Lessons from the Type II Family of G-Protein-Coupled Receptors


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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 CGRP 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 aCGRP 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

Key words: adrenomedullin, calcitonin-gene-related peptide, calcitonin receptor, calcitonin-receptor-like receptor, heterodimeric complex.

Abbreviations used: AM, adrenomedullin; CGRP, calcitonin-gene-related peptide; CRLR, calcitonin-receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; RAMP, receptor-activity-modifying protein; s. salmon.

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Table I

Definition of ligand specificity of the CTR and the CRLR by RAMPs

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<td>CGRP and amylin</td>
<td>CTR I</td>
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<td>Amylin</td>
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<td>CGRP</td>
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<td>AM and CGRP</td>
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with increased plasma insulin and glucose levels [10]. Therefore AM is physiologically important, the effects of the removal of αCGRP and βCGRP are open to debate, and CT and amylin are predominantly pharmacologically relevant.

Receptors for the related peptides CT, CGRP, AM and amylin are linked to cAMP formation, and to a lesser extent to the phospholipase C signalling pathway [1]. Arterial dilatation in different species and tissues occurs through relaxation of the smooth musculature due to cAMP and endothelial NO synthesis. In 1991, the structure of a pig CT receptor (CTR) was revealed through molecular cloning [11]. Homologous rat and human CTR-like receptors (CRLRs) described in 1993 were classed as orphan receptors until the discovery of the receptor-activity-modifying proteins (RAMPs) in 1997 [12-14]. RAMPs 1, 2 and 3, through interaction with CTR or CRLR, are required for the recognition of CGRP, AM and amylin by these receptors (Table 1). Here we review current knowledge on the interactions of CTR and CRLR as examples of G-protein-coupled receptors that require accessory proteins, the RAMPs, for functional expression.

Molecular structures of CTR, CRLR and RAMP1–RAMP3

CTR and CRLR are cell surface receptors with seven putative transmembrane domains. The conserved cysteine residues in the N-terminal extracellular region are characteristic of type II G-protein-coupled receptors [11–13] (Figure 1).

Three human CTR isotypes have been recognized [1,15–17]. CTR1 (Swiss-Prot accession no. P30988) contains 16 amino acids in the first intracellular loop that are absent from CTR2 (GenBank accession no. 137217). Radioligand binding to CTR1 and CTR2 is similar, but
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coupling to the adenylate cyclase and phospholipase C pathways is impaired with CTR1. CTR3 (GenBank accession no. AAC50301) lacks 47 N-terminal amino acids, including the signal sequence, but radioligand binding and cAMP formation of CTR3 transfected into COS cells are indistinguishable from those of CTR2 analysed under the same conditions. A physiologically relevant signalling pathway involves G1-activated adenylate cyclase; 100-fold higher concentrations of CT are required to activate the phospholipase C pathway.

The rat and human CRLRs (Swiss-Prot accession nos. Q63118 and Q16602 respectively) have 50% and 54% amino acid sequence identity with the rat and human CTRs respectively [12,13]. CRLR-encoding mRNAs have been localized in the lung, heart and kidney, which present both CGRP and AM receptor binding sites [13,18]. However, transfection of human and rat CRLRs into mammalian cells initially failed to elicit CGRP- or AM-induced responses [13]. They were therefore considered to be orphan receptors, with the exception of two situations. Transfection of CRLR-encoding cDNA alone into aortic endothelial (RAEC) or osteoblastic UMR-106 cells revealed an AM receptor [19,20]. CGRP was not recognized. Moreover, stable transfection of CRLR-encoding cDNA into HEK293 cells and subcloning led to the expression of a CGRP receptor [21]. The same cDNA transfected into other cells was non-functional [13]. Thus the RAEC, UMR-106 and HEK cell backgrounds were essential for the functional expression of AM or CGRP receptors.

Discovery of the RAMPs

Injection into Xenopus oocytes of cRNA from a library derived from human neuroblastoma cells with an endogenous CGRP receptor led to the identification of a protein that enhanced the cAMP response to CGRP [14] (Figure 1, Table 1). RAMP1 thus identified (Swiss-Prot accession no. O60894) is a 148-amino-acid protein with a 26-amino-acid N-terminal signal peptide, an extracellular domain of approx. 90 amino acids, a single transmembrane domain of 20 amino acids and a C-terminal tail of 10 amino acids. In mammalian cells, RAMP1 is also essential for CRLR to function as a CGRP receptor. The cellular background postulated in HEK cells that were responsive to the transfected CRLR alone presumably corresponds to RAMP1 [21] (see above).

Homology searches for expressed sequence tags related to RAMP1 have led to the discovery of sequences encoding RAMP2 and RAMP3 (Swiss-Prot accession nos. O60895 and O60896 respectively) [14]. Fewer than 30% of the amino acids of RAMP1, RAMP2 and RAMP3 are identical. RAMP1 and RAMP3 have six N-terminal conserved cysteine residues in the extracellular domain, four of which are also present in RAMP2 (Figure 1). RAMP2 and RAMP3 have up to four N-glycosylation sites, whereas RAMP1 is not glycosylated. DPPXX and LVVWXSK sequences adjacent to the transmembrane domain are conserved in all the RAMPs. The relative positions of the conserved cysteines in the extracellular domain of the RAMP resemble those in CTR and CRLR, even though the amino acid sequences show otherwise little identity [1].

In COS cells, which have no detectable endogenous RAMPs, co-transfection of CRLR- and RAMP2-encoding cDNAs revealed an AM receptor that cross-reacted with CGRP at high concentrations [20,22]. This receptor complex resembles those of RAEC and NG108-15 cells, which are antagonized by ADM-(22-52), but not by CGRP-(8-37) [19,23]. Another AM receptor, e.g. of rat astrocytes, recognizes CGRP and is antagonized by CGRP-(8-37) [3,23]. The role of RAMP3 is less clear. RAMP3 confers AM or AM/CGRP responsiveness to the CRLR [14,22]. Inhibition by RAMP3 of RAMP1-induced CGRP receptor expression suggests an additional regulatory role for RAMP3 [19]. Similarly, RAMP2-evoked AM receptor expression was inhibited by RAMP1, consistent with competitive interactions between the different RAMPs and the CRLR [20].

High-affinity binding sites for amylin, CGRP and salmon (s)CT have been detected in the nucleus accumbens of the rat central nervous system [24]. Along these lines, certain cells that express CTR bind amylin, CT and CGRP specifically [25-27]. Co-transfection of RAMP1- or RAMP3- and CTR2-encoding cDNAs resulted in the expression of functional amylin receptors [28,29]. RAMP1 yielded a receptor that bound CGRP, amylin and sCT, much like the receptor binding sites found in the nucleus accumbens [24]. With co-transfected RAMP1 and CTR2, CGRP stimulated cAMP formation at a 1000-fold lower concentration than with CTR2 alone [28]. This response was preferentially antagonized by sCT-(8-32), and not by CGRP-(8-37) [30]. The RAMP1-CRLR complex, on the other hand, was antagonized by CGRP-(8-37), and therefore re-
presents another CGRP receptor isotype. On co-transfection of RAMP3- and CTR2-encoding cDNAs a different amylin receptor subtype was revealed that did not recognize CGRP to any great extent [28].

**The receptor-RAMP complex**

Heterodimers of RAMP and CTR or CRLR that define amylin, CGRP and AM specificity have been recognized at the cell surface with the use of a membrane-impermeable protein cross-linker [14,30]. Importantly, 125I-labelled human CT cross-linked to CTR2 alone resulted in a protein of lower molecular mass than the 125I-CGRP-CTR2-RAMP1 complex. The ligand-RAMP-receptor complexes were identified by co-immunoprecipitation of fully functional epitope-tagged (for antibody recognition) RAMP and receptors. Localization of the complexes at the cell periphery was revealed by confocal and cell surface immunofluorescence microscopy [30,31]. In the absence of cross-linker, CRLR or CTR was co-immunoprecipitated with RAMP1. RAMP1 was no longer associated with the receptors after denaturing gel electrophoresis under reducing and non-reducing conditions. Heterodimers were therefore not generated through covalent interactions such as the formation of disulphide bonds between cysteine residues.

The human CRLR is core-glycosylated in the absence and presence of RAMP2 or RAMP3. When co-expressed with RAMP1, the same CRLR is increased in size, and resistance to endoglycosidase H indicates mature glycosylation [14]. Drosophila Schneider 2 cells co-transfected with CRLR and RAMP1 or RAMP2 reveal RAMP-independent uniform mature glycosylation, with CGRP and AM receptor functions similar to those of mammalian cells transfected with the same constructs [32]. Thus differential glycosylation of CRLR in the presence of RAMP1 or RAMP2 does not define CGRP or AM receptor specificity.

The importance of the N-glycosylation of CTR and CRLR was analysed through treatment of cells with tunicamycin and site-directed mutagenesis of N-glycosylation consensus sites. Tunicamycin decreased the sizes of CTR and CRLR towards those of the protein backbone, and specific binding of 125I-labelled CT, CGRP or AM was reduced in parallel [33,34]. Mutations of the two N-terminal Asn residues of the human CRLR reduced cell surface expression, and specific radioligand binding was impaired (Figure 1) [34]. However, substitution of the third C-terminal Asn with Thr, Ala, Gln or Pro abolished CRLR function, whereas an Asn→Asp mutant remained fully functional [31] (Figure 1, arrow). N-glycosylation and cell surface expression of the Asp mutant were comparable with those of the wild-type receptor. This C-terminal Asn residue is therefore a 'hot-spot' for the interaction of CGRP and AM with the CRLR-RAMP ternary complex.

The extracellular domains of RAMP1 and RAMP2 chimaera define, at least in part, the specificity of CRLR for CGRP or AM [35]. Removal of the transmembrane domain of RAMP1 obliterates cell surface expression of the RAMP, and CRLR is inactive [36]. With the truncation of the intracellular C-terminus, on the other hand, receptor binding was maintained. Removal of the intracellular phosphorylation consensus site of RAMP1 therefore did not affect CGRP receptor function.

**Other associated proteins and orphan receptors**

The receptor component protein (GenBank accession no. AAC52509) confers CGRP responsiveness in *Xenopus* oocytes [37]. However, a potential functional role for receptor component protein in mammalian cells remains to be clarified.

Non-reproducible results have implied interactions of the receptors L1 (also named G10D) and RDC1 (Swiss-Prot accession nos. P31392 and P11613 respectively) with AM or CGRP [38,39]. These receptor proteins are type I G-protein-coupled receptors, and the sequence identity with the CTR and CRLR is below 10%. Ligand binding and stimulation of cAMP by AM and CGRP examined in different cells transfected with the two receptors in several laboratories did not confirm the initial claims ([40]; results not shown). To all intents and purposes, therefore, L1 and RDC1 remain orphan receptors with no known ligands.

**Outlook and perspectives**

Ligand–receptor–RAMP complexes have been identified in cultured cells transfected with fully functional epitope-tagged constructs using *bona fide* techniques of cell biology, such as immunoprecipitation with and without cross-linker, cell surface immunofluorescence and confocal microscopy. The physiological relevance of the receptor–RAMP paradigm largely remains to be confirmed in an *in vivo* context. Along these lines,
endogenous RAMP2 in rabbit vascular endothelial cells and in osteoblastic UMR-106 cells has conferred AM ligand specificity to the transfected CRLR [19,20].

The wide tissue distribution of RAMP mRNAs suggests that these accessory proteins interact with structures other than the CTR or CRLR. Reduced cell surface expression of an angiotensin II receptor in the presence of RAMP1 or RAMP3 remains to be confirmed [41].

The expression and interaction of the proteins has not been analysed to any great extent in vivo, due to a lack of suitable antibodies against endogenous CTR, CRLR and RAMPs. Transgenic and knockout mice may help to address these functional aspects of the RAMPs. Evidence has been obtained that certain RAMPs interact competitively with CRLR [19,20]. As a consequence, differential regulation of expression of the three RAMPs in tissues and cells may fine tune their responsiveness to AM and CGRP with regard to overlapping biological actions such as vaso-dilatation.

Not all known receptors for CGRP and AM form identifiable receptor–RAMP heterodimers. In the central nervous system, CGRP binding is highest in the cerebellum, where CRLR-encoding mRNA is undetectable by in situ hybridization [18]. αCGRPr- or βCGRPr-prefering receptors localized by ligand receptor autoradiography in distinct periventricular areas of the human brain have so far not been identified [42]. There is no molecular counterpart of a postulated CGRP2 receptor, while the CGRP1 receptor is antagonized by CGRP-(8–37), like the CRLR–RAMP1 complex [43]. An AM receptor isotype that is more potently antagonized by CGRP-(8–37) than by AM-(22–52) in rat astrocytes remains to be cloned [23].

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References
Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction

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Abstract

The calcitonin-gene-related peptide (CGRP) receptor component protein (RCP) is a 148-amino-acid intracellular protein that is required for G-protein-coupled signal transduction at receptors for the neuropeptide CGRP. RCP works in conjunction with two other proteins to constitute a functional CGRP receptor: calcitonin-receptor-like receptor (CRLR) and receptor-activity-modifying protein 1 (RAMP1). CRLR has the stereotypical seven-transmembrane topology of a G-protein-coupled receptor; it requires RAMP1 for trafficking to the cell surface and for ligand specificity, and requires RCP for coupling to the cellular signal transduction pathway. We have made cell lines that expressed an antisense construct of RCP and determined that CGRP-mediated signal transduction was reduced, while CGRP binding was unaffected. Furthermore, signalling at two other endogenous G-protein-coupled receptors was unaffected, suggesting that RCP was specific for a limited subset of receptors.

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Calcitonin-gene-related peptide (CGRP)

CGRP is a 37-amino-acid, carboxy-amidated neuropeptide [1–3]. CGRP belongs to a family of related neuropeptides that includes calcitonin, adrenomedullin and amylin, the members of which are characterized by an N-terminal disulphide ring structure [4]. CGRP is expressed in neurons with a wide anatomical distribution in both the central and peripheral nervous systems [5,6], in sensory neurons projecting to the spinal cord [7], in motoneurons at the neuromuscular junction [8,9] and in the vasculature [10].

As might be predicted from such a broad anatomical distribution, CGRP has been implicated in a wide range of physiological functions. In the central nervous system CGRP mediates the development of tolerance to opiates, and this effect can be blocked by CGRP antagonists [11,12]. In the spinal cord peripheral noxious stimulation results in CGRP release from primary afferent fibres in the dorsal horn, causing slow depolarization and prolonged excitation of dorsal horn cells,