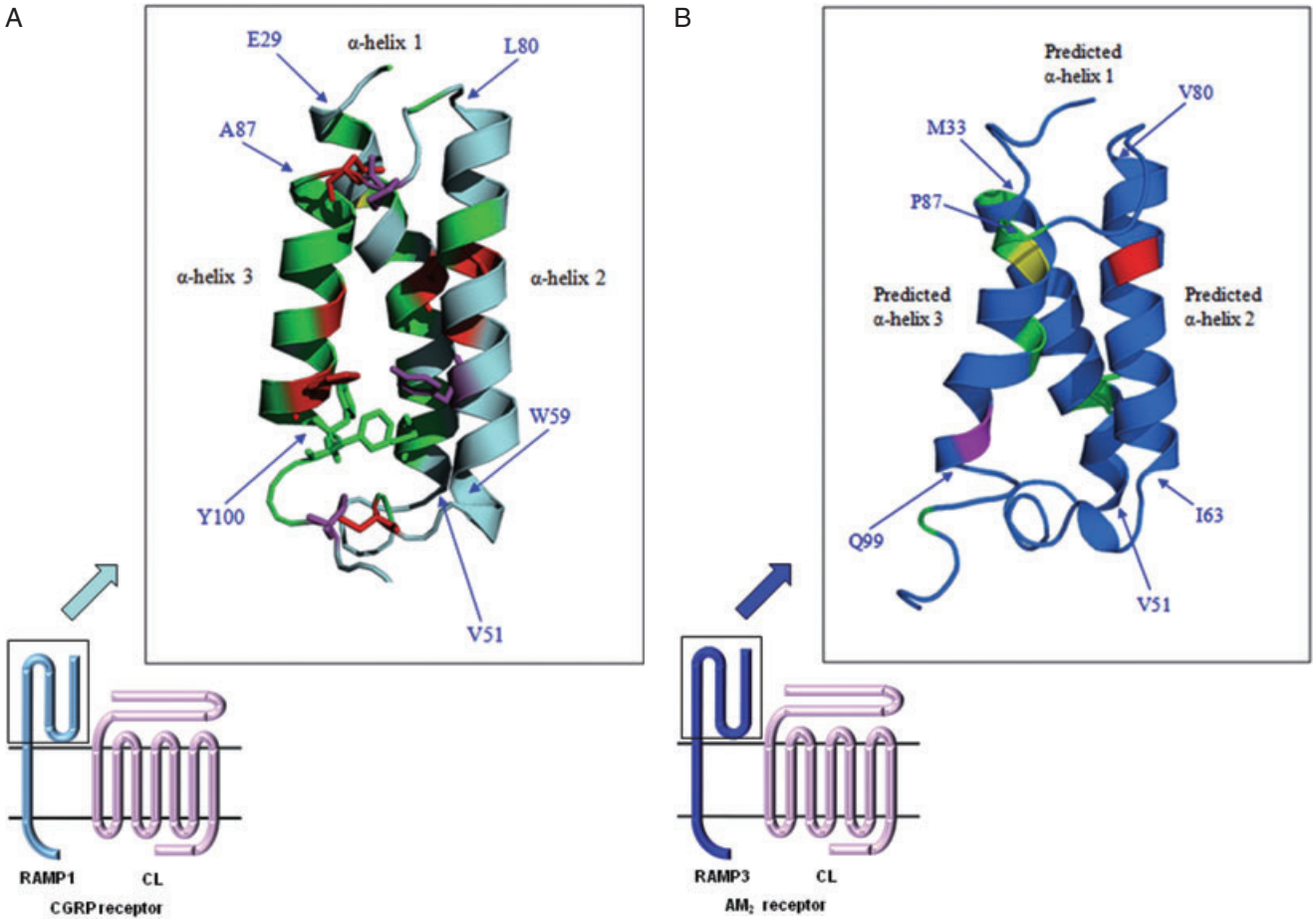

Figure 2 (A) Schematic representation of CGRP receptor. The expanded portion shows the crystal structure of the human RAMP1 N-terminus ($RAMP1_{27–107}$). The structure is taken from Kusano *et al.* (2008). (B) Schematic representation of AM_2 receptor. The expanded portion shows the structure of the human RAMP3 N-terminus ($RAMP3_{28–107}$) as modelled by Bailey *et al.* (R.J. Bailey *et al.*, unpublished). Residues at the boundaries of each helix are labelled on the diagrams. Differential colouring reflects the effects of point mutations introduced at a number of residues that have been characterized for either CGRP responses at the CGRP receptor (A) or AM responses at the AM_2 receptor (B). Green, peptide potency was not affected; red, peptide potency was reduced by <100-fold; purple, peptide potency was reduced by >100-fold; yellow, peptide potency was enhanced by <100-fold. In addition, residues shown in stick form indicate that the point mutations also reduced cell surface expression of the receptor complex. Pale blue (A) or dark blue (B) represents the residues that have not been characterized by point mutation in RAMP1 and RAMP3 respectively.

two disulphide bonds, which have a more important role in stabilizing the RAMP1 structure (C40–C72 and C57–C104), are conserved across three RAMPs. Further, the equivalent sites in RAMPs 2 and 3 to the intramolecular interaction sites identified in the crystal structure of RAMP1 extracellular domain are also mostly hydrophobic (Kusano *et al.*, 2008). A homology model for $hRAMP3_{28–107}$ has been produced based on the RAMP1 crystal structure (R.J. Bailey *et al.*, unpublished) (Figure 2B). This model provides an indication of how the structures of RAMP1 and RAMP3 compare, but a crystal structure of RAMP3 is needed to confirm similarities and differences between these proteins.

Beyond the valuable insight provided by the RAMP1 crystal structure, as mentioned earlier, the N-terminus of the RAMP has been shown experimentally to be the major determinant of receptor pharmacology, in conjunction with CL or CTR (Fraser *et al.*, 1999; Zumpe *et al.*, 2000; Hilairet *et al.*, 2001b; Udawela *et al.*, 2006b). It is thought that the RAMP and receptor work together to form the appropriate binding site for the individual ligands (Sexton *et al.*, 2009). Several studies have been carried out on this region, aiming to elucidate the residues that are important for receptor function and expression. In the following sections, mutagenesis data available from the literature for RAMPs are summarized and discussed in conjunction with the newly available structural information (for RAMPs 1 and 3).

RAMP1 N-terminus

Among the three RAMPs, RAMP1 is the best understood member, mainly with CL, as the CGRP receptor. The importance of the RAMP1 N-terminus in conferring receptor pharmacology was first demonstrated in a study employing RAMP1/RAMP2 chimeras (Fraser *et al.*, 1999). The respective roles of individual residues or regions within the RAMP1 N-terminus are inferred from subsequent studies employing individual amino acid substitutions, deletion mutants and

chimeras between RAMP1 and RAMP2. Overall, the mutations that induced a change in either CGRP receptor function or expression generally locate in α-helices 2 and 3 of the RAMP1 N-terminus, which suggests that α-helices 2 and 3 are the major determinants of CL and CGRP interactions (e.g. Y66, L69, T73, H97; see below).

Helix 1 and loop 1 (joining helices 1 and 2)

There have been attempts to identify important regions within helix 1 and loop 1 using deletion or chimera mutants. Several such mutants (deletions 28–33, 34–39, 41–45, 46–50, 51–55 and RAMP1/2 chimera 27–50) generally resulted in reduced peptide (hAM and hαCGRP) responses, which were mostly paralleled by some degree of loss in receptor expression at the cell surface (Kuwasako *et al.*, 2001; 2003a). Site-directed mutagenesis has been, in addition, employed to study the role of individual residues in RAMP1 to CGRP receptor function in these regions. These mutants and their effects are summarized in Table 2 and Figure 2A. Apart from the mutations introduced at two (C40 and C57) of the three cysteine residues (C27, C40 and C57), which showed a reduced CGRP potency and reductions in cell surface expression, the rest of the point mutations introduced in these regions elicited little effect in either CGRP response or cell surface expression. The data together indicate that α-helix 1 of RAMP1 may have only a minor role in directly contributing to CL and CGRP interactions.

Helix 2 and loop 2 (joining helices 2 and 3)

Several small deletion mutants covering regions 59–65, 67–71, 74–76, 78–80 and 83–86 in α-helix 2 and loop 2 have been characterized for their effects on CGRP and AM responses (Kuwasako *et al.*, 2001; 2003a). Although the data generated from these mutants are difficult to interpret as the change in pharmacology may have partially been a result of loss in cell surface expression, it has been suggested that residues 78–80 may specifically contribute to AM pharmacology as the CGRP receptor containing the deletion had a greater loss in AM than CGRP responses (Kuwasako *et al.*, 2003a). Regions 78–80 reside at the end of α-helix 2, and residues within this region lie either on or close to the ligand-binding pocket proposed from the crystal structure of the RAMP1 N-terminus. Thus, the differential effects observed with this deletion mutant on AM and CGRP responses may suggest different contributions of these residues to the AM and CGRP binding pockets, either in a direct or indirect manner.

Point mutants generated in α-helix 2 and loop 2 have provided some experimental support for the residues that may be important for receptor/ligand interactions (Table 2 and Figure 2A). W74 is the only residue that has been studied among the residues proposed to be important for CGRP interactions by the crystal structure. Although W74 has been demonstrated to be important for the high affinity and selectivity of the antagonist BIBN4096BS for the human CGRP receptor, it did not affect CGRP potency when mutated to alanine, lysine or glutamic acid (Mallee *et al.*, 2002; Hay *et al.*, 2006a; Qi *et al.*, 2008). These data suggest that W74 may be in the vicinity of CGRP binding residues, but that it is not directly involved in binding CGRP.

Some neighbouring residues of the proposed CGRP binding interface showed the importance of this region to CGRP receptor function. Mutant Y66A almost completely abolished CL trafficking, suggesting that considerable structural perturbation was introduced by the mutation and that Y66 may also directly contribute to the CL binding interface (Simms *et al.*, 2009). In contrast, L69A and T73A reduced CGRP potency without affecting the cell surface expression, indicating that they may contribute to CGRP interactions, most likely in an indirect manner given their position (not solvent exposed) in the crystal structure.

Helix 3 and region joining helix 3 and TM domain

A study employing deletion mutants generated within α-helix 3 and region joining helix 3 and TM domain (88–90, 91–94, 96–100, 101–103, 105–107, 109–112 and 113–118) has suggested that 91–103 might be important for conferring high-affinity CGRP binding, whereas residues 88–90 may specifically contribute to AM pharmacology (Kuwasako *et al.*, 2003a). Interestingly, when locating these residues in the crystal structure, residues 91–103 overlie the proposed CL interaction site. This indicates that the change in pharmacology observed was more likely to be related to the loss in the cell surface expression (as reported in the original study), resulting from disruption of the CL binding interface. Regions 88–90 reside at the beginning of α-helix 3, opposite but still in close proximity to the proposed ligand-binding pocket by the crystal structure. Thus, the region may contribute to the AM-binding pocket in either a direct or indirect manner.

Numerous point mutations that cover most of the residues in α-helix 3 and region joining helix 3 and TM domain have been characterized (Table 2 and Figure 2A). These data provide experimental support for some of the residues that have been suggested to be important for CL or CGRP interactions from the RAMP1 crystal structure. F93A RAMP1/CL in one study showed significantly reduced cell surface expression, but the CGRP response was not changed (Simms *et al.*, 2009). In another study, reduced total CGRP binding was further observed at F93A RAMP1/CL (Kuwasako *et al.*, 2003a). On the other hand, F93I led to reduced CGRP potency in the absence of any change in cell surface expression (Qi *et al.*, 2008). It, therefore, seems unlikely that F93 is directly involved in constituting a CL binding interface as suggested by Kusano *et al.* (2008). H97, which was also predicted to locate at the CL binding interface by the crystal structure, caused a significant reduction in both cell surface expression and CGRP-evoked cAMP production when mutated to alanine (Simms *et al.*, 2009). In another study, H97A led to a significantly reduced total CGRP binding, and small reductions in CGRP potency and cell surface expression (Kuwasako *et al.*, 2003a). These observations are broadly consistent with each other, generally supporting the importance of H97 in CL association. Another residue at the proposed CL binding interface, F101, showed an interesting response when

Table 2 Summary of mutagenesis data for the human RAMP1 N-terminus, expressed with CL

Mutation	*Effect*	*Cell type*	*Reference*
Helix 1			
C27A	↔hαCGRP potency	Cos7	Simms *et al.* (2006)
N31A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
A34E	↑hαCGRP potency, ↑hαCGRP E_{max}, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
L35A	↔hαCGRP potency, ↔ CSE	Cos7	Simms *et al.* (2009)
E38A	↔hαCGRP potency, ↑hαCGRP E_{max}, ↔ CSE	Cos7	Simms *et al.* (2009)
C40A	↓hαCGRP potency	Cos7	Simms *et al.* (2006)
C40A	↓↓CSE	Cos7	Simms *et al.* (2009)
L41A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
T42A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
Q45A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
V46D	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
V46A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
M48A	↔hαCGRP, ↔hAM potency, ↓CSE	Cos7	Simms *et al.* (2009)
E49A	↔hαCGRP potency, ↔ CSE	Cos7	Simms *et al.* (2009)
Loop 1			
W56A	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Simms *et al.* (2009)
C57A	↓hαCGRP potency	Cos7	Simms *et al.* (2006)
C57A	↓↓CSE	Cos7	Simms *et al.* (2009)
Helix 2			
Y66A	↓↓hαCGRP response, ↓↓CSE	Cos7	Simms *et al.* (2009)
L69A	↓hαCGRP potency, ↔ CSE	Cos7	Simms *et al.* (2009)
C72A	↓hαCGRP potency	Cos7	Simms *et al.* (2006)
C72A	↓↓CSE	Cos7	Simms *et al.* (2009)
T73A	↓hαCGRP potency, ↔ CSE	Cos7	Simms *et al.* (2009)
W74A	↔hαCGRP potency, ↔hβCGRP potency, ↔ CSE	Cos7	Hay *et al.* (2006a)
W74K	↔hαCGRP potency, ↔hβCGRP potency, ↔ CSE	Cos7	Hay *et al.* (2006a)
W74E	↔hαCGRP potency; ↑hAM potency, ↑$AM_{15–52}$, ↑AM2 potency, ↔ CSE, ↑$AM_{13–52}$-specific binding and affinity	Cos7	Qi *et al.* (2008)
Loop 2			
C82A	↔hαCGRP potency	Cos7	Simms *et al.* (2006)
P85A	↓hαCGRP potency, ↓hαCGRP E_{max}, ↓CSE	Cos7	Simms *et al.* (2009)
Helix 3			
N86A	↓hαCGRP potency, ↓CSE	Cos7	Simms *et al.* (2009)
A87P	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
E88L	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
V89A	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
D90A	↔hαCGRP potency, ↔CSE	Cos7	Simms *et al.* (2009)
R91A	↔hαCGRP potency, ↔ total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
F92A	↔hαCGRP potency, ↓ total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
F93I	↓hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
F93A	↔hαCGRP potency, ↓CSE	Cos7	Simms *et al.* (2009)
F93A	↔hαCGRP potency, ↓ total hαCGRP binding, ↓CSE	HEK293	Kuwasako *et al.* (2003a)
L94A	↔ hαCGRP potency, ↑hαCGRP E_{max}, ↔CSE	Cos7	Simms *et al.* (2009)
L94A	↔hαCGRP potency, ↑total hαCGRP binding, ↑CSE	HEK293	Kuwasako *et al.* (2003a)
V96A	↔hαCGRP potency, ↔ total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
H97A	↓hαCGRP potency, ↓hαCGRP E_{max}, ↓↓CSE	Cos7	Simms *et al.* (2009)
H97A	↔hαCGRP potency, ↓ total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
G98A	↔hαCGRP potency, ↑total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
R99A	↔hαCGRP potency, ↔ total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
Y100A	↔hαCGRP potency, ↓ total hαCGRP binding, ↓CSE	HEK293	Kuwasako *et al.* (2003a)
Region joining helix 3 and TM domain			
F101A	↔hαCGRP potency, ↓hαCGRP E_{max}, ↓CSE	Cos7	Simms *et al.* (2009)
F101A	↔hαCGRP potency, ↓↓total hαCGRP binding, ↓CSE	HEK293	Kuwasako *et al.* (2003a)
R102A	↔hαCGRP potency, ↔total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
S103A	↔hαCGRP potency, ↔total hαCGRP binding, ↔CSE	HEK293	Kuwasako *et al.* (2003a)
S103N	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
C104A	↓↓hαCGRP response, ↓↓total hαCGRP binding, ↓↓CSE	HEK293	Kuwasako *et al.* (2003a)
C104A	↓hαCGRP potency	Cos7	Simms *et al.* (2006)
C104A	↓↓CSE	Cos7	Simms *et al.* (2009)
D113A	↓hαCGRP potency,↔CSE	Cos7	Simms *et al.* (2009)
P114A	↓hαCGRP potency, ↓CSE	Cos7	Simms *et al.* (2009)
P115A	↔hαCGRP potency, ↓CSE	Cos7	Simms *et al.* (2009)

CSE, cell surface expression; ↔, unchanged compared to WT; ↓, reduced compared to WT; ↑, increased compared to WT; ↓↓, reduced to basal level; E_{max}, maximum response of agonist-evoked cAMP production (not changed, unless specified in the table).

mutated to alanine. Cell surface expression and CGRP binding were significantly reduced, accompanied by a reduced maximum response of CGRP-evoked cAMP production; however, CGRP potency was not affected (Kuwasako *et al.*, 2003a; Simms *et al.*, 2009). It is likely that F101A reduced the ability of RAMP1 to associate with CL, which supports the involvement of F101 in constituting the CL binding interface; however, the receptor function seemed to be retained or even enhanced at F101A mutant receptors.

In general, the residues identified on the crystal structure which have been tested thus far are clearly of importance for either CL interaction or ligand binding. Further experimental data are needed to define the exact residues that are involved in specific high-affinity ligand binding.

RAMP2 N-terminus

The structure–function relationships in the RAMP2 N-terminus are less well defined. Consistent with RAMP1, large chimeras involving exchanging the entire N-terminus of between RAMPs 1 and 2 suggest that the receptor pharmacology is mainly determined by the N-terminus of RAMP2 (Fraser *et al.*, 1999). Data generated from some smaller RAMP1/2 chimeras have narrowed down the pharmacology-determining region to 77–101 (Kuwasako *et al.*, 2001). Some deletion mutants within this region were also studied (deletions 66–69, 70–75, 76–78, 79–82, 83–85, 86–89, 86–92, 90–92, 93–96, 97–99 and 100–103); however, changes observed with the functional data were mostly paralleled by the poor cell surface expression of the mutant AM_1 receptors (Kuwasako *et al.*, 2001). This suggests that these mutations may have introduced gross alterations in the overall receptor structure. Nevertheless, the authors speculated that regions 86–92 were critical for peptide binding (Kuwasako *et al.*, 2001). An alanine scan was additionally performed at residues 86–92, but none of the individual mutations caused a change in the AM-evoked response (Table 3). In addition, the equivalent regions in rat RAMP2 (93–99) have been demonstrated to be important for AM affinity and potency in the rat AM_1 receptor (Kuwasako *et al.*, 2002). Deletion of this region had a significant impact on both AM binding and potency when co-expressed with CL.

Site-directed mutagenesis has also been carried out to investigate the role of individual residues in RAMP2 to AM_1 receptor function. The importance of the extracellular cysteine residues has been demonstrated by alanine substitution. The four mutant receptors containing C68A, C84A, C99A and C131A RAMP2 all failed to be transported and expressed at the cell surface, and thus failed to respond to AM (Kuwasako *et al.*, 2003b). This is likely to be due to destabilization of the RAMP2 structure. Besides cysteines, the extracellular histidine residues have also been studied for their involvement in AM binding (Kuwasako *et al.*, 2008). H124A and H127A seemed to alter the overall protein structure that led to poor cell surface expression, and failure to bind or respond to AM. H124, which is conserved among the RAMP family (H97 in RAMPs 1 and 3), is suggested to be directly involved in forming the interaction site with CL, as H97 in RAMP1 is identified as one of the three residues that constitute the CL binding interface (Kusano *et al.*, 2008). H127 is also likely to contribute to the CL binding interface, as it resides in close proximity to H124; the mutation at this position may have altered and thus disrupted the interface. On the other hand, H102A had little effect on the receptor expression and function. H71A displayed reduced AM binding and potency in the absence of a change in the cell surface expression. It is therefore possible that H71 in RAMP2 may be involved in AM binding, either directly or indirectly.

Unlike RAMP1, the interpretation of the mutagenesis data generated at RAMP2 residues is constrained at the present time by the lack of structural information for RAMP2.

RAMP3 N-terminus

Like RAMP2, there are only limited structural/functional data available for RAMP3. The N-terminal cysteine residues have

Table 3 Summary of mutagenesis data for the human RAMP2 N-terminus, expressed with CL

Mutation	*Effect*	*Cell system*	*Reference*
C68A	↓↓Specific hAM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2003b)
C84A	↓↓Specific hAM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2003b)
C99A	↓↓Specific hAM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2003b)
C131A	↓↓Specific hAM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2003b)
H71A	↓Specific hAM binding, ↓hAM potency, ↔CSE	HEK293	Kuwasako *et al.* (2008)
E101W	↓hαCGRP response, ↓hβCGRP response, ↓↓hAM response, ↓↓CSE	Cos7	Qi *et al.* (2008)
W86A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
M88A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
I89A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
S90A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
R91A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
P92A	↔hAM potency	HEK293	Kuwasako *et al.* (2001)
H102A	*↔Specific hAM binding, ↔hAM potency, ↔CSE	HEK293	Kuwasako *et al.* (2008)
H124A	↓↓Specific hAM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2008)
H127A	↓↓Specific AM binding, ↓↓hAM potency, ↓↓CSE	HEK293	Kuwasako *et al.* (2008)

*The significance is not specified in the original publication; therefore, changes indicated do not necessarily achieve statistical significance. CSE, cell surface expression; ↔, unchanged compared to WT; ↓, reduced compared to WT; ↑, increased compared to WT; ↓↓, reduced to basal level; E_{max}, maximum response of agonist-evoked cAMP production (not changed unless specified in the table).

Table 4 Summary of mutagenesis data for the human RAMP3 N-terminus, expressed with CL

Mutation	*Effect*	*Cell system*	*Reference*
Predicted helix 1			
E35A	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
D46V	↔hαCGRP potency, ↔hAM potency, ↓CSE	Cos7	Qi *et al.* (2008)
Predicted helix 2			
E74W	↓ hAM potency	Cos7	Hay *et al.* (2006a)
E74Q	↓ hAM potency	Cos7	Hay *et al.* (2006a)
E74D	↔hAM potency	Cos7	Hay *et al.* (2006a)
E74K	↓ hAM potency	Cos7	Hay *et al.* (2006a)
E74Q	↔hαCGRP potency, ↔CSE	Cos7	Qi *et al.* (2008)
E74D	↔hαCGRP potency, ↓CSE	Cos7	Qi *et al.* (2008)
E74K	↔hαCGRP potency, ↔CSE	Cos7	Qi *et al.* (2008)
E74W	↔hαCGRP potency, ↓hAM potency, ↓$hAM_{15–52}$ potency, ↓AM2 potency, ↓$hAM_{13–52}$-specific binding and affinity, ↔CSE	Cos7	Qi *et al.* (2008)
Predicted helix 3			
P87A	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
L88E	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
A89V	↔hαCGRP potency, ↑hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
I93F	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
H97A	↓↓hAM potency, ↓↓specific hAM binding, ↔CSE (WT RAMP3 had significant CSE)	HEK293	Kuwasako *et al.* (2008)
N103S	↔hαCGRP potency, ↔hAM potency, ↔CSE	Cos7	Qi *et al.* (2008)
H110A	↔hAM potency, ↔specific hAM binding, ↔CSE (WT RAMP3 had significant CSE)	HEK293	Kuwasako *et al.* (2008)

CSE, cell surface expression; ↔, unchanged compared to WT; ↓, reduced compared to WT; ↑, increased compared to WT; ↓↓, reduced to basal level; E_{max}, maximum response of agonist-evoked cAMP production (not changed unless specified in the table).

been examined in mouse RAMP3. When mouse RAMP3 was co-transfected with CL individual mutations of each cysteine to serine mostly led to reduced cell surface expression (Flahaut *et al.*, 2003), suggesting that some structural perturbation may have been introduced to the receptor complex by the mutations. However, the effects of mutations at cysteine residues in RAMP3 do not seem as large as those observed in human RAMPs 1 and 2 (Kuwasako *et al.*, 2003b; Simms *et al.*, 2006). Data generated by site-directed mutagenesis at other positions in human RAMP3 are summarized in Table 4 and Figure 2B.

Predicted helix 1

Mutagenesis has only been performed on two residues (E35 and D46) in predicted α-helix 1 of RAMP3. The mutations at these two positions induced no significant change in either CGRP or AM response. There was a small, but significant, reduction in the cell surface expression for mutant D46V, while mutant receptor containing E35A retained its expression level at the cell surface.

Predicted helix 2

The equivalent residues to 86–92 in human RAMP2 were also investigated in human RAMP3 (59–65) (Kuwasako *et al.*, 2001). Deletion of residues 59–65 of human RAMP3 reduced AM potency and diminished specific AM binding when co-expressed with CL. However, whether this effect seen was due to altered cell surface expression was unclear. The equivalent regions in rat RAMP3 (58–64) have also been characterized (Kuwasako *et al.*, 2002). Deletion of these seven residues led to a significant reduction in both AM binding and potency at AM_2 receptor, but cell surface expression was not affected.

Site-directed mutagenesis has also been conducted to determine the role of individual residues (Figure 2B, Table 4). Position 74 stands out from these studies. As mentioned earlier, W74 in RAMP1 has been demonstrated to be important for the high affinity and selectivity of BIBN4096BS for the human CGRP receptor; the equivalent residue E74 in RAMP3 also plays an important role in ligand binding. Very interestingly, E74W AM_2 displayed a reduced AM potency in the absence of any change in CGRP potency (Hay *et al.*, 2006a). Furthermore, the opposite effect was seen with the reciprocal W74E CGRP receptor, where AM potency was selectively enhanced and CGRP potency was not affected. To our knowledge, E74 is the only single residue in RAMP3 that has been demonstrated to be important for a specific peptide binding event.

Predicted helix 3

Site-directed mutagenesis has been performed at seven positions within predicted α-helix 3 (Table 4 and Figure 2B). These include two extracellular histidine residues (Kuwasako *et al.*, 2008). Alanine substitution at H110 did not affect the receptor function or expression when co-expressed with CL. On the other hand, H97, which is the equivalent residue to H97 in RAMP1, could potentially be involved in forming the CL binding interface. Consistent with this hypothesis, H97A caused a reduction in both AM binding and potency. However, the cell surface expression was difficult to interpret, as the V5-tagged WT RAMP3 showed significant cell surface expression in the absence of CL (Kuwasako *et al.*, 2008). It can be speculated that co-transfection of H97A RAMP3 may have reduced CL translocation to the cell surface.

Conclusions

Receptor–ligand interaction mechanisms for the calcitonin family of peptides are challenging to understand due to the multi-subunit composition of the receptors and potentially complex stoichiometry (Hilairet *et al*., 2001b; Heroux *et al*., 2007). Structure–function studies of the RAMPs have provided valuable information for understanding ligand and receptor interactions for these complexes. That said, whether the residues in RAMPs directly confer receptor-specific pharmacology through ligand interaction or act allosterically to modify the receptor conformation to allow ligand binding is still an open question. Although this review has focused on CL/RAMP complexes, the potential role of different RAMP regions and residues should also be considered for other RAMP receptor complexes. The availability of more structural information for RAMPs will enable a better interpretation of the mutagenesis data; on the other hand, any predictions based on the structural information need experimental support. The parallel analysis of structural and functional data should allow a more confident elucidation of the residues that are involved in the ligand and receptor interactions with RAMPs.

Acknowledgements

The authors are supported by the Health Research Council of New Zealand, Lottery Health Commission (New Zealand), the University of Auckland and Maurice and Phyllis Paykel Trust. We thank Dr David R. Poyner for reading the manuscript and his helpful suggestions.

Conflict of interest

There is no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition (2008 revision). *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Benítez-Páez A (2006). Sequence analysis of the receptor activity-modifying proteins family, new putative peptides and structural conformation inference. *In Silico Biol* **6**: 467–483.

Benítez-Páez A, Cárdenas-Brito S (2008). Dissection of functional residues in receptor activity-modifying proteins through phylogenetic and statistical analyses. *Evol Bioinform* **4**: 153–169.

Bomberger JM, Parameswaran N, Hall CS, Aiyar N, Spielman WS (2005). Novel function for receptor activity-modifying proteins (RAMPs) in post-endocytic receptor trafficking. *J Biol Chem* **280**: 9297–9307.

Bouschet T, Martin S, Henley JM (2005). Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane. *J Cell Sci* **118**: 4709–4720.

Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A *et al*. (2003). Novel receptor partners and function of receptor activity-modifying proteins. *J Biol Chem* **278**: 3293–3297.

Flahaut M, Pfister C, Rossier BC, Firsov D (2003). N-glycosylation and conserved cysteine residues in RAMP3 play a critical role for the functional expression of CRLR/RAMP3 adrenomedullin receptor. *Biochemistry* **42**: 10333–10341.

Fraser NJ, Wise A, Brown J, McLatchie LM, Main MJ, Foord SM (1999). The amino terminus of receptor activity modifying proteins is a critical determinant of glycosylation state and ligand binding of calcitonin receptor-like receptor. *Mol Pharmacol* **55**: 1054–1059.

Hay DL, Christopoulos G, Christopoulos A, Sexton PM (2006a). Determinants of 1-piperidinecarboxamide, *N*-[2-[[5-amino-l-[[4-(4pyridinyl)l-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl) (BIBN4096BS) affinity for calcitonin gene-related peptide and amylin receptors – the role of receptor activity modifying protein 1. *Mol Pharmacol* **70**: 1984–1991.

Hay DL, Poyner DR, Sexton PM (2006b). GPCR modulation by RAMPs. *Pharmacol Ther* **109**: 173–197.

Heroux M, Hogue M, Lemieux S, Bouvier M (2007). Functional calcitonin gene-related peptide receptors are formed by the asymmetric assembly of a calcitonin receptor-like receptor homo-oligomer and a monomer of receptor activity-modifying protein-1. *J Biol Chem* **282**: 31610–31620.

Hilairet S, Belanger C, Bertrand J, Laperriere A, Foord SM, Bouvier M (2001a). Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), and beta-arrestin. *J Biol Chem* **276**: 42182–42190.

Hilairet S, Foord SM, Marshall FH, Bouvier M (2001b). 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 Biol Chem* **276**: 29575–29581.

Kunz TH, Mueller-Steiner S, Schwerdtfeger K, Kleinert P, Troxler H, Kelm JM *et al*. (2007). Interaction of receptor-activity-modifying protein 1 with tubulin. *Biochim Biophys Acta* **1770**: 1145–1150.

Kusano S, Kukimoto-Niino M, Akasaka R, Toyama M, Terada T, Shirouzu M *et al*. (2008). Crystal structure of the human receptor activity-modifying protein 1 extracellular domain. *Protein Sci* **17**: 1907–1914.

Kuwasako K, Shimekake Y, Masuda M, Nakahara K, Yoshida T, Kitaura M *et al*. (2000). Visualization of the calcitonin receptor-like receptor and its receptor activity-modifying proteins during internalization and recycling. *J Biol Chem* **275**: 29602–29609.

Kuwasako K, Kitamura K, Ito K, Uemura T, Yanagita Y, Kato J *et al*. (2001). The seven amino acids of human RAMP2 (86–92) and RAMP3 (59–65) are critical for agonist binding to human adrenomedullin receptors. *J Biol Chem* **276**: 49459–49465.

Kuwasako K, Kitamura K, Onitsuka H, Uemura T, Nagoshi Y, Kato J *et al*. (2002). Rat RAMP domains involved in adrenomedullin binding specificity. *FEBS Lett* **519**: 113–116.

Kuwasako K, Kitamura K, Nagoshi Y, Cao Y-N, Eto T (2003a). Identification of the human receptor activity-modifying protein 1 domains responsible for agonist binding specificity. *J Biol Chem* **278**: 22623–22630.

Kuwasako K, Kitamura K, Uemura T, Nagoshi Y, Kato J, Eto T (2003b). The function of extracellular cysteines in the human adrenomedullin receptor. *Hyperten Res* **26**: 25–31.

Kuwasako K, Cao YN, Nagoshi Y, Kitamura K, Eto T (2004). Adrenomedullin receptors: pharmacological features and possible pathophysiological roles. *Peptides* **25**: 2003–2012.

Kuwasako K, Kitamura K, Nagata S, Kato J (2008). Functions of the extracellular histidine residues of receptor activity-modifying proteins vary within adrenomedullin receptors. *Biochem Biophy Res Commun* **377**: 109–113.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N *et al*. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin receptor-like receptor. *Nature* **393**: 333–339.

Mallee JJ, Salvatore CA, LeBourdelles B, Oliver KR, Longmore J, Koblan KS *et al*. (2002). Receptor activity-modifying protein 1 determines

the species selectivity of non-peptide CGRP receptor antagonists. *J Biol Chem* **277**: 14294–14298.

Morfis M, Tilakaratne N, Furness SGB, Christopoulos G, Werry TD, Christopoulos A *et al*. (2008). Receptor activity modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. *Endocrinology* **149**: 5423–5431.

Parameswaran N, Spielman WS (2006). RAMPs: the past, present and future. *Trends Biochem Sci* **31**: 631–638.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W *et al*. (2002). International Union of Pharmacology: XXXII. The mammalian calcitonin gene-related peptides, AM, AMY, and calcitonin receptors. *Pharmacol Rev* **54**: 233–246.

Qi T, Christopoulos G, Bailey RJ, Christopoulos A, Sexton PM, Hay DL (2008). Identification of N-terminal receptor activity-modifying protein residues important for calcitonin gene-related peptide, adrenomedullin, and amylin receptor function. *Mol Pharmacol* **74**: 1059–1071.

Sexton PM, Albiston A, Morfis M, Tilakaratne N (2001). Receptor activity modifying proteins. *Cell Signal* **13**: 73–83.

Sexton PM, Poyner DR, Simms J, Christopoulos A, Hay DL (2009). Modulating receptor function through RAMPs: can they represent drug targets in themselves? *Drug Discov Today* **14**: 413–419.

Simms J, Hay DL, Wheatley M, Poyner DR (2006). Characterization of the structure of RAMP1 by mutagenesis and molecular modeling. *Biophys J* **91**: 662–669.

Simms J, Hay DL, Bailey RJ, Konycheva G, Bailey G, Wheatley M *et al*. (2009). Structure function analysis of RAMP1 by alanine mutagenesis. *Biochemistry* **48**: 198–205.

Steiner S, Muff R, Gujer R, Fischer JA, Born W (2002). The transmembrane domain of receptor-activity-modifying protein 1 is essential for the functional expression of a calcitonin gene-related peptide receptor. *Biochemistry* **41**: 11398–11404.

Udawela M, Christopoulos G, Morfis M, Christopoulos A, Ye SY, Tilakaratne N *et al*. (2006a). A critical role for the short intracellular C terminus in receptor activity-modifying protein function. *Mol Pharmacol* **70**: 1750–1760.

Udawela M, Christopoulos G, Tilakaratne N, Christopoulos A, Albiston A, Sexton PM (2006b). Distinct receptor activity-modifying protein domains differentially modulate interaction with calcitonin receptors. *Mol Pharmacol* **69**: 1984–1989.

Zumpe ET, Tilakaratne N, Fraser NJ, Christopoulos G, Foord SM, Sexton PM (2000). Multiple ramp domains are required for generation of amylin receptor phenotype from the calcitonin receptor gene product. *Biochem Biophys Res Commun* **267**: 368–372.