The activation of the CGRP receptor

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Abstract

The CGRP (calcitonin gene-related peptide) receptor is a family B GPCR (G-protein-coupled receptor). It consists of a GPCR, CLR (calcitonin receptor-like receptor) and an accessory protein, RAMP1 (receptor activity modifying protein 1). RAMP1 is needed for CGRP binding and also cell-surface expression of CLR. CLR is an example of a family B GPCR. Unlike family A GPCRs, little is known about how these receptors are activated by their endogenous ligands. This review considers what is known about the activation of family B GPCRs and then considers how this might be applied to CLR, particularly in light of new knowledge of the crystal structures of family A GPCRs.

CGRP (calcitonin gene-related peptide) and CLR (calcitonin receptor-like receptor)

CGRP is an abundant 37-amino-acid neuropeptide that is especially found in the sensory nervous system. CGRP is released during neurogenic inflammation; it has been shown to be particularly important in migraine but it is also likely to be significant in many other pathological conditions [1]. CGRP belongs to a family of peptides that also includes calcitonin, amylin and the AMs (adrenomedullins). The peptides all have a disulfide-bonded ring at their N-termini, which is important for receptor activation; the cysteine residues are found at positions 2 and 7 in CGRP. The receptor for CGRP is a family B GPCR known as CLR. This is an unusual GPCR in that it has no known endogenous ligand; instead it is absolutely dependent on co-expression with one of three accessory proteins (RAMPs; receptor activity modifying proteins) in order for it to bind CGRP or allied peptides. RAMP1 forms part of the CGRP receptor; RAMP2 and RAMP3 form part of the AM1 and AM2 receptors [2,3]. The receptor also requires a peripheral membrane protein, CGRP-RCP (receptor component protein) for coupling to G, [4].

Family B GPCRs

Family B, or secretin-like GPCRs, form receptors for approximately 15 medium-sized peptides, including PTH (parathyroid hormone), secretin, glucagon, GLP-1 (glucagon-like peptide 1), GIP (glucose-dependent insulinothropic polypeptide), CRF (corticotropin-releasing factor), PACAP (PACAP/VIP) receptors [5]. The receptors have an extracellular N-terminus of approximately 100 amino acids that is responsible for binding the C-terminus of the ligand; a number of crystal structures exist to show these complexes [6]. Activation is likely to involve interactions between the N-terminus of the ligands and the TM (transmembrane) and ECL (extracellular loop) regions of the receptors [7] but the details of this remain obscure. There is currently no crystal structure for a family B GPCR. Although there are now a number of structures for family A GPCRs, both active and inactive conformations [8], these are of limited use for studying family B GPCRs owing to the lack of sequence conservation between the two families. Although there are a few motifs that seem to be shared between the two, such as the disulfide bond between ECL2 and the top of TM3 and an eighth helix [9], other well-characterized motifs found in family A GPCRs such as the DRY motif at the base of TM helix 3 and the NPXXY motif in TM helix 7 are absent. This makes it extremely difficult to even align the two families, let alone identify shared activation mechanisms. Nonetheless, given that they activate the same range of effectors as family A GPCRs, it is reasonable to assume that there must be some shared mechanisms, particularly at the cytoplasmic side of the membrane, as they activate a common set of G-proteins. Homology modelling has been recently facilitated by the proposal that a plant GPCR can be used to make correlations between family A and family B GPCRs [10]. Furthermore, the very recent publication of the crystal structures for a number of family A peptide hormones [11–15] also suggests common themes as to how GPCRs can recognize peptides [16].

The N-terminus of CLR and receptor activation

Crystal structures of the complex between the N-termini of CLR and RAMP1 are available, either on their own or with two small molecule antagonists bound [17]. As yet,
Figure 1 | Proposed binding cleft for CGRP in the N-terminus of the CGRP receptor

Red, CLR; green, RAMP1; grey, superimposed structures of bound PTH, PTH-related protein, GLP-1 and GIP. Residues implicated in the binding of CGRP on CLR or RAMP1 are shown in stick form.

Figure 2 | Model of CLR

Green, residues in TM2, TM3 and the Cys-Trp motif in ECL2 that alter CGRP potency; orange, Asn-Asn-Gln motif in TM 2/3/7; black, Pro321 in TM6; blue, Arg-His-Glu-Thr motif in TMs 2/3/6; red, Try-Leu-His motif in TM3.

there is no structure showing bound CGRP, but site-directed mutagenesis indicates that it is likely to sit in a pocket where one edge is formed by the N-terminal helix of CLR and the loop between helices 2 and 3 of RAMP1 [18,19]. This is consistent with the binding of GLP-1 and GIP to their receptors (Figure 1). There may be a bend in the middle of CGRP to orientate its C-terminus towards RAMP1. It remains unclear as to whether the N-terminus of CLR plays any role in receptor activation or is simply an anchor for the ligand. In the past, there has been considerable interest in the concept of an activator sequence in the N-terminus that interacts with the TM bundle, although this idea is no longer current and it has previously been suggested that an interaction between the N-terminus and ECL3 keeps the receptor in an inactive form [20–22]. There is clearly a precedent for the N-terminus activating the TM bundle with family C GPCRs, albeit with much smaller ligands that cannot reach into the helix domain. Conformational changes have been seen within the N-terminus of the CLR–RAMP1 complex during antagonist binding [23] and so some role in modulating receptor activity would not be surprising.

The ECLs

The ECLs are likely to have a particularly important role in the activation of family B GPCRs. They may be expected to play at least some role in the recognition of the N-terminal activator region of the native peptide agonists; they may also interact with the regions immediately C-terminal to these regions. A recent mutagenesis study of the GLP-1 receptor has shown how ECL2 plays an important part in determining the signalling bias of that receptor to individual G-proteins or β-arrestin [24]. The roles of ECL1 and ECL3 in CLR have been examined in recent studies [25,26] and some of this work has also been reviewed recently [27]. ECL1 appears to be of more significance for CGRP binding to the CGRP receptor than is ECL3; a hydrophobic patch at the interface between TM2 and ECL seems to be of especial significance and this is close to another key group of residues at the top of TM3 (Figure 2). By contrast, ECL3 is more significant for AM binding to CLR at the CGRP, AM1 and AM2 receptors. No single residue was identified in this study as being of particular significance for AM binding and receptor activation; the authors suggest that the loop as a whole was important for AM function, by promoting activation of Gs [26]. The data suggest that even within the CGRP receptor, different endogenous peptides may interact in different ways, with ECL1 being important for CGRP and ECL3 being important for AM. However, as will be seen below, there is also evidence for some conservation of the binding pocket, perhaps closer to the TM helices.

We have previously reported preliminary data suggesting that ECL2 is of particular importance for CGRP binding [27]. In particular, there is a highly conserved Cys-Trp motif, also found in ECL2 of a number of family A GPCRs [28], where the tryptophan is to be in close proximity to
bound CGRP, GLP-1 and CRF [27]. The last two contacts have been deduced from photoaffinity studies, where spatial resolution is limited; all three peptides may occupy broadly similar positions when bound to their receptors but higher resolution studies are needed.

A major problem when considering the role of ECLs in family B GPCRs is that it is very difficult to predict their structure. This is a particular problem for ECL2. A variety of structures for this loop have been seen in the crystal structures of family A GPCRs and its length makes modelling especially challenging [28]. This is coupled with a more general problem, in that the precise orientation of the TM helices is unknown; indeed, even in family A GPCRs there is considerable variation in the form and angles of the upper reaches of the helices between different receptors, and this is exacerbated by possible crystal packing artefacts [8]. Recent progress has been made with surface cysteine accessibility studies of the glucagon receptor to determine the geometry of ECL1; the results suggest that the non-helical section of the loop is limited to 15 residues and the first six of these form a $\beta$-sheet [29]. For ECL2, in family A GPCRs, all peptide receptors examined to date show a common fold, with the loop existing predominantly as a $\beta$-hairpin bending away from the TM bundle (as opposed to non-peptide receptors where there is often an $\alpha$-helix and the loop tends to either form a lid or at least partially obstruct the pocket between the TM helices) [11–15]. It is plausible that a similar arrangement exists for family B GPCRs, particularly given that the calcitonin family of ligands have six or seven amino acid rings at their N-termini and so binding would be impeded if the loop obstructed the binding site [16].

The orientation of the N-terminus of CGRP with respect to its receptor is unknown. Generally, for family B GPCRs, there is evidence for two broad types of binding, with the N-terminus of the ligand either extended across the tops of the ECLs, or orientated perpendicularly to the membrane so that it penetrates into the TM bundle. There is photoaffinity cross-linking data to support the former arrangement for the binding of PTH; using a similar approach the latter model has been proposed to apply to the binding of secretin and GLP-1 [28]. Mutagenesis data for the CGRP receptor indicates that His$^{219}$, Leu$^{220}$, and Leu$^{222}$ in TM helix 3 all influence CGRP; together with Leu$^{195}$, Val$^{198}$ and Ala$^{199}$ of TM helix 2. These residues are likely to extend down to the middle third of the TM bundle [25]. It is unclear if this means that the peptides themselves penetrate this far into the TM bundle, as it is possible to extend CGRP at its N-terminus without little or no loss of affinity [32] and AM has a substantial N-terminal extension before the disulfide-bonded ring. It is possible that the N-terminus of both CGRP and AM are tilted, so that the base of the disulfide ring sits deep within the TM pocket whereas the N-terminus is free to escape over the ECLs, or the more deeply located residues may exert their actions indirectly. Within the N-terminus of all members of the CGRP/calcitonin family of peptides, there is a conserved Thr$^6$ in CGRP and it appears likely that this is essential for biological activity. Thus this probably represents a conserved activation switch that, in turn, implies that all of these peptide ligands bind in a similar orientation to the TM bundle [27].

### The TM bundle

The crystal structures of active rhodopsin and the $\beta$-adrenoceptor have been of great help in determining how family A GPCRs are activated; in particular, the complex of the $\beta$-adrenoceptor with $G_\alpha_s$ has allowed a detailed analysis of G-protein contacts. The structures have confirmed that a concerted series of conformational changes take place, which culminate in the relative separation of the C-terminal ends of TM helices 3 and 6 to open up a binding pocket for $G_\alpha_s$.

A conserved arginine in TM helix 3 [arginine (3.50)], part of the Asp-Arg-Tyr motif, probably forms an ionic lock with a glutamate at the base of TM helix 6 [glutamate (6.30)] in the fully inactive receptor; TM6 is appreciably kinked owing to a proline midway down its length [proline (6.50)] and during activation a rotation of this helix breaks the lock to open up the binding pocket and allows the arginine to interact with $G_\alpha_s$ [8].

Family B GPCRs have a number of conserved proline residues, including a proline towards the middle of TM helix 6 that could potentially act as the equivalent of proline (6.50). In the CGRP receptor this is Pro$^{321}$, which is likely to be approximately two turns higher up the helix than proline (6.50) in family A GPCRs [10], where it is highly conserved. Furthermore, its mutation to alanine in the CGRP receptor disrupts receptor activation; although there is a reduction in CGRP affinity, there is an additional effect on efficacy, as would be expected for mutating a residue involved in ligand binding. If a kink is reintroduced in TM helix 6 in the mutated receptor one turn below Pro$^{321}$ (but not in other positions), the receptor regains its activity [33]. Thus this element of the activation mechanism is conserved between family A and family B GPCRs.

The formal sequence equivalent of the Asp-Arg-Tyr motif is likely to be the Tyr-Leu-His motif at the base of TM helix 3. In the VPAC1 receptor, mutation of Tyr-Leu produced subtle effects on G-protein coupling [34]. In CLR, alanine mutation of either Tyr$^{256}$/Leu$^{257}$ or His$^{258}$ causes substantial reductions in cell-surface expression; in the case of His$^{258}$ these are likely to mask any effects on coupling [35,36]. However, it seems unlikely that the Tyr-Leu-His motif is the functional equivalent of the Asp-Arg-Tyr motif. A turn above the Tyr-Leu-His sequence is Glu$^{257}$; this is absolutely conserved in all family B GPCRs. It appears to face Arg$^{257}$ and His$^{258}$ at the base of TM helix 2, residues that are equally well conserved, and it has been suggested that, together, they may form the functional equivalent of the Asp-Arg-Tyr motif [37]. The basic residues in TM helix 2 may hydrogen-bond with Thr$^{358}$ at the base of TM helix 6; again, another absolutely conserved residue in family B GPCRs [10]. Mutagenesis of either of the basic residues or the threonine either reduces receptor activation or, more significantly, causes constitutive activity in many GPCRs, depending on the nature of the group introduced [38–42]. These results match our own unpublished work and...
are consistent with this polar lock being the Asp-Arg-Tyr equivalent. Interestingly, the ionic lock is not present in the 6-opioid receptor but is replaced by a somewhat analogous polar lock between arginine (3.50) and threonine (6.34) [13]. A further switch has been identified in the VPAC1 receptor between an absolutely conserved glutamate in TM helix 7 (Glu376 in CLR), an equally conserved asparagine in TM helix 3 (Asn295 in CLR) and an arginine in TM helix 2 that has been proposed to interact with Glu1 of VIP [43]. In CLR (and the CTR) this arginine is changed to asparagine (Asn187). It is not clear whether this lock fulfills the same role in CLR as our mutagenesis studies only indicate a role for Gln376 [97]. It is probable that the significance of individual switches depends on the receptor or ligand.

**Conclusion**

In the absence of a crystal structure showing CGRP bound to its receptor, details of this process must out of necessity be speculative. Nonetheless, it is possible to make a number of testable hypotheses based on mutagenesis and molecular modelling. In particular, we propose that the N-terminus of CGRP is likely to make important contacts with the upper reaches of TM helices 2 and 3 and associated ECLs, with Thr6 of the ligand being of particular importance. These are coupled with movement of TM helices 3 and 6, broadly as described for family A GPCRs. However, rather than an ionic lock between TM helices 2 and 3, there is probably a polar lock between TM helices 2 and 6, with additional contacts causing a concerted movement of TM helix 3 with 2.

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**References**


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