Structure/function analysis of the second extracellular loop of the CGRP receptor

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CGRP is a 37 amino acid neuropeptide which binds to the family B G protein-coupled receptor (GPCR) calcitonin receptor-like receptor (CLR) and the accessory protein receptor activity modifying protein 1 (RAMP1), resulting in vasodilation and inflammation (Walker et al, 2010). In family A GPCRs the extracellular loop 2 (ECL2) domain has roles in ligand binding and activation however this domain and the overall mechanism of activation in family B GPCRs is not well understood (Barwell et al, 2011). Modelling from our group suggested that ECL2 starts at an alanine at position 271 (A271) at the top of transmembrane region 4 (TM4) and extends to an isoleucine (I294) at the top of TM5. There is thought to be a disulphide bond formed between a central cysteine residue of ECL2 (C260) and C190 of TM3.

In this work, all 24 residues of the ECL2 domain of the CGRP receptor have been mutated to alanine (or alanine to leucine) using site-directed mutagenesis of a mammalian CLR expression plasmid with an N-terminal HA-tag described previously (Conner et al, 2006). The constructs have been transiently co-transfected into COS7 cells with a RAMP1 plasmid and the effect of these mutations on receptor activation was measured through CGRP-mediated cAMP accumulation and internalisation, as described previously (Conner et al, 2006). Concentration response curves to CGRP were fitted to a sigmoidal function using Graphpad Prism 4 to estimate basal and maximum cAMP responses and also pEC_{50} values. Statistical analysis was by one-way ANOVA followed by Dunnett's test to compare mutants against controls.

This research has identified five alanine mutations of ECL2 (R274A, Y278A, D280A, W283A and T288A) that resulted in a significant, greater than 10-fold, reduction in EC_{50} cAMP signalling ($P<0.05$; N=3 in all cases). Four further mutations (S285A, L290A, L291A and Y292A) resulted in a significant, smaller (less than 10-fold) reduction in signalling ($P<0.05$; N=3 in all cases). Two potential, constitutively active mutants (CAMs; N281A and I294A) have been identified, however further analysis is needed to confirm this effect. A tyrosine to alanine substitution (Y277A) had significantly reduced signalling (approximately 10-fold; N=3) but retained wild type binding affinity (data not shown). Of these mutants, 5 have significantly ($P<0.01$; N=3 in all cases) reduced CGRP-mediated receptor internalisation with D280A, C282A and W283A alanine mutants totally abolishing receptor internalisation (presumably due to binding deficiency).

In summary, this research has identified key residues within the ECL2 domain of CLR involved in CGRP ligand binding and activation. This also confirms the key involvement of the ECL2 domain in the ligand-mediated activation of the calcitonin-family of family B GPCRs, which until recently, had only been described in family A GPCRs.

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