Ligand binding and activation of the CGRP receptor

A.C. Conner*, J. Simms†, J. Barwell†, M. Wheatley‡ and D.R. Poyner†[1]

*School of Medicine, University of Swansea, Swansea SA2 8PP, U.K., †School of Life and Health Sciences, Aston University, Birmingham B4 7ET, U.K., and ‡School of Biosciences, University of Birmingham, Birmingham B15 2TT, U.K.

Abstract

The receptor for CGRP (calcitonin gene-related peptide) is a heterodimer between a GPCR (G-protein-coupled receptor), CLR (calcitonin receptor-like receptor) and an accessory protein, RAMP1 (receptor activity-modifying protein 1). Models have been produced of RAMP1 and CLR. It is likely that the C-terminus of CGRP interacts with the extracellular N-termini of CLR and RAMP1; the extreme N-terminus of CLR is particularly important and may interact directly with CGRP and also with RAMP1. The N-terminus of CGRP interacts with the TM (transmembrane) portion of the receptor; the second ECL (extracellular loop) is especially important. Receptor activation is likely to involve the relative movements of TMs 3 and 6 to create a G-protein-binding pocket, as in Family A GPCRs. Pro^{321} in TM6 appears to act as a pivot. At the base of TMs 2 and 3, Arg^{151}, His^{155} and Glu^{211} may form a loose equivalent of the Family A DRY (Asp-Arg-Tyr) motif. Although the details of this proposed activation mechanism clearly do not apply to all Family B GPCRs, the broad outlines may be conserved.

Introduction

The CGRP (calcitonin gene-related peptide) receptor is an unusual GPCR (G-protein-coupled receptor). Not only is it a Family B GPCR, but also it is a heterodimer between a conventional seven-TM (transmembrane) helix GPCR-like protein [known as CLR (calcitonin receptor-like receptor)] and a single TM protein, RAMP1 (receptor activity-modifying protein 1). RAMP1 facilitates the expression of CLR at the cell surface; however, it is also required for ligand binding to the receptor and so must be intimately associated with the ligand-binding site [1]. Association with the homologous proteins RAMP2 or RAMP3 gives receptors for the related protein AM (adrenomedullin) [1]. The present paper will review what is known about how CGRP interacts with its receptor and how the receptor activates G-proteins.

CGRP, CLR and RAMP1

The structure of CGRP

CGRP is a 37-amino-acid peptide that is found throughout the nervous system, particularly in sensory nerve fibres. It belongs to the CT (calcitonin) family of peptides and has a number of key structural features (Figure 1). At the N-terminus there is a disulfide-bonded loop between residues 2 and 7; this is essential for receptor activation as its removal gives an antagonist, CGRP-(8–37) [2]. There then follows a region of amphipathic α-helix from residues 8 to 18, terminating in a β-turn. Thereafter the peptide appears largely unstructured in solution, until the final ten amino acids are reached. Here, the presence of a β-turn centred on Pro^{28} and a possible γ-turn at Pro^{34} appear to be needed for high-affinity binding [3]. The final residue of the peptide is amidated and this may be a contact point with the receptor [4].

CGRP appears to be similar to many other ligands for Family B GPCRs in that it can be split into discrete activation and binding domains. Accordingly, it is likely that it follows the pattern seen for the other family members where the N-terminal activation domain is associated with the TM portion of CLR, whereas the C-terminal binding domain associates with the extracellular N-terminus of CLR [5]. This is consistent with the interaction with RAMP1; the TM helix of this receptor contributes relatively little to the specificity of the interaction with CLR, whereas the N-terminus (of approx. 100 amino acids) is much more important [6]. Accordingly, we assume that the residues 1–7 of CGRP most likely interact with the ECLs (extracellular loops) of the receptor, whereas residues 8–37 interact with the N-termini of CLR and RAMP1.

Modelling RAMP1 and CLR

In order to understand further how CGRP might interact with its receptor, it is essential to understand the structure of RAMP1 and CLR. For the N-terminus of CLR, this is facilitated by the availability of an NMR structure of the N-terminus of the CRF (corticotropin-releasing factor) receptor, another Family B GPCR [7]. This has allowed the construction of a straightforward homology model. It should, however, be noted that the extreme N-terminus of the CRF receptor was not present in the NMR structure and so this must be added using *ab initio* techniques.

Key words: adrenomedullin, calcitonin receptor-like receptor, DRY motif, G-protein, receptor activity-modifying protein 1 (RAMP1), receptor activation.

Abbreviations used: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CRF, corticotropin-releasing factor; CT, calcitonin; ECL, extracellular loop; GPCR, G-protein-coupled receptor; ICL, intracellular loop; RAMP, receptor activity-modifying protein; TM, transmembrane.

[1]To whom correspondence should be addressed (email D.R.Poyner@aston.ac.uk).

Figure 1 | Alignment of human α-CGRP and allied peptides
Shaded residues are identical with those found in human α-CGRP.

```
CGRP        AC-DTATCVTHRLAGLLSRSGGVVKNNFVP-TNVGSKAF
Amylin      KC-NTATCATQRLANFLVHSSNNFGAILSS-TNVGSNTY
AM          GC-RFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY
CT          -CGNLSTCMLGTYTQDFNKFHTF------PQTAIGVGAP
```

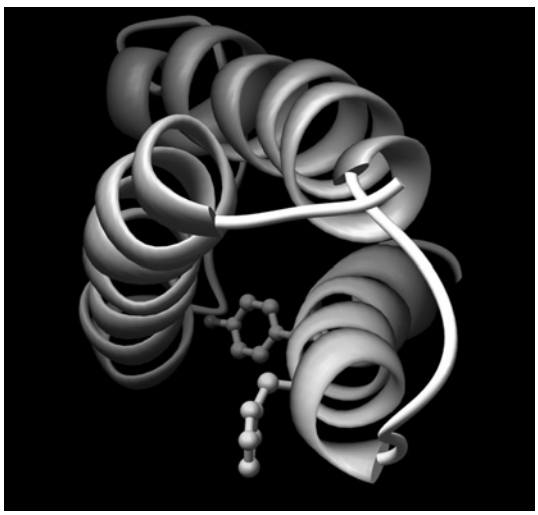
To model the TM portion of CLR, the boundaries of the individual helices were first identified by alignment with other Family B GPCRs. These were then aligned with rhodopsin by comparing locations of the most conserved residues in each helix. As a control, a random sequence based on CLR composition was constrained to the final alignment. Some 200 models were generated for both CLR and the control sequences with different orientations of residue side chains using MODELLER v.6, and each was scored for stability. The best structure was selected. The structures were inserted into a DPPC (dipalmitoyl phosphatidylcholine) bilayer, and 3 ns Molecular Dynamics simulations were carried out. The CLR model relaxed to a stable structure, whereas the control did not. As a further refinement, the active state of CLR was modelled by incorporating distant constraints derived from biophysical measurements on rhodopsin and the β-receptor. As a further control, the sequence of bovine rhodopsin was constrained to the structure of bacteriorhodopsin and underwent Molecular Dynamics simulations. The resulting TM bundle had a root-mean-squared deviation of 3.1 Å (1 Å = 0.1 nm) compared with the crystal structure of rhodopsin, demonstrating the method could accurately fold rhodopsin [8,9].

There are no suitable templates on which to base an homology model of RAMP 1 and so an *ab initio* method was followed. RAMP1 contains six cysteine residues and the disulfide bond pattern arrangement was first identified by mutagenesis. Following this, a consensus secondary structure was produced using a number of predictive software packages. Subsequently, 25 000 models were produced and scored; the best 20 (scored for stability) were essentially identical. This structure was then subject to a Molecular Dynamics simulation. The final structure was a trihelical bundle, with the TM domain forming a fourth helix (Figure 2) [10].

CGRP binding

Any model for the binding of CGRP to its receptor must necessarily be speculative; there is no information available from photoaffinity cross-linking studies and little mutagenesis has been carried out. As a consequence, we have carried out extensive mutagenesis of the extracellular domains of RAMP1 and CLR, by alanine scans and also production of chimaeras. For RAMP1, mutants that reduce CGRP binding seem to be largely concentrated in helices two and three (residues 60–64 and 86–94). Some of these are almost certainly involved in stabilizing intrahelical packing; however, it is unlikely that all fulfil this role. The modelling suggests that the cavity between these helices is a potential interaction

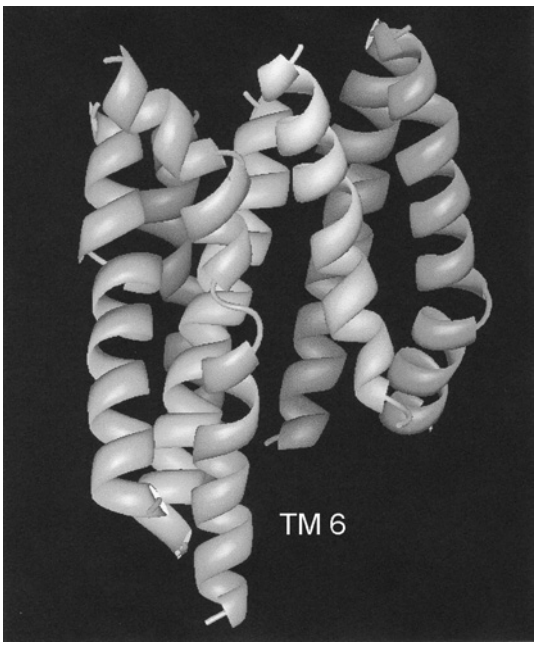
Figure 2 | Structure of human RAMP1
It has previously been shown that Phe^{93} and Tyr^{100} are important in cell-surface expression [23]. The *ab initio* model of hRAMP1 (human RAMP1) reveals that these residues are located in a cleft between helix 1 and helix 3, potentially stabilizing its structure.



site for CLR. For the most part, the predicted N-terminus of CLR has a compact structure with few obvious contact points for RAMP1. However, the extreme N-terminus, the equivalent of which is not present in the CRF receptor structure, is predicted to form an α-helix and could associate with RAMP1. We have examined this part of the molecule by alanine-scanning mutagenesis. There is a significant epitope involving residues from Asn^{39} to Met^{42} where mutagenesis reduces CGRP binding. Intriguingly, alanine substitution of several residues further proximal to this section appears to increase CGRP binding. Our data are consistent with a role for the extreme N-terminus of CLR in CGRP binding, either making direct contacts with the C-terminal portion of the peptide or by stabilizing its binding site, perhaps via interactions with RAMP1.

The N-terminus of CGRP is expected to make contact with the TM bundle of CLR and the ECLs. Of particular relevance is a photoaffinity cross-linking study involving CT, which is part of the same peptide family as CGRP and AM. Here, residue 8 at the N-terminus of the peptide cross-links to ECL3. Interestingly in the antagonist fragment sCT8-32, residue 8 cross-links to part of the N-terminus, reflecting either a difference in agonist/antagonist interactions or a difference between human and salmon CT. The authors speculate that the rest of the N-terminus may interact with TM6 of the receptor [11]. We have investigated ECL2 by means of an alanine scan. This has identified Arg^{252}, Tyr^{255}, Tyr^{256}, Asp^{258}, Cys^{260}, Trp^{261}, Ser^{263} and Thr^{266} as all being required for CGRP stimulation of cAMP production. These residues represent half the likely total length of ECL2, suggesting that it is very significant for CGRP binding. A role for ECL2 in receptor activation is consistent with what is seen in other GPCRs. In rhodopsin, it is believed to act as a lid

Figure 3 | Structure of the TM domain of human CLR
The kink in TM6 is due to Pro^{321}.



over the covalently bound retinal, suppressing basal activity [12]. It has also been shown to penetrate deeply into the TM bundle of the dopamine D_2 receptor [13]. Mutations in ECL2 are frequently associated with constitutive activity [14], and agonist-dependent conformational changes in ECL2 have been reported for the 5-HT_4 receptor (5-hydroxytryptamine type 4 receptor) [15].

Activation of CLR

Introduction

Although much work has been carried out on activation of Family A GPCRs, the activation of Family B GPCRs is much less well understood. Our working hypothesis has been that there are likely to be broad similarities between the activation of the two families. In particular, it is likely that there is a concerted movement of TMs 3 and 6 to open a binding pocket for G-proteins, as has been reported for Family A.

The role of TM6 and ICL (intracellular loop) 3

We have shown that Pro^{321} in TM6 is critical for receptor activation [8]. This introduces a kink into the helix which is removed in the mutant P321A, with the effect of reducing activity (Figure 3). If the kink is restored one turn above (P321A/I325P), then full receptor activity returns. This suggests that Pro^{321} acts as a hinge and that movement about this helix is required for receptor activity. This has clear resonances with models of activation proposed for Family A GPCRs and it is likely that relative movements of TMs 2, 3, 6 and 7 reorder the ICLs to create a G-protein-binding pocket [16]. The proximal part of ICL3 probably forms the wall of this pocket, with Ile^{290} particularly important. ICL2 swings away from the core of the receptor; Lys^{227} may interact directly with G_s [17]. Unlike with rhodopsin, we see no changes in the position of ICL1 or TM2.

TM3 and a DRY motif?

One of the most distinctive features of the Family A GPCRs is the DRY motif. This has traditionally been considered to be a means of constraining the receptor in the ground state by restricting the movements of residue side chains; upon activation, these form new arrangements allowing the movement of the TM regions. However, it is now clear that with some receptors, it functions in a different way and is more directly involved with receptor activation. There is no simple equivalent of the DRY motif in any Family B GPCR; however, there are a number of highly conserved residues at the base of TMs 2 and 3 which might form a functional equivalent. We have concentrated on three of these; Arg^{151} at the base of TM2, His^{155} further up TM2 and Glu^{211} in TM3. Alanine substitutions have shown that all three of these residues are important in coupling to G_s. We have started to explore the structural requirements at each site; it appears that the nature of the charge is important, but we need to investigate substituents such as asparagine and glutamine to fully elucidate the charge and geometric requirements. Double-mutant cycle analysis compares the effects of two single mutations to the corresponding double mutation to determine whether amino acids are functionally coupled; in this case, lack of additivity implies that they are part of the same pathway (e.g. [18,19]). It is unwise to over-interpret data arising from this approach in our system, as cAMP production is an indirect measure of receptor–G_s interactions. However, none of the double mutants approaches additivity and in some cases where we have reversed the positions of amino acids, there is limited recovery of function. Combined with modelling (Figure 2), our data are consistent with the three residues forming a functional triplet, consistent with a previous suggestion that, in Family B GPCRs, residues in TMs 2 and 3 may combine to form the motif [20]. It is likely that movements of ICL2 and 3 expose residues at the base of TMs 2 and 3 to G-proteins in the course of receptor activation (Figure 2). Our models suggest that there is a network of conserved amino acids in the central TM domain of the receptor that stabilize active and inactive conformations; this needs to be tested via further mutagenesis.

A universal model of activation of Family B GPCRs?

It is attractive to think that the mechanisms that we propose to be important for activation of CLR are conserved in other Family B GPCRs. However, at least at a detailed level, this is unlikely to be true. For example, in VPAC1 (vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1), mutation of the equivalent of Pro^{321} to alanine increases receptor activity [21]. In the secretin receptor, mutation of the equivalent of Glu^{211} to alanine has no effect; in contrast, a conserved asparagine residue found approximately one turn higher up in the helix reduces activation [22]. Thus

the detailed activation pathway is receptor-specific and may also be influenced by the presence of other factors such as scaffolding proteins or the phosphorylation status of the receptor. On the other hand, the general features of an activation pathway involving a concerted movement of the TM regions to create a G-protein-binding pocket are likely to be conserved. It seems unlikely that there are many different ways in which a GPCR can interact with a G-protein such as G_s. It may be that a number of ways are possible of producing what are essentially very similar active conformations of a GPCR; depending on the exact architecture of any given GPCR, only some of these may be significant for a particular receptor.

References

1 McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. and Foord, S.M. (1998) Nature **393**, 333–339
2 Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T. and Fujita, T. (1989) Am. J. Physiol. **256**, E331–E335
3 Conner, A.C., Hay, D.L., Howitt, S.G., Kilk, K., Langel, Ü., Wheatley, M., Smith, D.M. and Poyner, D.R. (2002) Biochem. Soc. Trans. **30**, 451–455
4 Carpenter, K.A., Schmidt, R., von Mentzer, B., Haglund, U., Roberts, E. and Walpole, C. (2001) Biochemistry **40**, 8317–8325
5 Hoare, S.R. (2005) Drug Discov. Today **10**, 417–427
6 Hay, D.L., Poyner, D.R. and Sexton, P.M. (2006) Pharmacol. Ther. **109**, 173–197
7 Grace, C.R., Perrin, M.H., Gulyas, J., Digruccio, M.R., Cantle, J.P., Rivier, J.E., Vale, W.W. and Riek, R. (2007) Proc. Natl. Acad. Sci. U.S.A. **104**, 4858–4863
8 Conner, A.C., Hay, D.L., Simms, J., Howitt, S.G., Schindler, M., Smith, D.M., Wheatley, M. and Poyner, D.R. (2005) Mol. Pharmacol. **67**, 20–31
9 Conner, A.C., Simms, J., Howitt, S.G., Wheatley, M. and Poyner, D.R. (2006) J. Biol. Chem. **281**, 1644–1651
10 Simms, J., Hay, D.L., Wheatley, M. and Poyner, D.R. (2006) Biophys. J. **91**, 662–669
11 Pham, V., Dong, M., Wade, J.D., Miller, L.J., Morton, C.J., Ng, H.L., Parker, M.W. and Sexton, P.M. (2005) J. Biol. Chem. **280**, 28610–28622
12 Schertler, G.F. (2005) Curr. Opin. Struct. Biol. **15**, 408–415
13 Shi, L. and Javitch, J.A. (2004) Proc. Natl. Acad. Sci. U.S.A. **101**, 440–445
14 Shi, L. and Javitch, J.A. (2002) Annu. Rev. Pharmacol. Toxicol. **42**, 437–467
15 Baneres, J.L., Mesnier, D., Martin, A., Joubert, L., Dumuis, A. and Bockaert, J. (2005) J. Biol. Chem. **280**, 20253–20260
16 Schwartz, T.W., Frimurer, T.M., Holst, B., Rosenkilde, M.M. and Elling, C.E. (2006) Annu. Rev. Pharmacol. Toxicol. **46**, 481–519
17 Conner, A.C., Simms, J., Conner, M.T., Wootten, D.L., Wheatley, M. and Poyner, D.R. (2006) Biochemistry **45**, 12976–12985
18 Vogel, W.K., Peterson, G.L., Broderick, D.J., Mosser, V.A. and Schimerlik, M.I. (1999) Arch. Biochem. Biophys. **361**, 283–294
19 Lee, W.Y. and Sine, S.M. (2005) Nature **438**, 243–247
20 Frimurer, T.M. and Bywater, R.P. (1999) Proteins **35**, 375–386
21 Knudsen, S.M., Tams, J.W. and Fahrenkrug, J. (2001) FEBS Lett. **503**, 126–130
22 Nachtergael, I., Gaspard, N., Langlet, C., Robberecht, P. and Langer, I. (2006) Cell. Signalling **18**, 2121–2130
23 Kuwasako, K., Kitamura, K., Nagoshi, Y., Cao, Y.N. and Eto, T. (2003) J. Biol. Chem. **278**, 22623–22630

Received 26 April 2007