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Themed Section: Secretin Family (Class B) G Protein-Coupled Receptors – from Molecular to Clinical Perspectives

REVIEW

Calcitonin and calcitonin receptor-like receptors: common themes with family B GPCRs?

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The calcitonin receptor (CTR) and calcitonin receptor-like receptor (CLR) are two of the 15 human family B (or Secretin-like) GPCRs. CTR and CLR are of considerable biological interest as their pharmacology is moulded by interactions with receptor activity-modifying proteins. They also have therapeutic relevance for many conditions, such as osteoporosis, diabetes, obesity, lymphatic insufficiency, migraine and cardiovascular disease. In light of recent advances in understanding ligand docking and receptor activation in both the family as a whole and in CLR and CTR specifically, this review reflects how applicable general family B GPCR themes are to these two idiosyncratic receptors. We review the main functional domains of the receptors; the N-terminal extracellular domain, the juxtamembrane domain and ligand interface, the transmembrane domain and the intracellular C-terminal domain. Structural and functional findings from the CLR and CTR along with other family B GPCRs are critically appraised to gain insight into how these domains may function. The ability for CTR and CLR to interact with receptor activity-modifying proteins adds another level of sophistication to these receptor systems but means careful consideration is needed when trying to apply generic GPCR principles. This review encapsulates current thinking in the realm of family B GPCR research by highlighting both conflicting and recurring themes and how such findings relate to two unusual but important receptors, CTR and CLR.

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Abbreviations
AM, adrenomedullin; Bpa, 3-(4-benzoylphenyl)alanine; BRET, bioluminescence resonance energy transfer; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; ECD, extracellular domain; ECL, extracellular loop; GLP-1, glucagon-like peptide 1; ICL, intracellular loop; PACAP, pituitary adenylate cyclase-activating polypeptide; PTH, parathyroid hormone; RAMP, receptor activity-modifying protein; RCP, receptor component protein; TM, transmembrane domain

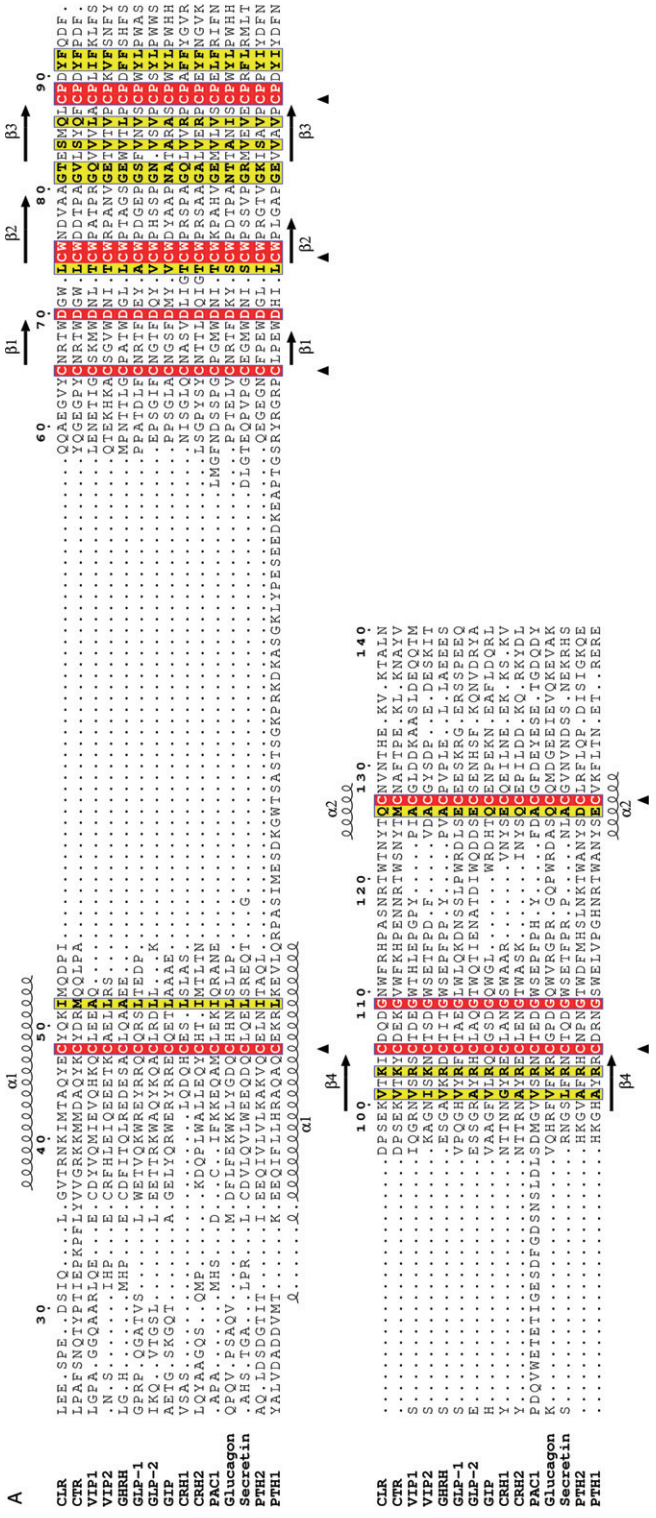
Introduction

The calcitonin (CT) family of peptides consists of six members: CT, amylin, adrenomedullin (AM), two distinct forms of calcitonin gene-related peptide (αCGRP and βCGRP), and AM2, also known as intermedin (Roh *et al.*, 2004). These peptides range from 32 to 52 amino acids in length and are responsible for a diverse array of functions. Consequently, their receptors have therapeutic relevance for many conditions, which include osteoporosis, diabetes, obesity, lymphatic insufficiency, migraine and cardiovascular disease.

The two GPCRs that are receptors for these peptides are the calcitonin receptor (CTR) and the calcitonin receptor-like receptor (CLR, historically known as CRLR). These belong to the subfamily of GPCRs known as the secretin (Fredriksson *et al.*, 2003) or 'B' family (Kolakowski, 1994) of GPCRs (Figure 1). CTR and CLR can form complexes with members

Figure 1

Amino acid sequence alignment of selected family B GPCRs generated using clustal-w and ESPript. (A) Aligned secondary structure of extracellular domains. Secondary structural elements of CLR and the PTH_1 receptor are shown above and below the alignment respectively. The signal peptide region has been omitted. (B) Alignment of the TM helices predicted by TMHMM for CLR (above) and the PTH_1 receptor (below), loops and C-terminus. Conserved regions are shown in solid red boxes and regions of similarity in yellow, cysteines forming disulphide bridges as determined by X-ray structure are indicated by a black triangle. Note that the entire sequences of the receptors were aligned but divided into parts (A) and (B) for the purposes of this figure.



of the membrane protein family called the receptor activity-modifying proteins (RAMPs), which consists of RAMP1, 2 and 3 in humans (McLatchie *et al.*, 1998). Orthologues of these RAMPs occur in most species; additional RAMPs are found in some fishes (Nag *et al.*, 2006). RAMP association with the CTR or with CLR generates multiple distinct receptor phenotypes with different specificities for the CT peptide family (Poyner *et al.*, 2002). CLR together with RAMP1 forms the CGRP receptor. In contrast, two AM receptors are formed by CLR and RAMP2 or RAMP3 respectively. CTR forms amylin receptors with RAMPs; the AMY receptors. The identification of the RAMP family was part of a revolution in GPCR research, which established that GPCRs are not merely monomeric units but are integral components of larger multifaceted signalling complexes.

Although CTR and CLR associate with RAMPs, they share common characteristics with other family B GPCRs. They possess a large N-terminal extracellular domain (ECD), three extracellular loops (ECL1, ECL2, ECL3), seven transmembrane (TM) domains, three intracellular loops (ICL1, ICL2, and ICL3) and an intracellular C-terminus (Figure 2). We review the current understanding of CTR and CLR activation, while taking into consideration recent advances in family B GPCR research.

The N-terminal ECD is essential for peptide binding

The concept that most simply encapsulates the mode of ligand binding and activation of family B GPCRs is known as the 'two domain model' (Hoare, 2005). In this model, the C-terminus of the peptide ligand is first captured by the receptor ECD and then delivered to the ECL and upper TM domain of the receptor to trigger receptor activation (Hoare, 2005). The ECLs and upper TM domain are often collectively referred to as the juxtamembrane region. This model has been confirmed by numerous studies using chimeric receptors [including secretin, parathyroid hormone (PTH), CTR, and the glucagon-like peptide 1 (GLP-1)] and their peptide ligands (Holtmann *et al.*, 1995; Moore *et al.*, 1995; Bergwitz *et al.*, 1996; Runge *et al.*, 2003).

As the N-terminal ECD is the initial peptide ligand binding component of the receptor, the structures of several family B GPCR ECDs have been studied in isolation. The family B GPCR ECDs all share the same overall fold consisting of two antiparallel β-sheets and an N-terminal α-helix that is stabilized by three disulfide bonds. The peptides that bind to these ECDs adopt an α-helical conformation and make several stabilizing hydrophobic and electrostatic interactions. Although a truncated pituitary adenylate cyclase-activating polypeptide (PACAP) peptide bound to its ECD initially revealed a unique docking mode, this has recently been revised and all family B peptides appear to bind in a similar

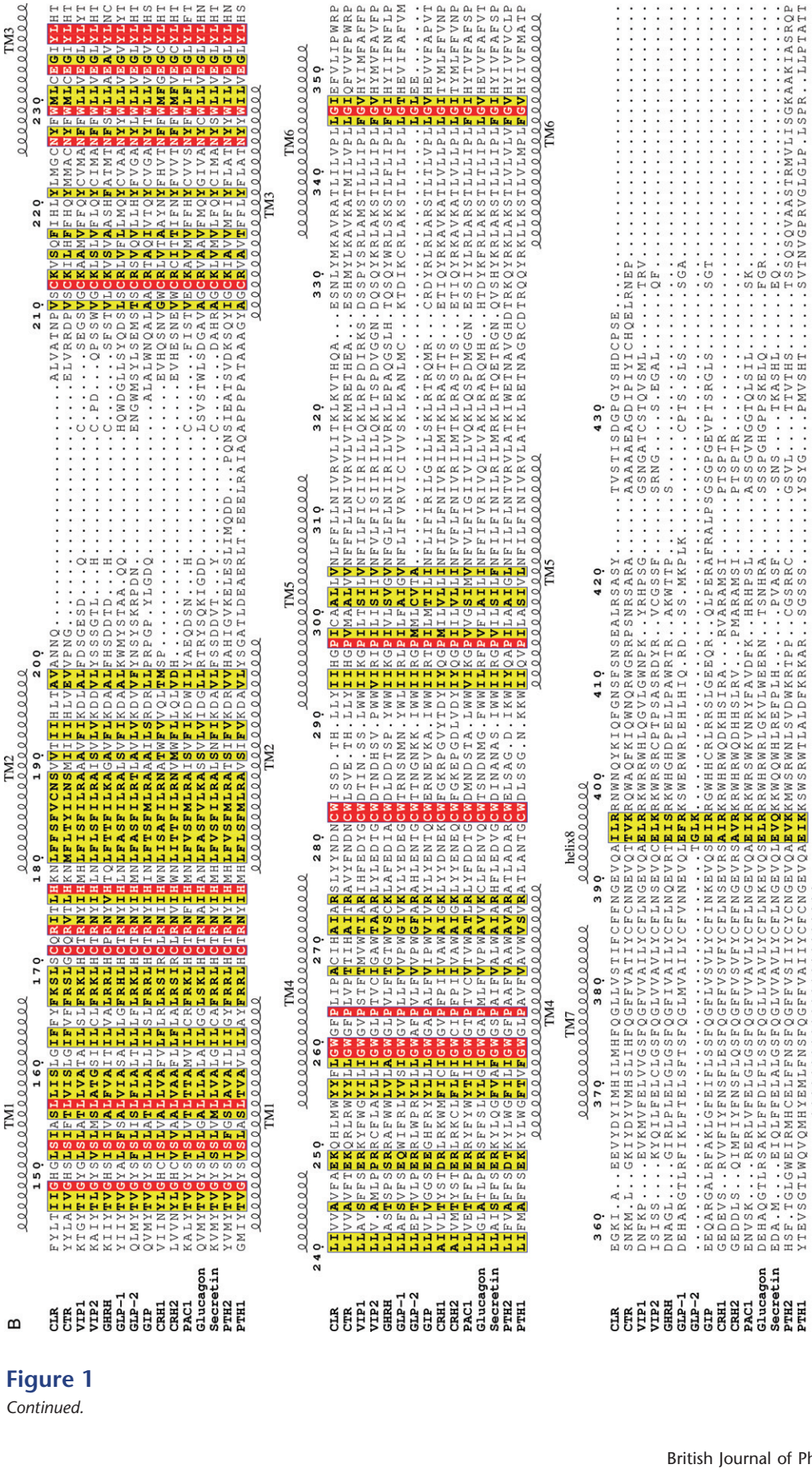


Figure 1

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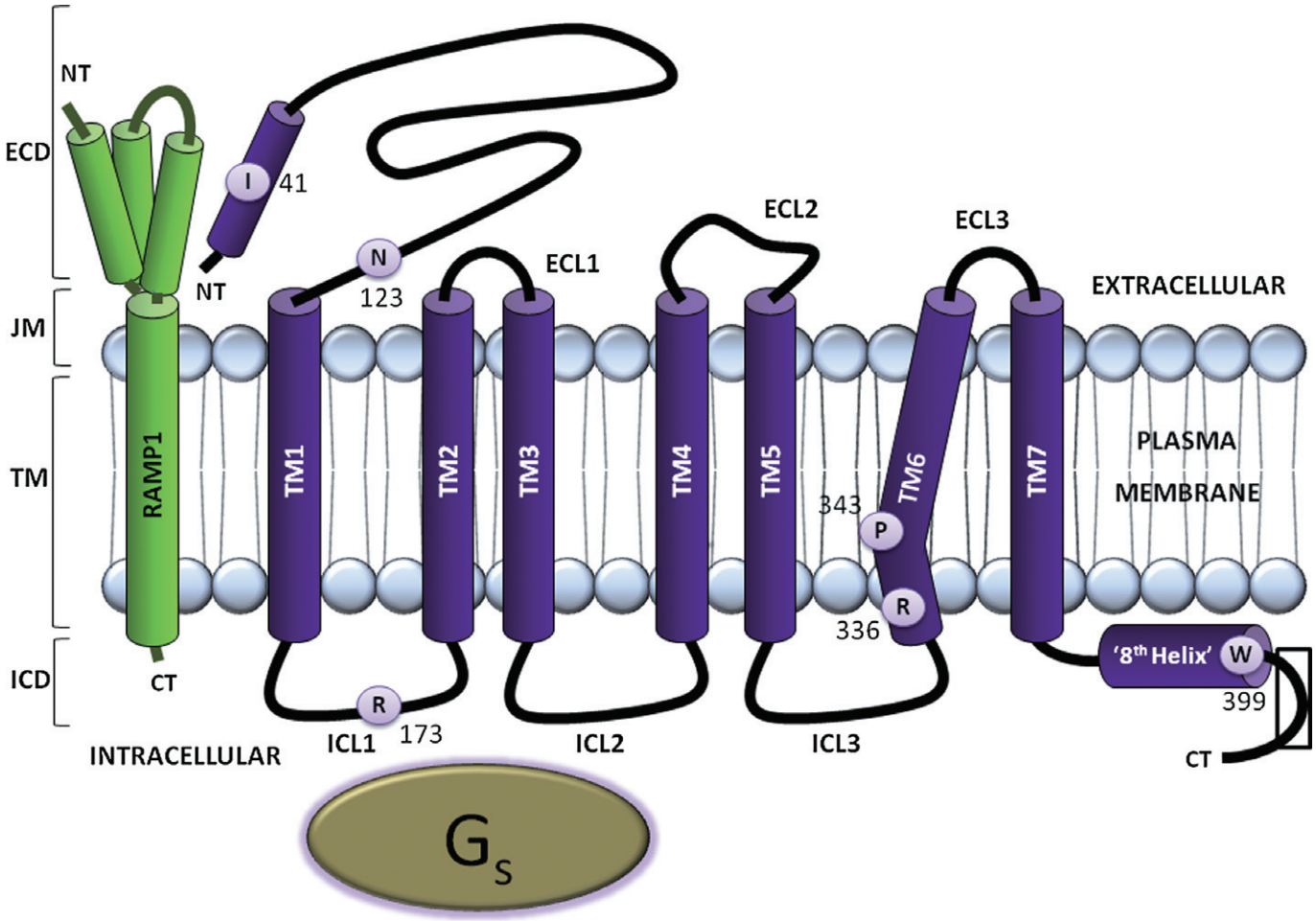


Figure 2

Schematic diagram of CLR (purple) with RAMP1 (green) (the CGRP receptor). Like other family B GPCRs, CLR is divided into functional domains: the N-terminal (NT) extracellular domain (ECD) is important for peptide binding; the extracellular loops (ECL) and upper transmembrane (TM) domain are collectively known as the juxtamembrane (JM) domain and are involved in peptide binding and receptor activation; the TM domain undergoes a conformational change upon activation and the intracellular loops (ICL) and receptor C-terminus (CT) are involved in interactions with intracellular proteins such as G proteins and β-arrestin. Amino acid residues are numbered from the start of the predicted N-terminal signal peptide. Some important residues, which have been discussed in the text or Table 1 are highlighted: I41 and N123 in the ECD, R173 in ICL1, P343 and R336 in TM6 and W399 in helix 8 of the C-terminus. The boxed region of the CLR C-terminus has been reported to be involved in receptor internalization. Helices in CLR and RAMP1 are represented as cylinders.

position to their respective ECDs, highlighting a similar peptide binding mode across this receptor family (for review see Parthier *et al.*, 2009; Kumar *et al.*, 2011).

The crystal structure of the ECD of the CGRP receptor elucidated a heterodimer between CLR and RAMP1 (ter Haar *et al.*, 2010). This crystal structure confirmed the function of several residues in RAMP1 or CLR previously suggested as being important for mediating interactions between the proteins (Figure 3A, Table 1) (ter Haar *et al.*, 2010). Although we do not yet have a structure, given the high degree of sequence homology between CTR and CLR, similar interactions presumably occur between CTR and RAMP1 (Figure 3B).

The CLR portion of the CGRP receptor shares similar structural characteristics with other family B GPCR ECDs, such as the PTH_1 and GLP-1 receptors. It is conceivable that the CLR component binds the CGRP peptide in a CLR/RAMP1 complex (and AM in the CLR/RAMP2 or CLR/RAMP3 complexes) in a similar manner to other family B GPCRs. Yet certain modifications to the mode of peptide binding are foreseeable in the presence of RAMPs as they do contribute to the orthosteric binding site (Moore *et al.*, 2010). As in the case of the CGRP receptor, both CTR and the RAMP components of the AMY receptors are likely to make contact with the peptides, although the exact peptide binding position within the ECD heterodimer is unclear.

Despite lacking a peptide-bound ECD structure for CT peptide family receptors, some insights have been gained from mutagenesis and photoaffinity labelling studies on these receptors. Similar to other family B GPCRs, residues within the ECD of CTR and CLR are critical for ligand binding (Dong *et al.*, 2004a; Banerjee *et al.*, 2006; Barwell *et al.*, 2010). The extreme N-terminus of CLR (residues

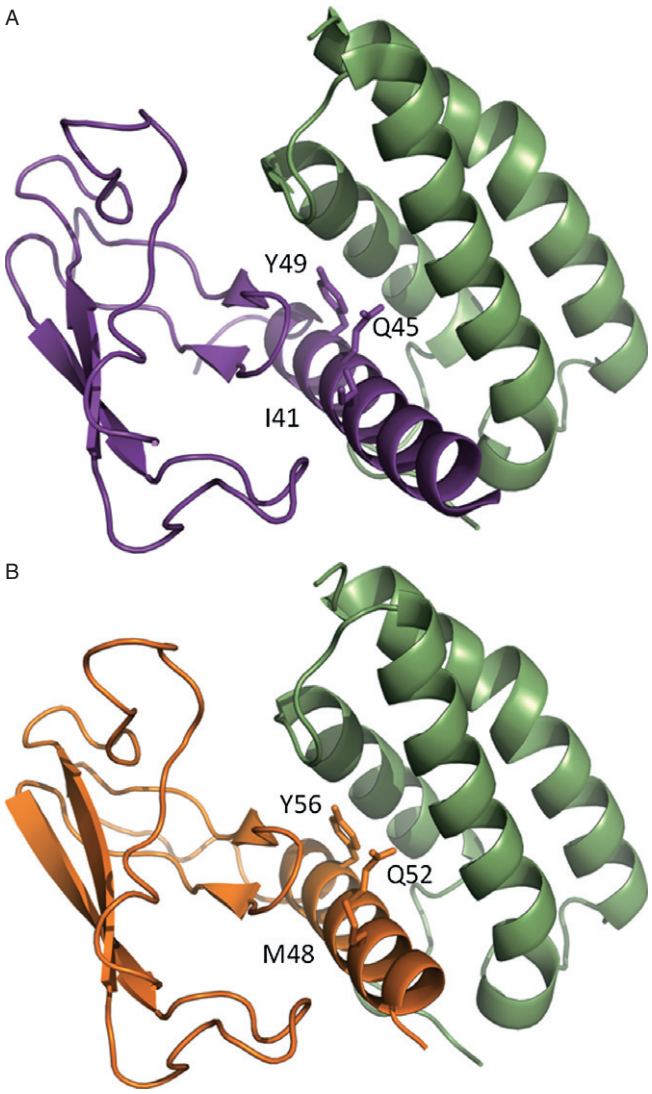


Figure 3

RAMP1 interaction with CLR and CTR via their ECDs. (A) CLR and RAMP1 X-ray crystal structure (Ter Haar *et al.*, 2010) and, (B) Homology model of CTR and RAMP1, generated using (A). CLR is in purple, RAMP1 in green and CTR in orange. Selected residues in the N-terminal α-helix of CLR, which are important for peptide (I41) or RAMP1 interactions are shown in stick format. The equivalent residues, which may share similar roles, are shown in CTR.

23–60) not only contributes to the selectivity of the interactions with either AM or CGRP (Koller *et al.*, 2002) but is required for RAMP1 association (Ittner *et al.*, 2005; Barwell *et al.*, 2010; ter Haar *et al.*, 2010). This is illustrated in Figure 3A. In the N-terminal α-helix of CLR, the residue I41 was demonstrated to be important for CGRP binding (Barwell *et al.*, 2010) (Table 1, Figure 3A). According to our model shown in Figure 3B, M48 of CTR is found in the equivalent position to I41 of CLR; it is possible that this residue may also be involved in ligand interactions. Mutation of M48 to isoleucine had little effect on salmon CT affinity and potency but this is a conservative substitution. In addition, it has been shown that salmon [Bpa8] $CT_{8\text{-}32}$ cross-links to residue M49 of the CTR (Pham *et al.*, 2005). This is also found in the N-terminal α-helix of CTR. [Bpa26] human CT cross-linked to T30 of CTR (Dong *et al.*, 2004a) indicating this region forms part of the peptide interaction site in these receptors.

Orientation of ECD relative to the juxtamembrane domain

In addition to the ECD, the juxtamembrane domain that incorporates both the ECLs and upper portions of the TM domain is involved in peptide ligand binding in family B GPCRs (Figure 2). Precisely how the ECD and juxtamembrane domain interact is unknown for any family B GPCR but experimental findings have provided some insights (Pham *et al.*, 2005; Parthier *et al.*, 2009; Dong *et al.*, 2010a). Vilardaga and co-workers (Vilardaga *et al.*, 1997) suggested a putative disulfide bond was present between the ECD and the top of TM2 in the rat secretin receptor. Second, the dimeric arrangement of the PTH_1 receptor ECD crystal structure provides possible novel constraints in the orientation of the ECD domain in respect to the TM domain bundle (Pioszak *et al.*, 2010). The authors postulate that there is a ~90° turn between the C-terminal α-helix located in the ECD and TM1. Unfortunately, these experimental findings are difficult to apply to CLR and CTR. Corresponding cysteine residues to the rat secretin receptor are not found in CLR and CTR. Additionally, CLR and CTR have to be able to accommodate a RAMP and so predictions on the orientation of the ECD based on other receptor systems may be misleading. Despite the lack of a direct distance restraint between the ECD and juxtamembrane domain of CLR and CTR, photoaffinity cross-linking experiments have provided some insight. As described above, salmon [Bpa8] $CT_{8\text{-}32}$ cross-links to residue M49 of the CTR within the predicted N-terminal α-helix of the CTR (Pham *et al.*, 2005). Bpa moieties show a preference for interacting with methionine residues (Wittelsberger *et al.*, 2006b) and given the low-resolution of such experiments it would be unwise to over-interpret the exact contact point. However, because human [Bpa8] CT binds to ECL3 (Dong *et al.*, 2004b), it has been hypothesized that the distal part of the receptor N-terminal α-helix may be in close proximity to ECL3 (Pham *et al.*, 2005).

Juxtamembrane domain – the pharmacophore required for receptor activation

The architecture of the juxtamembrane domain in family B GPCRs remains elusive. The juxtamembrane domain is found at the membrane-water interface, consequently this region has its own unique physico-chemical environment and its properties have to be differentiated from bulk solvents and lipid bilayers (Liang *et al.*, 2005). This is highlighted by protein phenomena such as snorkelling (i.e. polar side chains point away from the membranes hydrophobic core) and anti-snorkelling (hydrophobic side chains tend to point towards

Table 1

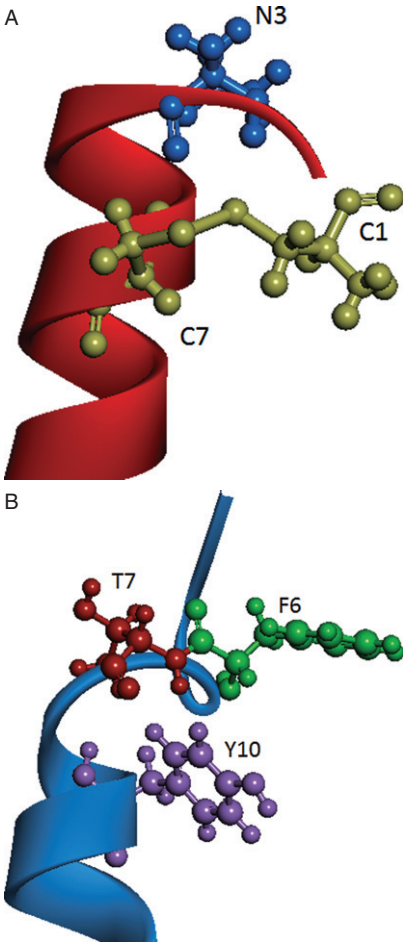
Mutations in CLR that change its function when complexed with RAMP1

Mutation	Location	Major Effect	Potential Mechanism	Reference
Point mutation				
L24A, L34A	ECD	6-8 fold reduction in CGRP affinity but not potency	Unknown	Banerjee *et al.*, 2006
I32A	ECD	Increases potency of CGRP ~10-fold	Unknown	Barwell *et al.*, 2010
G35A	ECD	Increases potency of CGRP ~10-fold	Unknown	Barwell *et al.*, 2010
T37A	ECD	Increases potency of CGRP ~10-fold	Unknown	Barwell *et al.*, 2010
I41A	ECD	Reduces binding of 75pM ^{125}I CGRP by >95%	Contact with CGRP?	Barwell *et al.*, 2010
A44V	ECD			
Q45A	ECD	Reduces cell surface expression of CLR by 44% (Q45A), 55% (C48A) and 66% (Y49A)	RAMP1 contact	Barwell *et al.*, 2010
C48A	ECD		Involved in disulphide bond in CLR	Barwell *et al.*, 2010
Y49A	ECD		RAMP1 contact	Barwell *et al.*, 2010
D70A, E, N	ECD	D70A, E reduced CGRP potency = 1000-fold, D70N reduced Emax by 75%, also reduction of RAMP1 association for all mutants	Stabilise CGRP binding site?	Ittner *et al.*, 2004
N123A	ECD/TM1	1000-fold decrease in CGRP potency, failure of ^{125}I CGRP to cross-link to receptor, normal cell surface expression of CLR but reduced immunoprecipitation of RAMP1 with CLR	Reduced stability of CLR/RAMP1 complex?	Gujer *et al.*, 2001
L169A	ICL1	3-fold reduction in CGRP potency	Stabilise Gs contact?	Conner *et al.*, 2006a
R173A	ICL1	21-fold reduction in CGRP potency	Stabilise Gs contact?	Conner *et al.*, 2006a
Y236A/L237A/H238A	TM3/ICL2	Reduced receptor expression (65% for Y236A/L237A, 90% for H238A)	Receptor trafficking?	Conner *et al.*, 2006a Conner *et al.*, 2006b
V245A/F246A	ICL2	4-fold reduction in CGRP potency	Stabilise Gs contact?	Conner *et al.*, 2006b
K249A	ICL2	11-fold reduction in CGRP potency	Stabilise Gs contact	Conner *et al.*, 2006b
I312A	TM5/ICL3	Reduced CGRP potency (68-fold for I312A, 2-fold for L316A)	Provide hydrophobic face for docking of Gs?	Conner *et al.*, 2006a
L316A	TM5/ICL3			Conner *et al.*, 2006a
R336A	ICL3/TM6	17-fold reduction in CGRP potency	Stabilises end of TM6 to allow Gs interaction?	Conner *et al.*, 2006a
P343A	TM6	14-fold reduction in potency, 10-fold reduction on binding	Mediates conformational change in TM6	Conner *et al.*, 2005
P353	ECL3	3-fold reduction in potency and binding	Stabilises CGRP binding site	Conner *et al.*, 2005
W399T/E	H8	Reduction in cell surface expression (40% W399T, 86% W399E)	Lipid association?	Conner *et al.*, 2008
Deletions				
Del 388–461	Entire C-terminus	Reduction in cell surface expression (90%) and internalisation (80%)	Removes H8 and ser/thr rich region	Conner *et al.*, 2008
Del 400–461	C-terminus beyond H8	80% reduction in internalisation only	Removes region rich in ser/thr residues	Conner *et al.*, 2008
Del 436–461	Extreme C-terminus	No effect on internalisation or expression		Conner *et al.*, 2008

The major functional change is noted; if receptor expression has been reduced, no other change is noted.
Del, deletion of the indicated residues.

Figure 4

The calcitonin family of peptides contain a conserved disulfide that mimics the helix N-cap motif. (A) N-terminal region of a human calcitonin analogue elucidated by solution NMR in the presence of sodium dodecyl micelles (Andreotti *et al.*, 2011; PDB 2JXZ). The peptide is depicted as a red ribbon and amino acids are labelled with single letter identification. The conserved disulfide bond and side chains of cysteine-1 and cysteine-7 are highlighted yellow. Asparagine-3 side chain is highlighted (blue) as it corresponds to the N′ residue as defined by Neumann *et al.* (2008). (B) Receptor-bound N-terminal region of PACAP(1–21)NH_2 (Inooka *et al.*, 2001). The peptide is depicted as a blue ribbon. Neumann *et al.* (2008) defined PACAP as having a simple IA type helix N-cap motif. Phenylalanine-6 (highlighted green) represents the N′ residue. Threonine-7 (highlighted red) represents the N-cap residue. Tyrosine-10 (highlighted purple) represents N3 residue. (C) A multiple sequence alignment of the helix N-cap regions of the calcitonin family of ligands [calcitonin, αCGRP, βCGRP, amylin, AM and AM-2 (also referred to as intermedin)] and PACAP. The calcitonin family peptides have been aligned using ClustalW and then aligned to PACAP based on the helix N-cap motif. Residues highlighted red in the PACAP sequence represent the N′, N-cap and N3 residues respectively. Residues highlighted red in the calcitonin family of ligands represent the conserved cysteine residues that participate in the disulfide bond and the equivalent N′ residue.



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PACAP	HSDGIFTDSYSRYRKQMAVKK
Calcitonin	...CGNLSTCMLGTYTQDFNK
alphaCGRP	..AC.DTATCVTHRLAGLLSR
betaCGRP	..AC.NTATCVTHRLAGLLSR
Amylin	..KC.NTATCATQRLANFLVH
Adrenomedullin	..GC.RFGTCTVQKLAHQIYQ
Arenomedullin-2	..GC.VLGTCQVQNLSHRLWQ

the hydrophobic core region of the membrane) along with the high prevalence of α-helices and irregular protein structures in this interface region, with a reduction of β-strands (Granseth *et al.*, 2005; Liang *et al.*, 2005). Furthermore, the domain contains loop regions, which are typically highly divergent both in length and amino acid composition (Peeters *et al.*, 2011). Accurate loop conformational prediction is difficult due to their inherent flexibility and is dependent on the length of the loop in question. Attempts to model the ECLs of the secretin receptor have produced several different solutions that cannot currently be resolved on the basis of experimental data (Dong *et al.*, 2010a). NMR techniques have been used to directly elucidate the conformation of isolated loops for the PTH_1 receptor (Piserchio *et al.*, 2000; Mierke *et al.*, 2007). This type of reductionist approach to study a loop in isolation has not been applied to CTR or CLR.

Conserved helix N-cap or equivalent in family B peptides

It has been suggested that family B peptide ligands contain a helix N-capping motif (Neumann *et al.*, 2008). This not only protects the peptide but may introduce a specific local fold to help facilitate receptor activation. Structures such as these have been found to exist for PACAP1-21$(NH)_2$ bound to the PACAP receptor (Inooka *et al.*, 2001) and also may be adopted by secretin (Dong *et al.*, 2010b). The CT family of peptides do not possess the conventional helix N-capping motif but instead possess a disulphide bond within their N-terminal domain (Figure 4). This modification is predicted to give rise to a conformation similar to that of a helix N-capped peptide (Neumann *et al.*, 2008).

The helix N-cap and juxtamembrane domain interface

There have been extensive efforts to determine precisely where the N-terminal regions of family B peptides dock into the juxtamembrane domain using receptor chimeras, photoaffinity labelling, disulfide trapping and site-directed mutagenesis studies. Several key studies are summarized in Table 2, which shed particular light on the role of the juxtamembrane domain and the orientation of the ligand with respect to the ECLs. For both the glucagon and PTH receptors, this is consistent with the ligand making extended contact with all three ECLs (Table 2). For the secretin receptor, a model based on cross-linking data suggests that the ligand may enter the TM domain vertically rather than horizontally (Dong *et al.*, 2010a). Triangulating current information to postulate a generic model for how the N-terminal domain of

Table 2

Ligand-juxtamembrane domain contacts in family B GPCRs

Receptor	Region involved in ligand contact	Technique	Reference
Secretin	ECL1, ECL2	secretin/$VPAC_1$ receptor chimaeras	Holtmann *et al.* (1996); Di Paolo *et al.* (1998); Di Paolo *et al.* (1999)
Secretin	ECL3	Photoaffinity cross-linking	Dong *et al.* (2010a)
PTH_1	ECL1, ECL2 and TM3	PTH_1/PTH_2 receptor chimaeras and mutagenesis	Turner *et al.* (1996); Bergwitz *et al.* (1997)
PTH_1	ECL1, TM2, ECL3 and TM6	Photoaffinity and disulphide cross-linking	Greenberg *et al.* (2000); Gensure *et al.* (2003); Wittelsberger *et al.* (2006a); Monaghan *et al.* (2008)
Glucagon	ECL1, ECL2 and ECL3	Glucagon/GLP_1 receptor and peptide analogues	Runge *et al.* (2003)
GLP1	ECL1, ECL2	Mutagenesis	Lopez de Maturana and Donnelly *et al.* (2002); Al-Sabah and Donnelly (2003); Lopez de Maturana *et al.* (2004)
GLP1	ECL1, ECL2	Photoaffinity cross-linking	Miller *et al.* (2011)
CRF1	ECL1, ECL2, ECL3	Mutagenesis, chimeric receptors	Liaw *et al.* (1997); Sydow *et al.* (1999); Gkountelias *et al.* (2009)
CRF1	ECL1, ECL2	Photoaffinity cross-linking	Assil-Kishawi and Abou-Samra (2002); Kraetke *et al.* (2005); Assil-Kishawi *et al.* (2008)

family B ligands dock into their cognate juxtamembrane domains is challenging.

Nevertheless, there are some existing models and data that provide insight into ligand binding and activation of family B GPCRs. The glucagon and PTH_1 receptor models are of particular interest (Runge *et al.*, 2003; Monaghan *et al.*, 2008) and both argue that the peptide has the capability to spread across the extracellular face of the TM bundle. In family A GPCRs, Rosenkilde and workers differentiated between the 'minor ligand pocket' (located between TM1, 2, 3 and 7) and the so-called 'major ligand pocket' (located between TM3, 4, 5, 6 and 7), which affect biased signalling (Rosenkilde *et al.*, 2010) (Figure 5). It is not known if such pockets exist in family B GPCRs, although the extracellular surface of the receptor must have distinct regions defined by the three ECLs and the N-terminal ECD. The PTH_1 receptor model suggests that the first residue of the peptide is located near TM5 and 6. By contrast the initial N-terminal residues of glucagon are predicted to be buried between TM1, 2 and 7; a model for GLP-1 binding to its receptor based on photoaffinity cross-linking also has the peptide docking at this part of the receptor (Miller *et al.*, 2011). Inspection of glucagon based on the predicted position of the helix N-cap reveals that it remains plausible that the glucagon N-cap motif can reach out beyond the TM1/2/7 region, given that K12 of glucagon is predicted to interact with a domain that incorporates ECL2 and the juxtamembrane regions of TM4 and 5 (Runge *et al.*, 2003). For GLP-1, the issue is less clear as the published model cannot be used to predict residue–residue contacts (Miller *et al.*, 2011). Yet, Chugunov *et al.* (2010) using a combination of pharmacology and molecular modelling to investigate the $VPAC_1$ receptor suggested that TM2, 3 and 7 propagated receptor activation and had a direct role in ligand binding as D3 of vasoactive intestinal peptide was able to form a salt bridge with R118 located on TM2 of the $VPAC_1$ receptor (Solano *et al.*, 2001). CTR and CLR are the only two members within family B GPCRs that do not have a positively charged residue at the equivalent R188 position, instead an asparagine in present (Figure 1).

These observations raise various pertinent questions. Do family B GPCRs have such well-defined minor and major binding pockets as family A GPCRs, given that the orientation of the helices in family B GPCRs is unknown? Is there a single mode of interaction for the endogenous peptide agonists at family B GPCRs? Is the helix N-cap a key to receptor activation? For family A GPCRs, it has been suggested that the major and minor binding pockets influence biased signalling by agonists; biased agonism has also been reported in family B GPCRs (Vilardaga *et al.*, 2001; Gesty-Palmer *et al.*, 2009). The architecture of the juxtamembrane domain remains elusive therefore the binding crevices within the TM domains can only be discussed speculatively, given the lack of structural data.

Interaction of CT family ligands with the juxtamembrane region of CLR and CTR

The precise way in which CT family peptides interact with their receptors remains unknown. Unsurprisingly, there is good evidence that the N-terminal portion of salmon CT interacts with the loop/TM domains of CTR to induce acti-

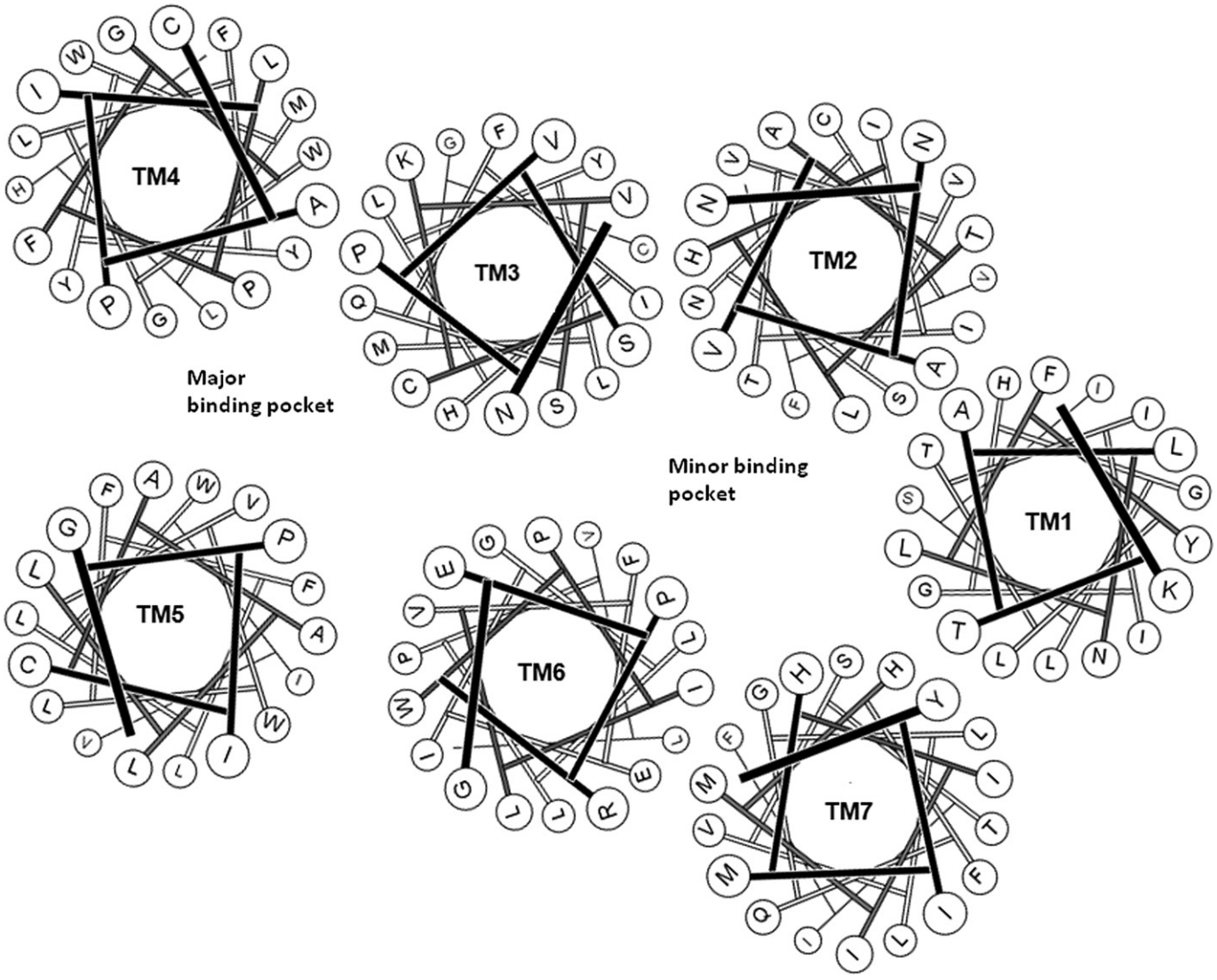

Figure 5

A speculative helical wheel representation of the CLR transmembrane bundle as viewed from the extracellular surface to show the analogous major and minor binding pockets found in family A GPCRs. Periodicity of each TM domain was approximated based on the Vohra *et al.* (2007) strategy coupled with a refinement procedure used to investigate speculative models of CLR based on bovine rhodopsin.

vation (Stroop *et al.*, 1996). It is interesting that chimeric receptors where the N-termini and TM bundles of the PTH_1 and CT receptors have been swapped can be activated by the corresponding chimeric ligands, suggesting that there is at least a degree of conservation in the way these receptors are activated (Bergwitz *et al.*, 1996). Beyond that, there is some evidence that may mean the peptides make extended contact across the juxtamembrane region. However, it is very difficult to distinguish between direct and indirect effects on the responses to ligands, particularly agonists where conformational changes remote from the actual binding site may be very important in modulating binding or efficacy.

Residues 16 and 19 of human CT or salmon $CT_{8\text{-}32}$ make photoaffinity contacts with the receptor N-terminus just above TM1 (Dong *et al.*, 2004a; Pham *et al.*, 2005). More recently, the potency of small-molecule agonists of CTR was shown to be severely impaired when Y150, L151, A152 and I153 of the receptor were deleted or mutated (Dong *et al.*, 2009). These residues are predicted to reside in the exofacial end of TM1 but it is not clear if the small-molecule agonists act in the same way as the endogenous peptide. For CLR, N123, just above TM1, is important in allowing a high-affinity interaction between the receptor and the peptide (Gujer *et al.*, 2001) (Table 1).

The rat CTR_{e2+} receptor has a 37 amino acid insert into ECL1 that disrupts ligand binding, indicating an influence for this loop in binding CT (Houssami *et al.*, 1995). It has been found that human [Bpa8] CT cross-linked with L368 of CTR, which is located in ECL3 (Dong *et al.*, 2004a,b). A non-peptide antagonist has been shown to require residues in TM7 for binding to CLR, consistent with an interaction for CGRP with or near this part of the receptor (Salvatore *et al.*,

2006), although an indirect, allosteric effect is also possible. Alanine-scanning mutagenesis of ECL1, 2 and 3 of CLR revealed that these domains are required for normal pharmacology (J. Barwell, Dr A.C. Conner and D.R. Poyner, unpubl. obs.), but the roles of these residues remain unclear.

The TM domains – the mystery continues

Unlike family A GPCRs the tertiary structure of the TM domain of family B remains elusive. Family B are remote homologues of the family A GPCRs sharing only a limited amount of amino acid conservation (Di Fabio *et al.*, 2008). Therefore, using standard conservation alignment procedures to construct a homology model is unachievable (Johnson and Overington, 1993; Rost, 1999). Yet, all GPCRs bind to similar effectors, for example, G-proteins, β-arrestins and a tentative hypothesis that GPCRs share a global geometric conformation could be assumed (Frimurer and Bywater, 1999). Indeed, there is some experimental support for this from studies of the PTH receptor constrained by Zn^{2+} binding sites (Sheikh *et al.*, 1999).

Novel alignment strategies have been employed to allow family B GPCR researchers to take advantage of the high-resolution family A crystal structures (Donnelly, 1997; Frimurer and Bywater, 1999; Bissantz *et al.*, 2004; Vohra *et al.*, 2007; Di Fabio *et al.*, 2008; Chugunov *et al.*, 2010). While sequence alignments share a degree of consensus, helices such as TM5 are particularly difficult to predict. Currently, TM models of Family B GPCRs remain speculative and must be used with caution. Experimental efforts have been used to determine distance restraints within family B TM domains. Engineered disulphide bonds between TM2 and TM7 as well as between TM5 and TM6 have been investigated in PTH_1 receptor (Thomas *et al.*, 2008; 2009). The use of reciprocal residue exchanges has found an important cluster of residues between TM 2, 3 and 7 in the $VPAC_1$ receptor (Chugunov *et al.*, 2010). Advancements in understanding helical packing will aid future TM domain modelling efforts.

Family B GPCRs have their own highly conserved motifs within their TM domain. Notable are the YLH motif located in TM3 and the VS/AxxY motif located in TM7 (Figure 1). It has been postulated that these motifs correspond to the functionally important E/DRY and NPxxY motifs found in family A GPCRs (Conner *et al.*, 2007; Langer and Robberecht, 2007). Yet on a mechanistic level how these family B motifs affect receptor function is unclear and it may be unwise to draw too many parallels with family A GPCRs without further data. For example, the YLH motif of family B GPCRs is expected to interact with TM2 (Frimurer and Bywater, 1999; Di Fabio *et al.*, 2008), rather than TM6, the expected interaction site if it were an exact equivalent of the E/DRY motif in family A GPCRs (Rovati *et al.*, 2007). Mutation of Y236 and L237 of the YLH motif in CLR prevents cell surface expression and therefore it is difficult to determine whether these residues are important for receptor activation (Conner *et al.*, 2005; Conner *et al.*, 2006b).

Analogous to family A GPCRs is the importance of proline residues within family B TM domains to induce receptor activation. The role of P343 in CLR has been addressed by mutation. Replacement by alanine impairs CGRP-mediated receptor activation, although the introduction of amino acids that permit some flexibility at this point or reintroducing the proline a turn below in the helix can restore function. The proline itself appears to introduce a marked bend in the helix (Conner *et al.*, 2005). Similar findings have been reported for this proline in the CTR (Bailey and Hay, 2007). Conserved TM prolines are also functionally important in the $VPAC_1$ receptor (Knudsen *et al.*, 2001). Thus at least for these receptors, there may be shared functions of TM prolines with family A GPCRs.

Intracellular face of the receptor

The intracellular face of GPCRs is a complex arrangement of ICLs and a C-terminal tail. The ICLs generally have roles in G-protein coupling (and hence desensitization) and receptor expression; the C-terminal tail in addition acts as a scaffold for other interacting proteins (Langlet *et al.*, 2005). A number of features are found in family B GPCRs. Thus the distal end of TM5 often extends down to form a hydrophobic helix that may facilitate G-protein coupling (Mathi *et al.*, 1997). There are other conserved motifs such as the R/K-xx-R/K motifs found in ICL3 of many family B GPCRs and which may also be important for G-protein coupling (Figure 1); however, their precise role is likely to depend both on the individual receptor and the cell in which it is expressed (Takhar *et al.*, 1996; Chan *et al.*, 2001; Couvineau *et al.*, 2003; Langer *et al.*, 2005).

The ICLs of CLR have been extensively investigated by site-directed mutagenesis to reveal hot spots needed for efficient Gs coupling (Table 1). There is some evidence that residues in ICL1, ICL2 and ICL3 of CLR are important for Gs-coupling (Conner *et al.*, 2006a,b). Their effects have been interpreted by molecular modelling but in the absence of a family B receptor structure, this is a very speculative approach. Very little is known about the role of the ICLs in the CTR. However, one human CTR variant [$CT_{(b)}$] contains an additional 16 amino acids in ICL1. This receptor shows poor internalization and has attenuated coupling to Gs and Gq compared with CTR that lack this insert (Moore *et al.*, 1995). Thus the extra residues in the loop may impair interactions with intracellular regulatory proteins.

Biophysical analysis of a C-terminal peptide mimetic that represented CLR from G389-N400 revealed the likely presence of a parallel membrane helix anchored to a liposome via W399, which is highly conserved in family B GPCRs (Figures 1 and 2) (Conner *et al.*, 2008). However, it is interesting to note that a crystal structure of the C-terminus of the PTH_1 receptor complexed to G-protein β1-γ2 subunits shows that the equivalent of this tryptophan contacts the G-protein (Johnston *et al.*, 2008). This tryptophan residue may be involved in protein–protein interactions; which may include a membrane interaction under the appropriate circumstances.

C-terminal tail truncations of CLR impair both cell surface expression and CGRP-mediated internalization when co-expressed with RAMP1; it was possible to identify distinct domains that mediated these functions (Table 1) (Conner *et al.*, 2008). The function of the CLR C-terminal tail

co-expressed with RAMP2 has also been investigated by mutagenesis (Kuwasako *et al.*, 2010; 2011). The authors concluded that the CLR, by virtue of its C-terminus, when co-expressed with RAMP2, is involved in both Gi and Gs coupling in response to AM. In particular, E390 was important for coupling to Gs.

For the rabbit CTR, the entire C-terminus can be deleted without altering coupling to Gs, but there is a reduction in ERK activation and Gq coupling (Seck *et al.*, 2005). By contrast, deletion of the C-terminus of the porcine CTR impairs both Gs and Gq coupling, in addition to receptor expression and internalization (Findlay *et al.*, 1994). It is unclear whether these differences reflect true species-specific differences between the receptors or whether they are because of experimental variables such as the coupling efficiencies of the cells used.

There is evidence that the RAMPs also influence receptor signalling. For example, Christopoulos and colleagues (Christopoulos *et al.*, 2003) noted that the $VPAC_1$ receptor/RAMP2 complex increased agonist mediated phosphoinositide hydrolysis, implying that RAMP2 may improve Gq accessibility. The RAMPs modulate signalling bias for AMY receptors (Morfis *et al.*, 2008). The C-termini of the RAMPs are particularly important for modulation of AMY receptor phenotype. The ability of the AMY receptors to stimulate a cAMP response was severely impaired when the C-terminal tail of the RAMPs was truncated (Udawela *et al.*, 2006). However, overexpression of Gs partially recovered the receptors ability to produce a cAMP response. Swapping the C-terminal tail between RAMPs 1 and 2 altered changed the signalling profile for AMY_1 and AMY_2 receptors (Udawela *et al.*, 2006). Interestingly, RAMP C-terminal truncations did not impair the cAMP response in CLR based receptors in this study (Udawela *et al.*, 2006).

A hydrophilic, 148 amino acid protein known as receptor component protein (RCP) has been proposed as a dynamic regulator of G-protein coupling, adding another level of sophistication to the CGRP system (Evans *et al.*, 2000). This is endogenously expressed in most cell lines used for the study of CLR and so antisense strategies have been used to examine the effect of reducing its expression, These have shown that reductions in RCP do not affect expression or high-affinity binding of CGRP and AM receptors but notably reduce signal transduction (Evans *et al.*, 2000). RCP has also been found to co-immunoprecipitate with CLR implying a direct interaction (Evans *et al.*, 2000). The tertiary structure of RCP has not been elucidated and its mechanism of action remains unclear. It is not known whether RCP interacts with CTR or other family B GPCRs.

Oligomerization of CTR and CLR

Both CTR and CLR oligomerize with RAMPs and this is essential for the formation of CGRP, AM and AMY receptors. The role of RAMPs has been extensively reviewed elsewhere (Hay *et al.*, 2006) and an account of the contacts between the ECDs of CLR and RAMP1 has been included in the recent paper that describes the structure of this complex (ter Haar *et al.*, 2010). In this section, CLR and CTR homodimers are considered, as well as the likely stoichiometry of their complexes with RAMPs. This point is of particular interest because, if the RAMPs associate with CLR or CTR homodimers, the resulting complexes will contain three or four proteins and must use a number of distinct dimerization interfaces on the GPCRs.

There is good evidence for the dimerization of some family B GPCRs having physiological significance (Gao *et al.*, 2009). The identity of the dimerization interfaces of family B GPCRs was initially investigated by evolutionary trace analysis (Vohra *et al.*, 2007). The results suggested that TM4 and TM6 are the most likely candidates to be involved in oligomerization. Since this publication it has become clear that TM4 is the GPCR dimerization site in the secretin receptor (Harikumar *et al.*, 2007; Gao *et al.*, 2009). On the other hand, RAMP3 association with the secretin receptor is mediated via TM6 and TM7 (Harikumar *et al.*, 2009). Thus the GPCR and RAMP dimerization interfaces on family B GPCRs are likely to be distinct.

It is clear that CTR is able to dimerize with itself and other family B GPCRs. Co-immunoprecipitation and Forster resonance energy transfer analysis revealed that two rabbit CTR isoforms (C1a and a Δ exon 13 variant) could form both homo- and heterodimers (Seck *et al.*, 2003). Interestingly, cell surface expression of the rabbit CTR C1a isoform was inhibited by the Δ exon 13 variant, suggesting that it acted as a dominant negative. A recent study compared human and rabbit CTR homodimerization (Harikumar *et al.*, 2010); human CTR was unable to produce a strong bioluminescence resonance energy transfer (BRET) signal unlike rabbit CTR. Mutating human CTR residues R236, T253 and V250 of the lipid-exposed face of TM4 to those that either corresponded to rabbit CTR or to residues found in the human secretin receptor, resulted in a significant BRET signal. Despite the lack of detectable BRET signal, functional studies with a human CTR TM4 mimic peptide showed that human CTR is likely to dimerize. This peptide caused a right-ward shift in cAMP production in both rabbit and human CTR expressing cells and inhibited rabbit CTR dimerization as assessed by BRET. This emphasizes the involvement of TM4 of family B GPCRs in receptor dimerization.

In the crystal structure of the CGRP receptor, CLR and RAMP1 form 1:1 heterodimers (ter Haar *et al.*, 2010), consistent with an earlier cross-linking study of full-length receptors (Hilairet *et al.*, 2001). However, a bimolecular fluorescence complementation with BRET approach suggested the CGRP receptor contained two CLRs with only one RAMP1 (Heroux *et al.*, 2007). This does not rule out the possibility that two individual RAMP molecules could bind to a CLR dimer (Heroux *et al.*, 2007; Sexton *et al.*, 2009). Although the stoichiometry of CGRP receptor is still unclear, the possibility that it contains a CLR dimer is thought provoking and in tune with the paradigm that family B GPCRs can form dimers.

Although the emphasis in this review has been on the role of TM domains in mediating dimerization, a stable dimer of the PTH_1 receptor ECDs has been crystalized and it has been proposed that the oligomerization state of this receptor is important for its activation (Pioszak *et al.*, 2010). The significance of this observation for CLR and CTR is unknown.

There also is evidence for intra-family hetero-oligomerization in family B GPCRs (Harikumar *et al.*, 2008). Determining the pharmacological profile of the various combinations of monomeric units along with their physiological relevance could aid drug design strategies immensely.

Concluding remarks

A full understanding of receptor activation requires the identification of the different states of the receptor along with understanding the molecular process that governs the trajectory between such states. Currently, understanding the CTR and CLR on this mechanistic level remains in its infancy. The lack of high-resolution structural data on the TM domain regions in these receptors coupled with discrepancies in the stoichiometric arrangement of the receptor complexes needs to be addressed. This will be no mean feat. The stoichiometry of the CTR and CLR complexes could be governed by a plethora of variables including cell-line specific factors, cell compartmentalization and lipid bilayer lateral movement. However, research in this area has remained vibrant, which in part may be ascribed to the potential pharmaceutical benefit, but also because CTR and CLR encompasses many novel and challenging aspects of GPCR research. These include dimerization, accessory proteins and elucidation of large diffuse pharmacophores. The ability to test and analyse whether certain concepts are generic or receptor specific, fuels hypotheses and guides future work. Consequently, the pursuit of mechanistic knowledge pertaining to CTR and CLR receptor activation remains both novel and exciting.

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Conflict of interest

None.

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