Interaction of calcitonin-gene-related peptide with its receptors


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Abstract

The receptor for calcitonin-gene-related peptide (CGRP) is a heterodimer formed by calcitonin-receptor-like receptor (CRLR), a type II (family B) G-protein-coupled receptor, and receptor-activity-modifying protein 1 (RAMP1), a single-membrane-pass protein. It is likely that the first seven or so amino acids of CGRP (which form a disulphide-bonded loop) interact with the transmembrane domain of CRLR to cause receptor activation. The rest of the CGRP molecule falls into three domains. Residues 28–37 and 8–18 are normally required for high-affinity binding, while residues 19–27 form a hinge region. The 28–37 region is almost certainly in direct contact with the receptor; 8–18 may make additional receptor contacts or may stabilize an appropriate conformation of 28–37. It is likely that these regions of CGRP interact both with CRLR and with the extracellular domain of RAMP1.

Introduction

Calcitonin-gene-related peptide (CGRP) is an abundant 37-amino-acid neuropeptide. It forms a family with calcitonin, amylin and adrenomedullin. While these peptides have only limited sequence identity, they share a number of structural features: a six (or seven for calcitonin)-amino-acid ring formed by a disulphide bond at their N-termini, an amphipathic α-helix and a C-terminal amide [1] (Figure 1). In view of the similarity of their structures, it is not surprising that they show cross-reactivity with each other’s receptors. The receptor for CGRP is a heterodimer between a type II G-protein-coupled receptor (GPCR), calcitonin-receptor-like receptor (CRLR), and a single-pass transmembrane protein, receptor-activity-modifying protein 1 (RAMP1). The receptor for adrenomedullin is formed by a dimer between CRLR and RAMP2, a homologue of RAMP1. A second adrenomedullin receptor is formed by the interaction of CRLR with a third member of the RAMP family, RAMP3 [2]. There is pharmacological evidence for a second subtype of CGRP receptor, the CGRP2 receptor, but its molecular nature remains unclear [3]. The CRLR-RAMP1 complex forms the CGRP1 receptor.

In view of the novel heterodimeric structure of the CGRP and adrenomedullin receptors, the molecular nature of the interaction between the peptides and their receptors is of considerable interest. This review will focus on the interaction of CGRP with RAMP1–CRLR, in order to suggest mechanisms that might be involved.

Structure of CGRP

CGRP has been examined by NMR and other physical techniques. Its N-terminus is constrained by a disulphide bond between residues 2 and 7. In aqueous solution, the rest of the molecule shows little secondary structure. However, in more hydrophobic solvents there is good evidence for an α-helix between residues 8 and 18 [4,5], followed by either a β- or a γ-turn in the region of residues 19–22 [6,7]. There then follows a largely unstructured section of the molecule. However, the C-terminus appears to show more order, with several studies noting turns in the regions of residues 28 and 30, and also 32 and 34 [5,6,8,9]. There is evidence that long-range interactions exist between different parts of the molecule; an NMR study concluded that alanine substitutions at positions 17, 20 and 21 altered the conformation of the C-terminus [10].

Structure–activity relationship for CGRP

N-terminus

CGRP is a good example of a peptide conforming to the ‘message–address’ concept [11]. Removal of the first seven amino acids gives CGRP-(8–37), a
fragment that still binds with high affinity but which is an antagonist [12]. Thus residues 1–7 (which includes the disulphide-bonded loop) activate the receptor. It has been suggested that two linear analogues of CGRP (in which the cysteines are derivatized with either acetamidomethyl or ethylamide groups) are selective agonists for the CGRP<sub>2</sub> receptor, but as these are partial agonists (at least under some circumstances), their use is controversial [3]. Little work has been done to probe the detailed structural requirements of this part of the molecule.

**Residues 8–18**

Residues 8–18 of CGRP form a potential amphipathic α-helix [13]. Its deletion causes an approx. 100-fold loss of affinity (Table 1). By contrast, this region can be replaced by various amphipathic sequences with relatively little loss of affinity [13].

While some disruption of helical structure may be tolerated by this region, gross changes, such as the introduction of a proline residue, lead to a large reduction in affinity [1].

The helix has two positively charged arginines at positions 11 and 18. Replacement of either of these individually with alanine produces only modest decreases in affinity (< 5-fold); however, a double alanine substitution causes a > 100-fold decrease in affinity ([14,15]; Table 1). Thus, while individually the residues can compensate for each other in promoting high-affinity binding, one or other must be present to allow an effective interaction with the receptor. In principle, the arginine side chains could interact either with other amino acids (either on the receptor or elsewhere on the peptide) or with solvent water molecules, orientating the helix so that its hydrophobic face is in contact with the receptor. This

**Figure 1**

Structure of human αCGRP

<table>
<thead>
<tr>
<th></th>
<th>1–7, receptor activation</th>
<th>8–18, receptor binding</th>
<th>19–27, hinge region</th>
<th>28–37, receptor binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ACDTATC</td>
<td>VTHRLAGLSRSGGVVKNFVPTNVGSKAFCNH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disulphide</td>
<td>α-helix</td>
<td>Turn</td>
<td>Turn</td>
<td>Turn</td>
</tr>
</tbody>
</table>

**Table 1**

Apparent pK<sub>a</sub> values for peptide antagonists on SK-N-MC (human CGRP<sub>1</sub>), L6 (rat CGRP<sub>1</sub>) and Col 29 (human CGRP<sub>2</sub>) cells

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>SK-N-MC</th>
<th>L6</th>
<th>Col 29</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Tyr&lt;sup&gt;6&lt;/sup&gt;]CGRP-(28–37)</td>
<td>5.27±0.07</td>
<td>&lt; 5.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>[Asp&lt;sup&gt;3&lt;/sup&gt;, Pro&lt;sup&gt;5&lt;/sup&gt;, Phe&lt;sup&gt;12&lt;/sup&gt;]CGRP-(27–37)</td>
<td>7.67±0.22</td>
<td>7.40±0.13</td>
<td>6.25±0.18</td>
</tr>
<tr>
<td>[Asp&lt;sup&gt;3&lt;/sup&gt;, Pro&lt;sup&gt;5&lt;/sup&gt;, Phe&lt;sup&gt;12&lt;/sup&gt;]CGRP-(28–37)</td>
<td>7.50±0.11</td>
<td>7.09±0.07</td>
<td>5.70±0.19</td>
</tr>
<tr>
<td>[Asp&lt;sup&gt;3&lt;/sup&gt;, Pro&lt;sup&gt;5&lt;/sup&gt;, Phe&lt;sup&gt;12&lt;/sup&gt;]CGRP-(8–18,28–37)</td>
<td>6.79±0.13</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGRP-(8–18,22–37)</td>
<td>7.06±0.12</td>
<td>8.30±0.20</td>
<td>6.60±0.22</td>
</tr>
<tr>
<td>CGRP-(8–21,28–37)</td>
<td>7.38±0.18</td>
<td>8.20±0.20</td>
<td>6.90±0.20</td>
</tr>
<tr>
<td>CGRP-(8–18,28–37)</td>
<td>6.42±0.26</td>
<td>n.d.</td>
<td>6.17±0.20</td>
</tr>
<tr>
<td>Mastoparan–CGRP-(28–37)</td>
<td>6.64±0.16</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGRP-(19–37)</td>
<td>5.89±0.20</td>
<td>6.1±0.24</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>[Ala&lt;sup&gt;11,18&lt;/sup&gt;]CGRP-(8–37)</td>
<td>6.60±0.30</td>
<td>5.3±0.04</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGRP-(8–37)</td>
<td>7.49±0.25</td>
<td>7.95±0.14</td>
<td>6.48±0.28</td>
</tr>
</tbody>
</table>

Values are means±S.E.M. (n = 3–5). n.d., not determined. Data are taken from [14,15], or D. R. Poyner and S. G. Howitt, unpublished work.
has been investigated by substituting the arginines with serines; these should allow hydrogen bonding with water molecules, but would not compensate for a loss of ionic interactions. [Ser"CGRP-(8-37) binds with 100-fold lower affinity than CGRP-(8-37), suggesting that the two arginines are involved in charge-charge interactions [16]. The spatial constraints restricting the hydrophobic side of the helix have been investigated by replacing the leucines at positions 12, 15 and 16 with phenylbenzoic acid residues. There is a large decrease in affinity when Leu" is substituted, suggesting that it fits into a restricted space.

**Residues 19-27**

Apart from a turn involving residues 19–22, no secondary structure has been demonstrated for this portion of the molecule. Deleting it in its entirety causes an approx. 10-fold decrease in the affinity of CGRP-(8-37), showing that it is of significance in binding. Addition of either residues 19–21 or residues 22–27 to CGRP-(8-18,28-37) restores affinity close to that of CGRP-(8-37) (Table 1). It seems that this part of the molecule is necessary as a spacer or hinge region, but there are no stringent constraints on its composition.

**Residues 28-37**

Residues 28–37, by themselves, show only weak binding to the CGRP receptor (Table 1). However, by making a few amino acid substitutions, a high-affinity antagonist can be generated [9,17] (Table 1). These appear to work by stabilizing a high-affinity conformation of the peptide. Presumably in full-length CGRP the helix comprising residues 8–18 induces this conformation in the C-terminus, either directly or as a consequence of its own receptor interactions. The key amino acids are Thr"Val"Gly"and Phe" with a C-terminal amide. Bends centred on residues 29 and 34 maximize the solvent exposure of the side chain of Thr" and place it 8 Å from the side chain of Val" and 17 Å from the side chain of Phe". It has been suggested that these amino acids are in direct contact with the receptor [9]; presumably Gly" plays a structural role in holding the peptide in an appropriate conformation, perhaps in a sterically confined pocket.

**Structure–activity studies on CRLR/RAMP1**

Little is known about how either CGRP or adrenomedullin interact with their receptors. In human CRLR, the first 18 amino acids are required for adrenomedullin (but not CGRP) binding [18]. Asp" close to the start of the first transmembrane helix, is a putative glycosylation site in human CRLR. It actually plays little part in glycosylation, but its mutation to alanine, threonine or proline (but not aspartic acid) impairs CGRP binding and signal transduction without greatly altering membrane expression [19]. Adrenomedullin binding and transduction are also impaired by mutating this site [20].

The above data relate entirely to ligand interactions with the extracellular N-terminus of CRLR. It would be surprising if CGRP and adrenomedullin made no contacts with other parts of CRLR, i.e. the extracellular loops or the transmembrane domain. It is possible that the ligand N-terminus may interact with the transmembrane regions, as conformational changes in the latter are responsible for receptor activation. There is considerable evidence for multiple contacts between other type II GPCRs and their ligands. For example, it has been suggested that parathyroid hormone makes contact with both the N-terminus and the juxtamembrane region of the receptor [21]. There are also key hydrophilic residues within the transmembrane regions in this and other receptors, which are essential for ligand binding and receptor activation [22]. There are over 20 hydrophilic amino acids within the transmembrane domain of CRLR, with a particular concentration in the first two transmembrane helices. In helix 1 there is a distinct hydrophilic face. Preliminary data from an alanine scan suggest that this is not involved in receptor activation; perhaps it presents the other face of the helix as a RAMP interaction site.

The mechanism of action of the RAMPs in CGRP/adrenomedullin binding is unknown. Studies with chimaeric RAMPs have shown that the N-terminus of RAMPl is the key determinant for expression of CGRP binding [23]; this would be expected to interact with the N-terminus of CRLR. In human RAMPl, substituting the tryptophan at position 74 with the lysine found in rat RAMPl confers the lower-affinity, rat CRLR–RAMP1 pharmacology for binding of the non-peptide CGRP antagonist BIBN4096BS [24]. Residues 86–92 of human RAMP2 and 59–65 of human RAMP3 are needed for adrenomedullin binding [25]; the data from that study suggest that the main epitope of RAMP1 needed for CGRP binding is between residues 76 and 124. The RAMPs may act allosterically on the N-terminus of CRLR, or they may physically form part of the
binding site. In support of the latter possibility, CGRP and adrenomedullin can be cross-linked to RAMP1 or RAMP2 in addition to CRLR [26,27]. Both CGRP and adrenomedullin have been reported to label 17 kDa bands in cross-linking experiments conducted on a variety of tissues; these bands are the size expected for RAMPs [28,29].

Little is known about the structure of RAMPs. We have recently expressed the extracellular domain of RAMP1 as a glutathione S-transferase fusion protein in Escherichia coli. The protein appears as inclusion bodies. These can be solubilized in 6 M guanidinium hydrochloride in the presence of a reducing agent and then diluted into a refolding buffer containing an oxidizing agent to give a protein that is soluble at concentrations in excess of 5 mg/ml. This will allow us to determine the structure of the extracellular domain of RAMP1.

A model of CGRP binding?

Given what is known about type II GPCR–ligand interactions and the structure of CGRP, it is tempting to suggest that there are several points of contact. Residues 8–18 and 27–37 of CGRP may interact mainly with a binding pocket made up of the N-termini of CRLR and RAMPL, whereas residues 1–7 and perhaps the N-terminal portion of residues 8–18 may interact with the juxtamembrane regions of CRLR.

CGRP$_2$ receptors

Little has been done to characterize the structural requirements for CGRP binding to CGRP$_2$ receptors. However, it is instructive to compare the affinities of various ligands for CGRP$_1$ and CGRP$_2$ receptors (Table 1). There is a correlation between the values, albeit with the affinities for CGRP$_2$ being about an order of magnitude lower than those for CGRP$_1$. Rorabaugh et al. [30] suggested that the differences between the receptors could be explained by tissue factors such as proteases. However, it is also possible that one or more of the main ligand–receptor contacts is deficient compared with the CGRP$_1$ receptor, resulting in the lower affinities.

It is clear that more work is required to understand the unique heterodimers formed by RAMPs with type II GPCRs.

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References


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Functional relevance of G-protein-coupled-receptor-associated proteins, exemplified by receptor-activity-modifying proteins (RAMPs)

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Abstract

The calcitonin (CT) receptor (CTR) and the CTR-like receptor (CRLR) are close relatives within the type II family of G-protein-coupled receptors, demonstrating sequence identity of 50%. Unlike the interaction between CT and CTR, receptors for the related hormones and neuropeptides amylin, CT-gene-related peptide (CGRP) and adrenomedullin (AM) require one of three accessory receptor-activity-modifying proteins (RAMPs) for ligand recognition. An amylin/CGRP receptor is revealed when CTR is co-expressed with RAMP1. When complexed with RAMP3, CTR interacts with amylin alone. CRLR, initially classed as an orphan receptor, is a CGRP receptor when co-expressed with RAMP1. The same receptor is specific for AM in the presence of RAMP2. Together with human RAMP3, CRLR defines an AM receptor, and with mouse RAMP3 it is a low-affinity CGRP/AM receptor. CTR–RAMP1, antagonized preferentially by salmon CT-(8–32) and not by CGRP-(8–37), and CRLR–RAMP1, antagonized by CGRP-(8–37), are two CCRP receptor isotypes. Thus amylin and CGRP interact specifically with heterodimeric complexes between CTR and RAMP1 or RAMP3, and CGRP and AM interact with complexes between CRLR and RAMP1, RAMP2 or RAMP3.

Introduction

Calcitonin (CT), CT-gene-related peptide (CGRP), adrenomedullin (AM) and amylin are related hormones and neuropeptides. They are characterized by N-terminal six- or seven-amino-acid ring structures linked by disulphide bonds and amidated C-termini, both of which are required for biological activity (for refs., see [1]). CT inhibits osteoclastic bone resorption and, among neural actions, has analgesic properties and inhibits food intake [2]. CGRP and AM are potent vasodilators, causing arterial hypotension (for refs., see [3]). CT and αCGRP are the products of a single gene, with precursor proteins encoded by two alternatively spliced mRNAs being expressed in a tissue-specific manner [1]. CT is expressed in thyroid C-cells, and αCGRP in the peripheral and central nervous system. βCGRP is the product of another closely related gene with stop codons in a CT-encoding non-functional exon. CT/αCGRP knockout mice were hypertensive [4]. Mice lacking the αCGRP gene alone were either hypertensive or had normal cardiovascular regulation and neuromuscular development [5,6]. However, double knockout mice lacking αCGRP and βCGRP remain to be developed. AM knockout mice die at mid-gestation, with extreme hydrops foetalis and cardiovascular abnormalities [7,8]. Heterozygote AM−/+ mice survived to adulthood, but had elevated blood pressure and diminished NO production [8]. Amylin, through inhibition of gastric emptying, improves hyperglycaemia in patients with Type I and Type II diabetes [9]. Amylin knockout mice, on the other hand, present...