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Relative antagonism of mutants of the CGRP receptor extracellular loop 2 domain (ECL2) using a truncated competitive antagonist ($CGRP_{8-37}$): Evidence for the dual involvement of ECL2 in the two-domain binding model.

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ABSTRACT: The second extracellular loop (ECL2) of the G protein-coupled receptor (GPCR) family is important for ligand-interaction and drug discovery. ECL2 of the family B cardioprotective CGRP receptor is required for cell signalling. Family B GPCR ligands have two regions; the N-terminus mediates receptor activation and the remainder confers high-affinity binding. Comparing antagonism of $CGRP_{8-37}$ at a number of point mutations of ECL2 of the CGRP receptor, we show that the loop facilitates interaction with the N-terminal loop but also residues C-terminal to this. This has implications for understanding family B GPCR activation and for drug design at the CGRP receptor.

More than a third of all therapeutic compounds target members of the G protein-coupled receptor (GPCR) super-family. Understanding how ligands bind to GPCRs is therefore key to modern molecular pharmacology. The second extracellular loop (ECL2) of the GPCR super-family is the largest and most structurally diverse of the ECLs and is vital for ligand binding and activation for almost all of these receptors [1-3]. Crystal structures of the largest sub-family of GPCRs (family A) show ECL2 in a variety of conformations, including beta-sheets, alpha helices and unstructured loops [4-6]. Family B GPCRs includes a smaller group of pharmaceutically important peptide-binding receptors. Conversely, the three available crystal structures of family B GPCRs suggest that ECL2 has no significant secondary structural components [7-9]. Despite this, every biochemical analysis done on the family B GPCR ECL2 regions have found it to be vital for the binding of the respective peptide ligands and the subsequent activation of various signalling pathways [10-12].

Family B GPCRs are all activated by relatively small peptide ligands (typically under 100 amino acids long). They have a diverse range of physiological effects (such as cardioprotection, bone resorption, migraine, digestion control, glucose homeostasis and steroid hormone responses), which has made them of considerable therapeutic interest [13]. These peptide ligands are thought to follow a broadly consistent binding mechanism, termed the two-domain model [14]. Briefly, the C-terminus of the ligand binds to the large extracellular N-terminus of its GPCR. This facilitates the binding of the N-terminus of the ligand to the ECL regions and transmembrane (TM) domain of the receptor. This in turn causes receptor activation and second messenger signalling. It is not known exactly where the ligand N-terminus binds or how that interaction results in the conformational changes that stabilise receptor activation. This information will help our understanding of ligand-binding and also provide a platform for the design of specific molecules for individual receptor structures.

One important member of the family B GPCRs is the CGRP receptor. This belongs to the calcitonin-like sub-family and is of considerable therapeutic interest due to the highly potent vasodilatory properties of the CGRP neuropeptide ligand. CGRP has cardioprotective properties of potential beneficial use in heart failure and hypertension [15,16] and has been implicated in the induction of migraine [17]. The CGRP receptor has also been studied extensively because of another unusual feature; it functions as an obligate heterodimer formed by a GPCR subunit (calcitonin receptor-like receptor; CLR) and a single TM span-

ning protein called receptor activity modifying protein 1 (RAMP 1) [18].

In our recent alanine scan of the CGRP receptor, we substituted 24 residues of the ECL2 region and found that 14 had significant differences in receptor signalling [12]. The largest differences occurred with mutation of residues in the N-terminal half of the loop, although the supporting *in silico* modelling data predicted the involvement of C-terminal residues in receptor-ligand interactions. This alanine-substitution analysis identified residues required for CGRP receptor function providing a platform for a more mechanistic analysis of ECL2. In the present study, six key residues identified from the alanine scan of the CGRP receptor were selected to investigate the affinity of a truncated version of CGRP missing the first seven residues ($CGRP_{8-37}$). $CGRP_{8-37}$ acts as a reversible, competitive antagonist with an affinity approximately 10-fold lower than full length CGRP. It is thought that CGRP adheres to the two-domain binding model whereby the 8-37 region of CGRP has a discrete binding region that facilitates a second binding event allowing the first seven residues of CGRP to act as the activation domain [14].

Specifically, we are asking whether the ability of $CGRP_{8-37}$ to antagonise a mutant receptor (where the interaction with the full length CGRP agonist is impaired) is reduced to the same degree as the potency of CGRP. If this the case, then it would imply that the mutated residue interacted with both CGRP and $CGRP_{8-37}$. By contrast, if the effect of $CGRP_{8-37}$ is the same for wild type (WT) and mutant receptors, it follows that the mutated residue is interacting just with the first seven residues of CGRP (either directly or by affecting a CGRP-independent activation process of the receptor). As labelled $CGRP_{8-37}$ is not readily available to allow a direct measure of its affinity, this is assessed indirectly by looking at the shift the antagonist causes in the concentration-response curve to CGRP. If the same parallel rightward shift is observed in both WT and mutant receptors, then the affinity and mode of antagonism is also the same.

For this study, we have targeted receptor mutations that either had the biggest reduction in cAMP signalling (R274A, D280A and W283A) or those that were predicted to make ligand contacts in our earlier study (I284A, D287A and T288A) [12].

The binding affinity, expressed as Log Kd (pKd) for $CGRP_{8-37}$ to the WT and mutant receptors was estimated either by the shift in the dose ratio where there was no significant change in maximum response (Emax) in the presence of the antagonist (pKd = (Log{[$CGRP_{8-37}$]/Dose-ratio -1} or by the method of Gaddum where the Emax was depressed [19]. Cos 7 cells were transiently transfected with the WT or mutant receptor construct in a mammalian expression vector pcDNA3.1- (Invitrogen, UK) and co-transfected with a pcDNA3/RAMP1 construct as described previously [12]. Cells were stimulated with CGRP agonist (over a concentration range of 10^{-12} M to 10^{-5} M) in the presence or absence of 10^{-7} M $CGRP_{8-37}$ antagonist and cAMP was measured using the FRET-based Lance-assay as described [20]. 10^{-7} M $CGRP_{8-37}$ was used as lower concentrations of $CGRP_{8-37}$ gave CGRP activation curves that were poorly resolved from the control in the absence of antagonist; higher concentrations gave incomplete curves (data not shown).

The signalling curves for this study are shown in figure 1. The Emax values of the alanine substitution receptor (+ $CGRP_{8-37}$) were compared to a normalised alanine substitution receptor – $CGRP_{8-37}$ (set to 100%) and compared using a one sample t test (table 1 and figure 2). The computed pKd values are shown in table 1.

I284A resulted in a significant reduction in the affinity of $CGRP_{8-37}$ (table 1). It is likely therefore that the reduction in cAMP signalling observed with the I284A mutant [12] is caused by a disruption of the receptor's ability to interact with the CGRP 8-37 region. There was also a small reduction in the affinity for W283A. Conversely, the shift in the CGRP concentration-response curve caused by the $CGRP_{8-37}$ antagonist at the R274A, D280A, and T288A receptors was not significantly different to that seen for the WT CGRP receptor. This suggests that the deleterious effects on signalling of these mutants previously seen [12] is not mediated by the CGRP 8-37 region and therefore, stabilises direct or indirect interactions between the CGRP receptor and the first seven residues of CGRP (the activation domain). However, for all of these mutants as well as W283A, $CGRP_{8-37}$ appeared to act non-competitively at the mutants, with significant reductions in Emax (Figure 2). D287A had no effect on CGRP potency, in contrast to our previous study [12].

The apparent change in mode of antagonism is unexpected. Caution is needed with this interpretation, as it was not possible to construct full concentration-response curves to CGRP in the presence of $CGRP_{8-37}$ for the mutants with lower CGRP potency, as the required concentrations of CGRP would be impractical. However, when we fit "expected" concentration-response curves in accordance with competitive inhibition assuming a full Emax (dotted line graphs in figure 1), then the shift in the curve for CGRP for the mutant receptors would be even greater than for the WT receptors; i.e. the affinity for $CGRP_{8-37}$ would have increased. In either interpretation, these mutations give unexpected effects.

There was no evidence of any non-competitive inhibition by $CGRP_{8-37}$ on the WT receptor, even when cells were transfected with only 10% of the normal CLR/RAMP1 cDNA (data not shown). Under these conditions, any receptor reserve will have been removed (the maximum stimulation of cAMP was reduced by 93%), which might otherwise mask non-competitive behaviour of antagonists. There was still a clear shift of the CGRP concentration response curve in the presence of 10^{-7} M $CGRP_{8-37}$ (shift 1.54 ± 0.35, n=3), but there was no significant change in the Emax in the presence of the antagonist (125 ± 25% of CGRP alone).

Of the residues examined in this study, only I284 is predicted to face bound CGRP in a model of CLR (Fig 3); interestingly it interacts with the 8-18 helix of the peptide. T288 may have a weak interaction with the extreme N-terminus of CGRP, but the other residues face into the binding pocket. The model must be treated with caution, as we have not included RAMP1, but the findings are in agreement with our functional assays. We speculate that the R274, D280 and W283 and (to a lesser extent) T288 are important for maintaining the architecture of the peptide-binding pocket. In support of this, there is a correlation between the reduction in CGRP potency at the mutants and the decrease in Emax in the presence of $CGRP_{8-37}$ (Fig 2b). We suggest that when R274, D280 and W283 and T288 are mutated, residues 1-7 of CGRP no longer fit easily into this pocket, whereas $CGRP_{8-37}$ binds relatively unimpaired. This may slow the association of CGRP to the mutated receptors, resulting in apparent non-competitive inhibition with $CGRP_{8-37}$ under the conditions of our assay.

This work is the first mutational evidence to map the interaction of CGRP within ECL2 of its receptor. The data suggests that the mode of interaction of $CGRP_{8-37}$ with the CGRP receptor is likely to be more complicated than predicted from a simple consideration of the two-domain model [14]. In a previous structure-activity study, we demonstrated that mutations to residues 8 and 9 of CGRP gave partial agonists, challenging a simplistic notion that $CGRP_{1-7}$ is the sole determinant of receptor activity [21]. The current data suggests that the ECL2 interaction also extends beyond the extreme N-terminus of the peptide; the model indicates that they can interact with ECLs 2 and 3 and so directly influence the TM bundle. This helps our understanding of receptor activation as well as emphasising the key role of ECL2 in the CGRP receptor.

Figure 1. Dose response curves of Cos7 cells transfected with WT CGRP receptor (WT CLR + R1) or alanine substitution receptor (mutant CLR + R1) incubated with and without $CGRP_{8-37}$ (10^{-7} M) stimulated with CGRP over a logarithmic concentration range of (10^{-12} M to 10^{-5} M). Raw cAMP values were normalized to WT (0M $CGRP_{8-37}$) basal and Emax values. The curves show means ± s.e.m values of 3-5 determinations. The broken lines in the graphs of the mutant receptors show the fit if it is assumed that the maximum response is unchanged in the presence of $CGRP_{8-37}$.

	pKd WT	pKd mutant
R274A	7.64 ± 0.10	7.40 ± 0.05
D280A	7.41 ± 0.01	7.43 ± 0.15
W283A	7.59 ± 0.03	7.13 ± 0.14*
I284A	7.77 ± 0.09	6.85 ± 0.12**
D287A	7.79 ± 0.33	7.65 ± 0.35
T288A	7.54 ± 0.18	7.87 ± 0.21

Table 1. Differences in the $CGRP_{8-37}$ pKd values at WT and mutant receptors Values are means ± s.e.m., *,** p value < 0.05, 0.01, unpaired Student's t-test)

n e)

Figure 2a. % Emax values of mutants in the presence of $CGRP_{8-37}$ relative to Emax values without antagonist. Significant differences were determined using a one sample t test. * p value < 0.05. b) Correlation between pEC_{50} for CGRP and change in Emax at alanine mutants.

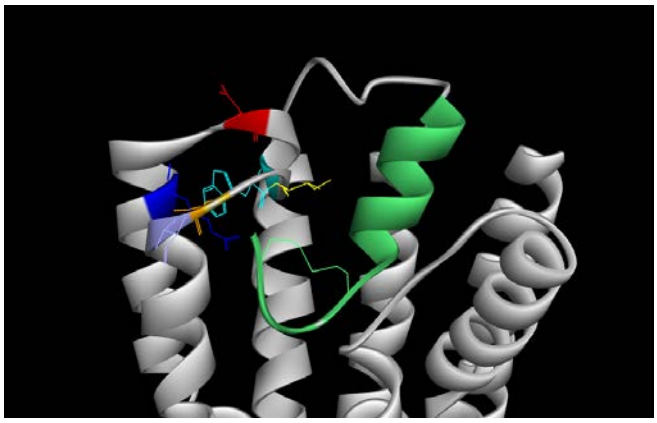

Figure 3. Model of the ECL2 region of CLR, showing the bound N-terminus (residues 1-18) of CGRP in green. R274, blue; D280, red; W283, light blue; I284, yellow; D287, orange; T288, purple.

ASSOCIATED CONTENT

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Notes

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ABBREVIATIONS

(Word Style "Section_Content"). CLR, calcitonin receptor-like receptor; CGRP, calcitonin gene related peptide; ECL, extracellular loop GPCR, G protein-coupled receptor; TM. transmembrane; WT, wild-type.

REFERENCES

1. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289: 739-745.

2. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, et al. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318: 1258-1265.

3. Chien EY, Liu W, Zhao Q, Katritch V, Han GW, et al. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. Science 330: 1091-1095.

4. Wu H, Wacker D, Mileni M, Katritch V, Han GW, et al. (2012) Structure of the human kappa-opioid receptor in complex with JDTic. Nature 485: 327-332.

5. Haga K, Kruse AC, Asada H, Yurugi-Kobayashi T, Shiroishi M, et al. (2012) Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. Nature 482: 547-551.

6. Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, et al. (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. Nature 454: 486-491.

7. Hollenstein K, Kean J, Bortolato A, Cheng RK, Dore AS, et al. (2013) Structure of class B GPCR corticotropin-releasing factor receptor 1. Nature 499: 438-443.

8. Siu FY, He M, de Graaf C, Han GW, Yang D, et al. (2013) Structure of the human glucagon class B G-protein-coupled receptor. Nature 499: 444-449.

9. Jazayeri A, Dore AS, Lamb D, Krishnamurthy H, Southall SM, et al. (2016) Extra-helical binding site of a glucagon receptor antagonist. Nature 533: 274-277.

10. Koole C, Wootten D, Simms J, Miller LJ, Christopoulos A, et al. (2012) Second extracellular loop of human glucagon-like peptide-1 receptor (GLP-1R) has a critical role in GLP-1 peptide binding and receptor activation. J Biol Chem 287: 3642-3658.

11. Gkountelias K, Tselios T, Venihaki M, Deraos G, Lazaridis I, et al. (2009) Alanine scanning mutagenesis of the second extracellular loop of type 1 corticotropin-releasing factor receptor revealed residues critical for peptide binding. Mol Pharmacol 75: 793-800.

12. Woolley MJ, Watkins HA, Taddese B, Karakullukcu ZG, Barwell J, et al. (2013) The role of ECL2 in CGRP receptor activation: a combined modelling and experimental approach. J R Soc Interface 10: 20130589.

13. Hollenstein K, de Graaf C, Bortolato A, Wang MW, Marshall FH, et al. (2014) Insights into the structure of class B GPCRs. Trends Pharmacol Sci 35: 12-22.

14. Hoare SR (2005) Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. Drug Discov Today 10: 417-427.

15. Brain SD, Grant AD (2004) Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev 84: 903-934.

16. Smillie SJ, Brain SD (2011) Calcitonin gene-related peptide (CGRP) and its role in hypertension. Neuropeptides 45: 93-104.

17. Russell FA, King R, Smillie SJ, Kodji X, Brain SD (2014) Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev 94: 1099-1142.

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

19. Kenakin TP (2014) A Pharmacology Primer: Techniques for more effective and strategic drug discovery: Academic Press, Elsevier.

20. Hunter MR, Glass M (2015) Increasing the flexibility of the LANCE cAMP detection kit. J Pharmacol Toxicol Methods 71: 42-45.

21. Hay DL, Harris PW, Kowalczyk R, Brimble MA, Rathbone DL, et al. (2014) Structure-activity relationships of the N-terminus of calcitonin gene-related peptide: key roles of alanine-5 and threonine-6 in receptor activation. Br J Pharmacol 171: 415-426.

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