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Receptor activity-modifying protein dependent and independent activation mechanisms in the coupling of calcitonin gene-related peptide and adrenomedullin receptors to Gs

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ABSTRACT

Calcitonin gene-related peptide (CGRP) or adrenomedullin (AM) receptors are heteromers of the calcitonin receptor-like receptor (CLR), a class B G protein-coupled receptor, and one of three receptor activity-modifying proteins (RAMPs). How CGRP and AM activate CLR and how this process is modulated by RAMPs is unclear. We have defined how CGRP and AM induce Gs-coupling in CLR-RAMP heteromers by measuring the effect of targeted mutagenesis in the CLR transmembrane domain on cAMP production, modeling the active state conformations of CGRP and AM receptors in complex with the Gs C-terminus and conducting molecular dynamics simulations in an explicitly hydrated lipidic bilayer. The largest effects on receptor signaling were seen with H295A$^{5.40b}$, I298A$^{5.43b}$, L302A$^{5.47b}$, N305A$^{5.50b}$, L345A$^{6.49b}$ and E348A$^{6.52b}$, F349A$^{6.53b}$ and H374A$^{7.47b}$ (class B numbering in superscript). Many of these residues are likely to form part of a group in close proximity to the peptide binding site and link to a network of hydrophilic and hydrophobic residues, which undergo rearrangements to facilitate Gs binding. Residues closer to the extracellular loops displayed more pronounced RAMP or ligand-dependent effects. Mutation of H374$^{7.47b}$ to alanine increased AM potency 100-fold in the CGRP receptor. The molecular dynamics simulation showed that TM5 and TM6 pivoted around TM3. The data suggest that hydrophobic interactions are more important for CLR activation than other class B GPCRs, providing new insights into the mechanisms of activation of this class of receptor. Furthermore the data may aid in the understanding of how RAMPs modulate the signaling of other class B GPCRs.

1. Introduction

Class B G protein-coupled receptors (GPCRs) are a small but physiologically and therapeutically important sub-group of the GPCR superfamily. They are involved in a diverse range of physiological responses such as vasodilation, stress, digestion and glucose homeostasis [2]. This has made them important drug targets for a range of human diseases including diabetes, obesity, cancer, cardiovascular disease and migraine [22].

Calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) are peptide ligands that bind to heterodimers of the class B calcitonin receptor-like receptor (CLR), in association with a single transmembrane receptor activity-modifying protein (RAMP1, 2 or 3). CGRP binds the CLR/RAMP1 complex with high affinity but AM can also bind to this receptor. AM binds to both CLR/RAMP2 (AM_1 receptor) and CLR/RAMP3 (AM_2 receptor) with high affinity. At the human AM receptors, CGRP binds very weakly [1,5,52]. CGRP and AM are potent vasodilators and have been implicated in cardiovascular disease [55]. The CGRP system is under intense drug scrutiny as a target for migraine [40]. Thus, understanding the activation mechanism of these receptors and how RAMPs affect this, is of considerable importance for drug development.

GPCR activation, once thought to be a simple on/off switch mechanism, has become increasingly defined by its complexity. A receptor is able to exist in multiple conformations, stabilized by

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different ligands, resulting in activation or inactivation of several possible signaling pathways and requiring the allosteric effects of a bound G protein to achieve maximum affinity for agonist binding [47,16,29,28].

Most understanding of the activation mechanism of GPCRs comes from class A GPCRs, where activation involves conformational changes in the transmembrane helices (TM). The movement of the extracellular ends of TM helices is a key process in the activation mechanism of GPCRs, as is the stabilization of conformational changes through inter-helical interactions of both polar and hydrophobic residues, often involving conserved motifs [47,18]. Individual agonists produce a variety of changes around their binding pockets, but these converge to produce changes in the upper half of the TM bundle, which are propagated to the cytoplasmic end of the bundle. The most significant movement is a rigid-body rotation of the bent TM6 (accompanied by some torsional changes in the vicinity of P5.50), thus opening the cleft required for G protein-binding [47,45].

Three published X-ray crystal structures of class B GPCRs, all in the inactive conformation [43,23,26], were available when this article was submitted; more recent cryo-electron microscopy structures of the active form are discussed below. These three class B X-ray structures show structural similarity with the class A crystal structures on the intracellular (G protein-binding) half of the TM bundle. The extracellular side however is more open. Despite this, molecular dynamics simulations of the corticotropin-releasing factor receptor 1 (CRF1R) suggested that activation of the receptor involved an outward movement of TM5 and 6, consistent with class A [42]. However, in the inactive class B X-ray structures TM6 is not uniformly bent as in class A; TM6 is relatively straight in the glucagon receptor [43,26]. Consequently, the anticipated outward movement of TM6 in class B GPCRs may have a more significant torsional component. A network of hydrophilic interactions between TM helices has been suggested in the class B glucagon-like peptide-1 receptor (GLP-1R) [60,58]. Conserved polar residues within the TM helices of the GLP-1R are important in coordinating either global receptor activation conformational changes or for fine-tuning responses, leading to biased signaling. The role of hydrophobic residues within the TM bundle in class B GPCRs largely remains to be addressed, but in class A GPCRs hydrophobic residues play an important role in facilitating the conformational change [47,18].

RAMPs can significantly alter the pharmacology and signaling of class B GPCRs but the mechanisms are poorly understood [53,57,11,54,21,48,35]. For CGRP and AM receptors, there is a direct interaction of the RAMP extracellular domains with the C-terminus of the peptide [10], but there is also evidence for RAMP affecting the GPCR extracellular loops (ECLs) [51]. Thus their effects on the entire GPCR need to be considered.

How RAMPs affect the activation mechanisms of class B GPCRs is not known. Furthermore, it is unclear whether CLR has unique features compared to other class B GPCRs, given its obligate requirement for RAMPs. We have addressed these questions in CGRP and AM receptors using an integrated experimental and computational approach, to provide a model with which to compare the effect of different RAMPs on the GPCR activation mechanism. We used structural models to select amino acids that we hypothesized are most likely to be involved in stabilizing conformational changes. Mutants were pharmacologically characterized and computational simulation of the inactive to activation transition of CLR/RAMP complexes with Gs was used to interpret the results. This has allowed us to suggest a mechanism for receptor activation leading to Gs coupling for CLR. This shows commonalities but also special features compared to other class B GPCRs.

2. Materials and methods

2.1. Materials

Human αCGRP and human AM (AM 1–52), were from American Peptide (Sunnyvale, CA, USA) or Bachem (Bubendorf, Switzerland). Forskolin was from Tocris Bioscience (Bristol, UK). LANCE cAMP assay kits and all reagents and plates were from PerkinElmer (Waltham, MA, USA). [^{125}I]-human alpha iodohistidyl^{10}-CGRP and [^{125}I]-human (13–52) iodotyrosyl^{52}adrenomedullin (^{125}I-AM) were also purchased from Perkin Elmer. All other chemicals were from Sigma.

2.2. Expression constructs and mutagenesis

Human CLR with an N-terminal haemagglutinin (HA) epitope was mutated using a method based on the Quik Change II site-directed mutagenesis kit (Stratagene, Cambridge, UK) and described previously [4]. Human RAMP 1 with an N-terminal myc epitope tag [56], human RAMP2 with an N-terminal FLAG epitope tag [38] and untagged human RAMP3 were also used [12].

2.3. Cell culture and transfection

Culture of Cos7 cells was performed as previously described [3]. These cells were originally obtained from the American Type Culture Collection and cells were used between passages 16 and 32. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 8% heat-inactivated foetal bovine serum and kept in a 37 °C humidified 95% air, 5% CO_2 incubator. For cAMP assays and cell surface expression ELISAs, cells were seeded into 96-well plates at a density of 15,000 cells per well (determined using a Countess Counter™, Invitrogen, Carlsbad, CA, USA) 1 day before transfection. Cells were transiently transfected using polyethylenimine (PEI) as described previously [3] using a 1:1 ratio of CLR to RAMP.

2.4. Cell surface expression ELISA

Cell surface expression of all RAMP/HA-CLR receptor complexes was assessed by measuring HA-CLR expression in an ELISA as previously described [12,4], with some modifications. Paraformaldehyde (8%, 100 μL) in PBS was added to each well of a 96 well plate containing transfected cells and the plate was incubated at room temperature with gentle shaking for 20 min. The cells were washed twice in PBS (100 μL per well). A 1% solution of BSA or 10% goat serum (100 μL) in PBS was added to each well to block nonspecific protein interactions and incubated at room temperature for 1 h. The wells were aspirated and 50 μL of anti-HA monoclonal primary antibody (Sigma H-9658), diluted 1:2000 in 1% BSA or 1% goat serum in PBS, was added to each well and incubated at room temperature for 1 h. The wells were aspirated and washed once in PBS before adding 50 μL anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma A-4416), diluted 1:2000 in 1% BSA or 1% goat serum in PBS at room temperature for 1 h. The wells were aspirated and washed twice in PBS before adding 50 μL of o-phenylenediaminedihydrochloride (OPD) solution and incubating this in the dark for 15 min. H_2SO_4 (50 μL, 0.5 M) was added to stop the reaction and absorbances were read at 490 and 650 nm. The wells were aspirated and washed twice in PBS. Cresyl violet working solution (50 μL) was added to each well and incubated at room temperature for 30 min. The wells were washed once in PBS and 1% sodium dodecyl sulphate was added and incubated at room temperature with