Short N-terminal regions of the calcitonin-like receptor define ligand specificity

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Short N-Terminal Regions of the Calcitonin-Like Receptor Define Ligand Specificity

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presented by

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2004
To Roland
and my family
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## Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>BS3</td>
<td>bis(sulfosuccinimidyl)suberate, membrane-impermeable protein cross-linker</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CD97</td>
<td>leucocyte cell-surface antigen</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>COS-7</td>
<td>monkey kidney cell line</td>
</tr>
<tr>
<td>CL</td>
<td>calcitonin-like</td>
</tr>
<tr>
<td>CLR</td>
<td>calcitonin-like receptor(s)</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>CRFR</td>
<td>corticotropin-releasing factor receptor(s)</td>
</tr>
<tr>
<td>CRSP</td>
<td>calcitonin receptor stimulating peptides</td>
</tr>
<tr>
<td>CT</td>
<td>calcitonin</td>
</tr>
<tr>
<td>CTR</td>
<td>calcitonin receptor(s)</td>
</tr>
<tr>
<td>CTR2</td>
<td>CTR isotype 2</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMR&lt;sub&gt;1&lt;/sub&gt;</td>
<td>EGF-module-containing mucin-like hormone receptor 1</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
</tr>
<tr>
<td>GHRHR</td>
<td>growth hormone-releasing hormone receptor(s)</td>
</tr>
<tr>
<td>GIPR</td>
<td>gastric inhibitory peptide receptor(s)</td>
</tr>
<tr>
<td>GLP1R</td>
<td>glucagon-like peptide 1 receptor(s)</td>
</tr>
<tr>
<td>GLUCR</td>
<td>glucagon receptor(s)</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor(s)</td>
</tr>
<tr>
<td>h</td>
<td>human</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney cell line</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>m</td>
<td>mouse</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PACAPR</td>
<td>pituitary adenyl cyclase-activating polypeptide receptor(s)</td>
</tr>
<tr>
<td>PTHR</td>
<td>parathyroid hormone receptor(s)</td>
</tr>
<tr>
<td>r</td>
<td>rat</td>
</tr>
<tr>
<td>RAMP</td>
<td>receptor-activity-modifying protein(s)</td>
</tr>
<tr>
<td>s</td>
<td>salmon</td>
</tr>
<tr>
<td>SecR</td>
<td>secretin receptor(s)</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>human neuroblastoma cell line</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VIPR1</td>
<td>vasoactive intestinal peptide receptor(s)</td>
</tr>
<tr>
<td>VIPR2</td>
<td>helodermin-preferring vasoactive intestinal peptide receptor(s)</td>
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</tbody>
</table>
Summary

Calcitonin (CT), α- and β-CT gene-related peptide (CGRP), adrenomedullin (AM), and amylin belong to the calcitonin family of peptides. Six or seven amino acid ring structures, formed by disulfide bonds between cysteine residues, and amidated carboxyl-termini in all these peptides are required for the biological actions. Calcitonin receptors (CTR) and initially orphan CT-like receptors (CLR) with 60% amino acid sequence similarity belong to the class B1 of G protein-coupled receptors (GPCR). The structure of the receptors for CGRP, AM and amylin were established following the discovery in 1998 of three single transmembrane-domain receptor-activity-modifying proteins (RAMP). The RAMP are required as accessory proteins for the functional expression of the CLR. CLR/RAMP1 and -2 heterodimers recognize CGRP and AM, respectively. With RAMP3 the CLR interacts with CGRP and AM. The CTR does not require RAMP to recognize CT, but the receptor associates with RAMP1 and RAMP3 to form mixed type CGRP/amylin and amylin receptors, respectively.

In the present thesis, mechanisms of interaction of the CLR with RAMP1 and RAMP2 and their ligands CGRP and AM have been investigated. Eighteen N-terminal extracellular amino acid residues adjacent to the signal sequence of the CLR share little sequence homology with the corresponding region of the CTR. The importance of this sequence for the function of the CLR has been studied. The 18 N-terminal amino acids of the human (h) CLR were either deleted (Δ18-hCLR) or substituted by the corresponding sequence of a porcine (p) CTR, to reveal a chimeric pCTR-hCLR. The Δ18-hCLR was inactive in the absence and presence of hRAMP1 or –2. But the pCTR-hCLR co-expressed with hRAMP1 recognized CGRP, while the interaction with AM in the presence of hRAMP2 was abolished.

In the mouse (m) CLR, progressive truncation from the N-terminus up to amino acid 20 resulted in a progressive decrease in AM recognition in the presence of mRAMP2. A detailed analysis was achieved with the introduction of deletion and alanine substitution mutations. This allowed us to identify the amino acid sequence 14 to 20 as an important structural domain within the mCLR required for its functional expression as a mCLR/mRAMP2 AM receptor. Importantly, the deletion of the amino acids 14 to 20 of the mCLR minimally affected the CGRP receptor function in the presence of mRAMP1. The substitution of the amino acids 14 to 20 by alanine residues partially restored the functional expression of the mCLR/mRAMP2 AM receptor. Moreover, the deletion of the amino acids 14 to 17 or 14 to 20 in the mCLR altered its structure in a way that chemical cross-linking was no longer possible.
In another approach, the functional role of the amino acids 64 to 74 of the mCLR with the sequence CNRTWDGWLCW was investigated. This sequence is highly conserved in family B1 GPCR. D$^{69}$ was mutated in the mCLR to alanine and glutamic acid. D69A and D69E mutations impaired the complex formation with RAMP1 and as a consequence abolished CGRP binding and signaling.

In conclusion, the extreme N-terminal 18 and 20 amino acids in the human and mouse CLR are important for high affinity interactions of CLR/RAMP1 and –2 heterodimers with CGRP and AM. The deletion of the amino acids TRNKIMT 14 to 20 in the mCLR suppressed the functional expression of the AM/CLR/RAMP complex, while maintaining CGRP receptor function with RAMP1. Moreover, aspartate$^{69}$ in the mCLR, which is conserved in family B1 GPCR is required for heterodimerization of the CLR with RAMP1 or –2 and as a consequence CGRP and AM receptor binding and signaling.
Kurzfassung


In der vorliegenden Arbeit wurden Interaktionsmechanismen des CLR mit RAMP1 und –2 und dessen Liganden CGRP und AM untersucht. Die an die Signalsequenz des CLR anschließenden 18 Aminosäuren bilden das extrazelluläre, N-terminale Ende und unterscheiden sich in ihrer Sequenz von der entsprechenden Stelle im CTR. Im menschlichen (h) CLR wurden diese 18 Aminosäuren entfernt (Δ18-hCLR) oder durch die entsprechende Aminosäuresequenz eines CTR des Schweins (pCTR) ersetzt. Daraus entstand der chimäre pCTR-hCLR. Der Δ18-hCLR war mit und ohne hRAMP inaktiv. Der chimäre pCTR-hCLR assoziiert mit hRAMP1 erkennt CGRP ähnlich dem Wildtyp. Der pCTR-hCLR/hRAMP2-Komplex geht mit AM keine erkennbare Interaktion ein.

Mit der zunehmenden Verkürzung des N-Terminus des murinen (m) CLR bis zur Aminosäure 20 wurde die AM-Rezeptorinteraktion vermindert. Die CGRP-Rezeptorfunktion wurde nur wenig beeinträchtigt. Mit der Deletion oder der Alanin-Substitution der Aminosäuren 14 bis 20 des mCLR wurde eine Rezeptordomäne identifiziert, die für die Erkennung des AM als Liganden durch den mCLR/mRAMP2 Komplex wichtig ist. Die gleiche Deletion der Aminosäuren 14 bis 20 führt zu keiner Beeinträchtigung des Signaling durch CGRP am Rezeptor/RAMP1 Komplex. Eine Stimulation von cAMP durch AM ist in mCLR(Δ14-20)/mRAMP2 exprimierenden Zellen nicht erkennbar, obwohl beide Proteine an der Zelloberfläche exprimiert sind. Der Ersatz der Aminosäuren 14 bis
20 durch Alanine führte zu einer 100-fach verminderten EC\textsubscript{50} des mCLR für AM. Ausserdem wurde die Struktur des mCLR durch das Fehlen der Aminosäuren 14 bis 17 und 14 bis 20 so verändert, dass der mCLR an der Zelloberfläche nicht mehr mit mRAMP2 über freie Aminogruppen gekrosslinkt werden konnte.

Die Aminosäuren 64 bis 74 des mCLR mit der Sequenz CNRTWDGWLCW bilden eine in der Familie B1 konservierte Domäne. D\textsuperscript{69} ist allen gemeinsam. D69A- und D69E-Mutationen führten zu einer verminderten Komplexbildung mit mRAMP1 und dadurch zum Verlust einer cAMP-Stimulierung durch CGRP.

Die Resultate können wie folgt zusammengefasst werden: die ersten 20 N-terminalen Aminosäuren des hCLR und die ersten 18 Aminosäuren des mCLR sind wichtig für die hoch affine Interaktion der CLR/RAMP1- und CLR/RAMP2-Komplexe mit CGRP oder AM. Die Deletion der Aminosäuren 14 bis 20 im mCLR verhindert die Bildung eines aktiven AM-Rezeptors mit mRAMP2, ohne jedoch die CGRP-Rezeptorfunktion mit assoziiertem mRAMP1 zu beeinträchtigen. Überdies ist das konservierte Aspartat\textsuperscript{69} im mCLR für die Heterodimerisierung des mCLR mit mRAMP1 oder –2, und somit für dessen Funktion als CGRP oder AM-Rezeptor unerlässlich.
General introduction

Calcitonin family of peptides

The calcitonin family of peptides comprises calcitonin (CT), α- and β-CT gene-related peptide (CGRP), adrenomedullin (AM) and amylin [1, 2]. These peptides have in common an amino(N)-terminal ring structure of six or seven amino acids linked by a disulfide bridge between two cysteine residues, and amidated carboxyl(C)-termini required for biological activity (Figure 1). As a result of limited structural homology, the peptides crossreact with the corresponding receptors which results in overlapping biological actions (Table 1) [3]. The N-terminally truncated forms of CT, αCGRP and AM lacking the ring structure are competitive antagonists [4].

More recently intermedin, AM2 and calcitonin receptor stimulating peptides (CRSP) have been identified. They also interact with receptors of the calcitonin peptide family [5-7].

Figure 1: Amino acid sequences of human CT, α- and βCGRP, AM and amylin. Vertical lines or bold letters indicate identical amino acids. Disulfide bonds between cysteine residues forming the ring structures are also indicated (bold lines).

Calcitonin

CT was discovered as a hypocalcaemic hormone in 1961 [8]. The peptide consists of 32 amino acids and is synthesized in the C-cells of the thyroid. A rise in serum calcium concentrations stimulates CT secretion. The primary transcript of the CT gene is spliced in a tissue-specific manner to reveal CT expression in the thyroid C-cells and αCGRP expression predominantly in the central and peripheral nervous system. The CT encoding mRNA consists of exons I-IV of the CT gene, with exon IV encoding CT. Proteolytic cleavage of the precursor protein yields CT and
N-terminal and C-terminal flanking peptides with unknown biological functions [9].

CT inhibits osteoclastic bone resorption and stimulates the urinary calcium excretion, which results in decreased serum calcium levels. Hence, CT regulates calcium homeostasis and maintains skeletal mass during periods of calcium stress like during growth, pregnancy, and lactation [2], [10]. CT inhibits bone resorption and this is used for the treatment of Paget's disease and osteoporosis [11]. In the kidney, CT stimulates the excretion of calcium, phosphate, sodium and chloride through inhibition of the tubular reabsorption. Central actions of CT include antinociception as well as the inhibition of gastric secretion and food intake [12].

Table 1: Origin and biological actions of CT, CGRP, AM and amylin

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Synthesis</th>
<th>Biology</th>
</tr>
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<tbody>
<tr>
<td>CT</td>
<td>thyroid C-cells</td>
<td>hypocalcaemic hormone; analgetic actions</td>
</tr>
<tr>
<td>CGRP</td>
<td>nervous system</td>
<td>vasodilatory and hypotensive actions; positive chronotropic and inotropic effects in the heart</td>
</tr>
<tr>
<td>AM</td>
<td>adrenal medulla</td>
<td>vasodilatory and bronchodilatory actions; inhibition of water intake upon intracerebro-ventricular administration</td>
</tr>
<tr>
<td>Amylin</td>
<td>pancreatic β-cells</td>
<td>inhibition of insulin secretion, glucose uptake and glycogen synthesis in skeletal muscle</td>
</tr>
</tbody>
</table>

**Calcitonin gene-related peptide**

αCGRP was discovered in 1982 as a novel protein product of the CT gene [13]. βCGRP was described in 1985, differing from αCGRP in 3 and 1 of the 37 amino acid residues in man and rat, respectively [14, 15]. CGRP is synthesized in neurons of the central and peripheral nervous system and released through stimulation of voltage-dependent calcium channels from afferent nerve fibers.

CGRP is likely the most potent vasodilatory peptide acting directly on vascular smooth muscle cells through stimulation of cyclic AMP levels [16-18], and indirectly on vascular endothelial cells where it stimulates
nitric oxide (NO) production. The local release of NO leads to cGMP production in adjacent vascular smooth muscle cells. There is evidence that the subsequent activation of a cGMP dependent protein kinase leads to activation of calcium-gated potassium channels [19]. The resulting cell membrane hyperpolarization causes an endothelium dependent vasodilatation.

In the heart, CGRP has positive chronotropic and indirect inotropic effects brought about through activation of the sympathetic nervous system [1]. Moreover, CGRP enhances the glomerular filtration rate, renal blood flow, and the secretion of renin [20-22]. The inhibition of gastric acid secretion by CGRP may be mediated by the release of gastric somatostatin and a decrease in acetylcholine transmission in the enteric nervous system [23-25].

**Adrenomedullin**

AM, a multifunctional regulatory peptide, is synthesized as prepro-adrenomedullin. Proteolytic cleavage of preproadrenomedullin yields pro-adrenomedullin N-terminal 20 peptide (PAMP) and AM [26]. A receptor for the hypotensive PAMP remains to be discovered.

AM was isolated in 1993 from human pheochromocytoma, a tumor arising from the adrenal medulla [27, 28]. Human and rat AM consist of 52 and 50 amino acids, respectively [2, 26, 29]. Human and rat AM unlike CT, CGRP and amylin, have 15 and 13 amino acids extensions, respectively, N-terminal to the intramolecular ring-structure. AM lacking these amino-terminal amino acid residues have the biological activity of the intact AM. In contrast peptides hAM_{22-52} and rAM_{20-50} lacking the 6 amino acids ring structure are antagonists.

AM is predominantly expressed in endothelial and vascular smooth muscle cells. It is also synthesized in numerous other tissues including the adrenal medulla, the kidney, lung, heart, spleen and the central nervous system [26, 30]. Thus, AM is considered as a multi-functional peptide involved in multiple physiological processes including vasodilatation, electrolyte balance, neurotransmission, cell growth and the regulation of hormone secretion [29]. Moreover, AM appears to contribute to septic shock [31].

Many actions of AM are mediated through the adenylyl cyclase/protein kinase A signaling pathway. But there is evidence for the activation of other signaling pathways resulting in increased intracellular calcium concentrations and formation of NO [32].
Amylin

Amylin is a 37 amino acid polypeptide, isolated from amyloid fibrils of a human insulinoma [33]. Amylin is synonymous with islet amyloid polypeptide (IAPP) or diabetes-associated peptide (DAP) [2]. Amylin is cleaved from a precursor molecule. It is co-localized and co-secreted with insulin from normal pancreatic β-cells. Proposed biological actions of amylin include suppression of food intake, gastric emptying, and argentine-stimulated glucagon secretion from pancreatic β-cells [34]. Amylin is a major component of amyloid deposits in the pancreas of patients with non-insulin-dependent (type II) diabetes mellitus and it has been associated with its pathogenesis [35]. Along these lines, high concentrations of amylin inhibit glucose-stimulated insulin secretion. Moreover, the suppression of the amylin activity by amylin receptor antagonists CGRP_{8-37} or amylin_{8-37} results in higher insulin levels [36].

G protein-coupled receptors

G protein-coupled receptors (GPCR) with seven transmembrane domains are a family of over 1000 proteins. Their predicted topology in the plasma membrane includes an extracellular N-terminus, three extracellular and three intracellular loops, and a cytoplasmatic C-terminus. The crystal structure of bovine rhodopsin serves as a model [37]. The GPCR have been classified into six families: the Rhodopsin family (A), the secretin receptor family (B), the metabotropic glutamate / pheromone receptor family (C), fungal pheromone receptors (D), cAMP receptors (Dictyostelium) (E), and frizzled / smoothened receptors (F) [http://www.gpcr.org]. Ligands of GPCR are exceptionally diverse and include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and proteases. Rhodopsin is activated by light. Upon ligand interaction, the GPCR activate heterotrimeric guanine nucleotide-binding proteins (G-proteins) that couple the receptors to signaling enzymes, e.g. adenylyl cyclases, phospholipases, and ion channels (Fig. 2).
Fig. 2: Activation of signaling cascades by GPCR. Binding of ligand causes a conformational change of the GPCR and interactions with the α-subunits of trimeric GTP-binding proteins $G_s$ and/or $G_q$ that activate membrane bound adenylyl cyclase and phospholipase C, respectively. Activation of the adenylyl cyclase results in the production of cAMP and activation of protein kinase A (PKA). Activated phosphoinositide-specific phospholipases C (PI-PLC) cleaves phosphatidylinositol bisphosphate (PIP$_2$) into inositol trisphosphate (IP$_3$) and diacylglycerol (DG). IP$_3$ releases Ca$^{2+}$ from the endoplasmic reticulum into the cytosol and DG activates protein kinase C (PKC), which catalyses the phosphorylation of target proteins.

**Homo- and heterodimerization of GPCR**

GPCR, unlike enzyme-coupled receptors were initially considered as monomeric proteins interacting with a specific ligand. Over 20 years ago, the concept of homo- and heterodimerization of GPCR was set up [38, 39]. Strong evidence for the existence of muscarinic/adrenergic dimers was obtained in 1993 [40]. Homo- or heterodimerization of GPCR is now well-established [41-45].

Homodimers of the family A of GPCR include the $A_1$ adenosine receptor [46], $\beta_2$ adrenoreceptor [47-49], $D_2$ dopamine receptor [50-52], and $\delta$-opioid receptor [48, 53]. Homodimerization increases the sensitivity of certain receptors for their ligands. Moreover, defective mutant receptors can regain their activity through dimerization [54].

The biological significance of GPCR heterodimerization has been demonstrated for the GABA$_B$ receptor isomers GABA$_B_1$ and GABA$_B_2$. 
Heterodimerization at the cell surface is required for GABA$\text{B}$ receptor function [55-57].

Heterooligomerization of GPCR reveals receptor complexes with novel actions. Dimerization of $\kappa$- and $\delta$-opiod receptors results in decreased affinity for either $\kappa$- or $\delta$-opioid receptor-selective agonists and antagonists, but the affinity for partially selective ligands is increased [58]. Moreover, heterodimerization of $\mu$- and $\delta$-opioid receptors changes the signaling pathway. The adenylyl cyclase activity decreases through stimulation of monomeric receptors. The dimers phosphorylate mitogen-activated protein kinase (MAPK) [59]. CCR2-CCR5 chemokine receptor heterodimers trigger cytosolic calcium response at 10- to 100-fold lower concentrations of the corresponding chemokines as compared to the CCR2 and CCR5 receptors alone. This suggests cooperation of the two ligands upon simultaneous binding to the CCR2-CCR5 heterodimers [60].

Heterodimerization between GPCR and ion channels has been demonstrated for the dopamine D$_5$ receptor and the GABA$\text{A}$ ion channel [61].

**Class B of G protein-coupled receptors**

GPCR exhibit limited amino acid sequence similarity between the individual families A, B, and C. High sequence homology is observed between the transmembrane domains. All GPCR are proposed to have a common evolutionary origin [62]. In the family B of GPCR, the secretin receptor was the first to be cloned [63]. Later at least 33 human genes encoding family B GPCR have been identified. They exist in animals, but not in plants, fungi or in prokaryotes. The family B of GPCR consists of three subfamilies: the peptide hormone receptors (B$_1$), the putative cell-surface signaling molecules (B$_2$), for example the $\alpha$-latrotoxin receptor, the epidermal growth factor (EGF)-module-containing, mucin-like hormone receptor (EMR$_1$) and the leucocyte cell-surface antigen (CD$_{97}$). The family B$_3$ consists of the receptors for methusaleh and related Drosophila proteins [64]. Here, the peptide hormone receptors are discussed. Their ligands include the peptides glucagon, pituitary adenylyl cyclase activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), secretin, growth-hormone-releasing hormone (GHRH), parathyroid hormone (PTH), and CT [64]. A phylogenetic tree of the receptors, based on amino acid sequence homology, is shown in Figure 3.

The class B of GPCR have in common N-terminal signal peptides. The N-terminal, extracellular domains together with the extracellular loop regions and the transmembrane domains are essential for ligand binding.
The extracellular N-terminus contains three or four cysteins and two tryptophan residues, which may be critical for ligand binding. Moreover the intracellular loop IC3 is important for the coupling to G proteins [64].

![Figure 3: Structural relationship within the family B₁ of GPCR. It includes the CLR, the CT receptor (CTR), the rat gastric inhibitory peptide receptor (GIPR), the glucagon receptor (GlucR), the glucagon-like peptide 1 receptor (GLP1R), the helodermin-preferring vasoactive intestinal peptide receptor (VIPR2), the pituitary adenyl cyclase activating polypeptide receptor (PACAPR), the vasoactive intestinal peptide receptor (VIPR1), the secretin receptor (SecR), the growth hormone-releasing hormone receptor (GHRHR), the parathyroid hormone receptor (PTHr), and the corticotropin-releasing factor receptor (CRFR) [65].](image)

**Calcitonin receptors**

A CTR was first cloned in 1991 from a porcine kidney epithelial cell line [66]. Subsequently, the structure of CT receptors has been identified in man, rat, mouse, flounder, pig, rabbit, guinea and frog. The cAMP- and phospholipase C signaling pathways are activated by the CTR. Activation of Phospholipase D has also been proposed [67].
Alternative splicing of primary gene transcripts reveals several CTR isoforms. A hCTR1 isoform of 490 amino acids differs from a more abundant hCTR2 by a 16 amino acid insert in the first intracellular loop. This insert impairs coupling to adenylyl cyclase and the phospholipase C. In a hCTR3 isoform 47 amino acids in the extra-cellular domain of the hCTR2 are deleted. In rat, unlike in man and pig, a CTR1b isotype differs from CTR1a by a 37 amino acid deletion in the first extracellular loop and is the rat homologue of the hCTR2. The rat CTR1b is predominantly expressed in the brain [67-69].

**Calcitonin-like receptors**

Cloned man, rat, mouse, flounder, salmon, fugu, pig, bovine and xenopus CLR share 30% sequence homology and 60% amino acid sequence similarity with the CTR. Initially, The CLR were published as orphan receptors of between 459 to 465 amino acids. [65, 70]. Isotypes within individual species have so far not been found.

Human embryonic kidney (HEK293) cells, stably transfected with cDNA encoding the hCLR exhibited high affinity $^{125}$I-CGRP binding and CGRP evoked stimulation of cAMP formation [71]. hCLR expressing monkey kidney (COS-7) cells and opossum kidney (OK) cells on the other hand, failed to bind $^{125}$I-CGRP and a cAMP response to CGRP was also not observed [65]. In 1998, McLatchie et al. demonstrated that the CLR requires a novel associated receptor-activity-modifying protein (RAMP1), endogenous in HEK293, but not in COS-7 and OK cells, to reveal a receptor for CGRP [72].

Northern blot analysis of RNA extracted from rat tissues revealed highest expression of CLR encoding mRNA in the heart, the lungs and the kidneys [65, 73]. During fetal and postnatal development of the rat CLR, mRNA was predominantly detected in the lungs, and in brain in the piriform cortex, in the central and basolateral amygdala and in the amygdalostriatal transition area [74]. A detailed immunohistochemical analysis in human tissues largely confirmed the distribution of CLR encoding mRNA in rat tissues [75]. In the lungs intense staining was observed in alveolar capillaries. In the cardiovascular system CLR immunostaining was found in the endothelium of blood vessels, in heart muscle cells and endocardium. The kidney showed staining of juxtaglomerular arteries, the glomerular capillaries and chief cells of the collecting duct. The trabecular veins and sinuses of the spleen were also intensely stained.
Receptor-activity-modifying proteins

The cDNA encoding human (h) RAMP1 was cloned from a cDNA library of the human neuroblastoma (SK-N-MC) cell line with well characterized CGRP receptors. The complementary RNA was expressed in Xenopus oocytes where it enhanced the cAMP formation mediated by endogenous CGRP receptors [72]. hRAMP1 consists of 148 amino acids, and has a single transmembrane domain. RAMP2 and RAMP3 encoding cDNAs, corresponding to expressed sequence tags found in public databases with the nucleotide sequence of RAMP1, were cloned from SK-N-MC and a human spleen library.

The amino acid sequences of the three RAMP in individual species are approximately 60% similar [72]. Meanwhile, the mouse and rat homologues of the three human RAMP have been identified [76, 77]. RAMP are type I transmembrane proteins of 148 to 174 amino acid residues with a signal peptide followed by an N-terminal extracellular domain, and with a short intracellular C-terminus. Six cysteins are conserved in the extracellular domains of RAMP1 and -3 in man, mouse and rat, and four are found in RAMP2. The cysteine residues that are conserved in RAMP1 and -2 are important for their function and may form intramolecular disulfide bridges [78, 79]. RAMP1, unlike RAMP2 and –3 lacks N-glycosylation sites in the N-terminal extracellular domain [80, 81]. As a result, RAMP1, in contrast to RAMP2 and –3, requires the CLR for the co-transport to the cell surface [72, 82-84].

mRNA encoding the three RAMP show a distinct tissue distribution. In man, RAMP1 mRNA is highly expressed in the skeletal musculature, pancreas and brain, but poorly in the kidneys and liver. RAMP2 and –3 are more ubiquitously expressed. RAMP2 mRNA is found in the lungs and RAMP3 mRNA is abundant in the heart, brain and kidneys of man, rat and mouse [81].

Biology of receptor/RAMP heterodimers

The CLR co-expressed with RAMP1 forms CLR/RAMP1 heterodimers that are receptors for CGRP, antagonized by CGRP<sub>8-37</sub> [72, 76, 85]. RAMP2, on the other hand, associates with the CLR to reveal a receptor for AM. CLR/RAMP3 heterodimers are mixed type CGRP/AM receptors [86].

CGRP and AM receptors are linked to cAMP formation [76, 85]. High nanomolar concentrations of CGRP and AM also increase cytosolic free calcium [83].
The novel concept of RAMP-defined ligand specificity was extended to the hCTR2 and the rCTR1a. Co-expression of human RAMP3 with the CTR revealed amylin receptors cross-reacting with CT and CGRP at high nanomolar concentrations [87, 88]. Moreover, the CTR co-expressed with RAMP1 is a receptor for CGRP and amylin antagonized by CT<sub>8-37</sub> [89, 90].

**Receptor/RAMP interactions**

Non-covalent interactions and the co-localization of RAMP1 with the CLR or the CTR at the cell surface has been demonstrated by co-immunoprecipitation and confocal microscopy, respectively [91, 92]. The CLR also associates with RAMP2 at the cell surface. Cell surface cross-linking of <sup>125</sup>I-CGRP to the CLR or the CTR and RAMP demonstrates in close proximity of the receptors and RAMP1 a <sup>125</sup>I-CGRP binding pocket [80]. Apparently, the associated RAMP1 and –2 modulate the CLR structure to adopt CGRP or AM selectivity [93]. The N-terminal extracellular regions of RAMP determine CGRP or AM selectivity [94, 95]. Interestingly, the over 100-fold higher affinity of the recently described small molecule CGRP antagonist, BIBN4096BS, for the hCLR/hRAMP1, but not the rCLR/rRAMP1 CGRP receptors is determined by tryptophan 74 in hRAMP1 that is a lysine in rRAMP1 [96].

In the N-terminal extracellular domain of the human CLR, Asn<sup>117</sup>, a putative N-glycosylation site, is important for the CGRP recognition of the hCLR/hRAMP1 complex. The Asn<sup>117</sup> to Asp substitution maintained the hCLR/hRAMP1 CGRP receptor activity, indicating that N-glycosylation of Asn<sup>117</sup> is not required for the functional expression of the hCLR/hRAMP1 receptor complex. But, the mutations of Asn<sup>117</sup> to Thr, Ala, Gln, or Pro inactivated hCLR/hRAMP1 heterodimers [97]. Altogether, this implies that the CLR and the RAMP contribute to high affinity interactions with CGRP and AM.

The phosphorylation of the CLR upon CGRP binding and receptor activation leads to the internalization of the CLR/RAMP1 heterodimers. Internalization is enhanced with β-arrestin. The ternary complex of CLR, RAMP1 and β-arrestin is internalized in clathrin-coated pits. Recycling of the internalized CLR/RAMP heterodimers to the plasma membrane is inefficient [83, 98].
Luebke et al. discovered a novel CGRP receptor component protein (CGRP-RCP) that appears to intracellularly associate with CLR/RAMP heterodimers and to facilitate the coupling to down-stream signaling effectors [99, 100]. This was demonstrated by co-immunoprecipitation of CLR/RAMP/CGRP-RCP complexes [101]. CGRP-RCP appears to be specific for the CGRP receptor, since it did not influence signaling of receptors for CT, amylin, neuropeptide Y, VIP and β-endorphin [99]. The distribution of the expressed RCP in the central and peripheral nervous system in rat correlates with that of CGRP receptors [102].
Aim of the thesis

The identification of RAMP1, -2 and –3 revealed a new principle of the function of GPCR. The CLR and the CTR of the family B1 of GPCR associate with individual RAMP to form heterodimeric receptors for CGRP, AM and amylin. This thesis focuses on the identification of subdomains of the CLR that are required for functional association with RAMP1 and –2 and the binding of CGRP and AM by respective CLR/RAMP1 and –2 heterodimers.

The CLR and the CTR, the closest homologues in the B1 family of GPCR, exhibit 60% overall amino acid sequence similarity, but the sequences of the amino acids 1 to 20 in the respective receptors are distinct. Moreover, the CTR, unlike the CLR, does not form an AM receptor with RAMP2. Thus, we have investigated the hypothesis that the N-terminal 20 amino acids adjacent to the signal sequence in the CLR constitute a subdomain that is important for AM receptor function. We have also studied the functional importance of N^{65}, a putative N-glycosylation site, and of D^{69} in the N-terminal extracellular domain of the mouse CLR. D^{69}, unlike N^{65}, is conserved in all family B1 GPCR.

The extreme N-termini of the human and mouse CLR up to amino acid 20 were progressively truncated or substituted by the corresponding sequence of the CTR, or targeted deletions or amino acid substitutions were introduced. Site directed mutagenesis yielded N65A, N65D, D69E, D69A and D69N substitutions. The effects of the mutations on cell surface expression and on complex formation of the CLR and RAMP1 and –2 and on CGRP and AM binding and signaling were investigated.
The extreme N-terminus of the calcitonin-like receptor contributes to the selective interaction with adrenomedullin or calcitonin gene-related peptide

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Abstract

The calcitonin (CT)-like (CL) receptor is a CT gene-related peptide (CGRP) receptor or an adrenomedullin (AM) receptor when co-expressed with receptor-activity-modifying proteins (RAMP) 1 or -2, respectively. The CL receptor shows 57% overall sequence identity with the CT receptor, but the homology is much lower in the extreme N-terminus. An N-terminal deletion mutant of the human (h) CL receptor (Δ18-hCL) and a chimeric receptor consisting of the N-terminal amino acids of the porcine (p) CT receptor fused to the Δ18-hCL receptor (pCT-hCL) were therefore analyzed. The Δ18-hCL receptor function was abolished when co-expressed with RAMP1 or -2. The pCT-hCL receptor was a fully functional CGRP receptor when co-expressed with RAMP1, but the RAMP2-dependent AM receptor function was impaired. Limited sequence similarities in the N-terminus of the pCT and the hCL receptors rescue CGRP but not AM receptor binding and signalling.

Key words: Adrenomedullin; Calcitonin gene-related peptide; Calcitonin-like receptor; Receptor-activity-modifying protein

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CL, calcitonin-like; CT, calcitonin; RAMP, receptor-activity-modifying protein
1. Introduction

Calcitonin (CT), CT gene-related peptide (CGRP), adrenomedullin (AM) and amylin are closely related hormones and neuropeptides consisting of 32 to 52 amino acids. They have in common six or seven amino acid ring structures formed by a disulfide bridge near the N-terminus, an adjacent alpha helix of up to 10 amino acids and amidated C-termini. The similarity of the peptides is responsible for their crossreactivity with the receptors. Biological actions include hypocalcaemia (CT), vasodilatation (CGRP and AM) and inhibition of gastric emptying (amylin).

The CT receptor and the CT-like (CL) receptor are closely related members of the B family of G protein coupled receptors with putative seven transmembrane domains [2-4]. The initially orphan CL receptor interacts with associated receptor-activity-modifying proteins (RAMP) [5-7]. The RAMP are proteins with an extracellular N-terminus of about 100 amino acids, a 20 amino acid single transmembrane domain and an intracellular domain of up to 10 amino acids. When co-expressed with RAMP1 the CL receptor recognized CGRP. With RAMP2 or -3 an AM specific receptor was observed. The CT receptor does not require any known RAMP to bind CT, but CGRP was only recognized when the CT receptor was co-expressed with RAMP1 [8]. CT and CL receptors form non-covalent heterodimeric complexes with the RAMP at the plasma membrane which define ligand specificity for CGRP, AM or amylin [8-12]. The interactions between receptors and RAMP have been investigated. As a result it was revealed that the Asn^{117} residue in the CL receptor can only be substituted by an Asp. Other substitutions in position 117 led to deficient or absent recognition of CGRP by the CL receptor/RAMP1 complexes [9].

In man the CT and the CL receptors show an overall amino acid sequence identity of 57%. But the extreme N-termini of the receptors up to the first conserved cystein residue show only limited similarity (Fig. 1). A naturally occurring truncated human (h) CT receptor isotype, lacking the signal sequence and the N-terminal 23 amino acids, has wild-type CT receptor function [13]. Here, the extreme N-terminus of the hCL receptor was therefore removed and examined as a potential region defining ligand specificity for CGRP or AM when co-expressed with hRAMP1 and -2, respectively. The corresponding deletion mutant of the hCL receptor (∆18-hCL) lost its function as a CGRP and AM receptor when co-expressed with hRAMP1 or -2. Substitution of the first 18 amino acids of the hCL receptor by the corresponding sequence of the porcine CT receptor (pCT-hCL) reconstituted hRAMP1 dependent CGRP recognition, but the hRAMP2 dependent AM response was impaired.
Fig. 1. Amino acid sequence alignment of the N-termini of wild-type and mutant hCL and pCT receptors. The sequences shown lack the putative signal peptides and include the first predicted N-glycosylation consensus site of the receptors (▼). Conserved cysteins (●) and similar (gray boxed) and identical (black boxed) amino acids are indicated. A conserved K/R-X-K/R motif found in mammalian CL and CT receptors is underlined.

2. Materials and Methods

2.1. Materials
Human αCGRP was from Bachem AG (Bubendorf, Switzerland), and hAM from Peptide Institute (Osaka, Japan). Na[125I] and Hybond ECL nitrocellulose membranes were from Amersham International (Little Chalfont, UK), and restriction enzymes from Promega (Madison, WI, USA) and Roche Diagnostics AG (Rotkreuz, Switzerland). Tissue culture supplies, LipofectAMINE and OptiMEM medium for transfections were from Invitrogen (Carlsbad, CA, USA). Other reagents unless indicated were from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. cDNA and mutagenesis
Expression constructs encoding hRAMP1- and hRAMP2-V5-His\textsubscript{6} (hRAMP1 and hRAMP2) were obtained as previously described [8]. The DNA encoding a pCT-hCL fusion receptor with C-terminal myc and His\textsubscript{6} epitope tags was cloned into the mammalian expression vector pcDNA3 as follows. The cDNA encoding the hCL receptor was subcloned into the EcoRI restriction site of plasmid pIC19H (Gene Bank accession no. VB 0091) [4]. An EcoRV/Hpal restriction fragment, containing the 5'-noncoding sequence and the sequences encoding the signal peptide and the amino acids 1 to 107 of the hCL receptor, was replaced by an EcoRV/Hpal DNA fragment encoding the Ile\textsuperscript{19} to Val\textsuperscript{107} sequence of the hCL receptor, amplified by PCR. This construct was digested with the EcoRV restriction enzyme and a DNA fragment encoding the signal peptide, and the amino acids 1 to 24 of the pCT receptor in frame with the hCL receptor sequence beginning with Ile\textsuperscript{19} was introduced. This fragment was also obtained by PCR and had an EcoRV and an adjacent BamHI restriction site at the 5'-end and a 3' blunt end. The pCT-hCL receptor
fusion gene was then digested with BamHI and HindIII restriction enzymes. This removed a DNA fragment that encoded the pCT-hCL fusion receptor lacking 187 amino acids at the C-terminus. The fragment was cloned into BamHI/HindIII digested pcDNA3 encoding myc and His6 epitope tags downstream of the HindIII restriction site. The resulting plasmid was digested with HindIII restriction enzyme and the missing 187 amino acids C-terminal portion of the pCT-hCL receptor was introduced with a PCR-amplified DNA fragment containing the corresponding coding sequence in between HindIII restriction sites in frame with the upstream pCT-hCL receptor and the downstream myc-His6 encoding sequences. The final pCT-hCL-myc-His6 (pCT-hCL) receptor construct was verified by sequencing.

The Δ18-hCL-myc-His6 receptor expression construct was derived from pCT-hCL-myc-His6 receptor. pCT-hCL-myc-His6 receptor construct was digested with BamHI, RcaI and HpaI restriction enzymes. The RcaI/HpaI fragment encoding the Met20 to Asn139 domain of the hCL receptor was isolated. A BamHI/RcaI DNA fragment encoding the 24 amino acids signal sequence of the pCT receptor in frame with the downstream hCL-myc-His6 receptor sequence from Met20 to Asn139 was amplified with Pfu DNA Polymerase (Amersham Pharmacia Biotech, Little Chalfont, UK). The BamHI/RcaI and RcaI/HpaI fragments isolated from pCT-hCL-myc-His6 receptor were cloned into the BamHI/HpaI digested pCT-hCL-myc-His6, receptor revealing Δ18-hCL-myc-His6 (Δ18-hCL) receptor. This construct was also verified by sequencing.

2.3. Cell culture, transfection and cAMP measurement
Chinese hamster ovary (CHO) cells were cultured in Ham F12 medium supplemented with 10% fetal calf serum. 24 or 48 h before transient transfection 5.2 or 2.6 x 10^4 CHO cells per cm^2 were seeded into 24-well-plates or 100 mm-dishes, respectively. The cells were transfected at 37°C for 4 h in 130 µl OptiMEM medium per cm^2 containing 0.5 µl LipofectAMINE and 105 ng DNA of indicated receptor and hRAMP1- or hRAMP2-V5-His6 expression constructs. In control experiments, 105 ng hRAMP1 or hRAMP2 expression constructs were transfected together with 105 ng pcDNA3 to keep the DNA concentration constant. The transfected cells were kept in tissue culture medium for two days prior to the experiments. cAMP was measured in extracts of cells as described [14].

2.4. Radioligand binding, crosslinking and protein gel autoradiography
Transiently transfected CHO-cells in 100 mm dishes were detached with 0.05% EDTA in PBS and collected by centrifugation at 200 x g for 5 min. [125I]-labeled hαCGRP and -hAM (7.4 10^13 Bq/mmol) were prepared as described [15,16]. The cells were incubated with 100 kBq [125I]hαCGRP
or [\(^{125}\)I]hAM in 0.4 ml HAM F12/DMEM mixture (1:1), 0.1% BSA on ice for 2 h. Subsequently, the cells were washed with PBS and incubated on ice for 1 h in 400 µl PBS containing 1 mM cross-linker BS\(^3\) (Pierce Biotechnology, Rockford, IL). Cross-linking was quenched by the addition of 1 M Tris-HCl (pH 8.0) to a final concentration of 50 mM. After centrifugation at 200 x g for 3 min the cells were lysed in 300 µl 50 mM HEPES (pH 7.5), 140 mM NaCl, 0.5% Triton X-100 (BioRad Laboratories, Inc., Hercules, CA), 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml aprotinin and 3 µg/ml leupeptin (lysis buffer). The cell lysates were cleared by centrifugation at 20,000 x g for 5 min. Myc-tagged receptor components were immunoprecipitated from the supernatants by sequential incubation at 4°C with 30 µl ImmunoPure immobilized protein G (Pierce Biotechnology, Rockford, IL) for 1 h to remove non-specifically adsorbing proteins, 3 µg myc antibodies (Invitrogen, Carlsbad, CA) for 2 h, and with 45 µl ImmunoPure immobilized protein G overnight on an end-over-end rotator. The precipitates were collected by centrifugation at 2700 x g for 3 min. The pellets were washed once with lysis buffer. The amounts of [\(^{125}\)I]-labeled peptides in the immunoprecipitates were measured in a \(\gamma\)-counter (Kontron, Zurich, Switzerland). Deglycosylation of proteins bound to ImmunoPure immobilized protein G was carried out at 37°C for 18 h with 2 U of N-glycosidase F (Roche Diagnostics AG, Rotkreuz, Switzerland) in 50 µl lysis buffer adjusted to 10 mM EDTA, 0.1% SDS, 0.5% octylglucopyranoside and 1% \(\beta\)-mercaptoethanol. The proteins were then eluted at 60°C for 5 min from the ImmunoPure immobilized protein G and the samples centrifuged at 10,000 x g for 3 min. Proteins in the supernatant were subjected to 10% SDS-PAGE and electrotransferred to nitrocellulose Hybond ECL membranes (BioRad Laboratories, Inc., Hercules, CA) in a Trans-Blot cell (BioRad Laboratories, Inc., Hercules, CA) at 10 V and 4°C overnight. Cross-linked [\(^{125}\)I]hαCGRP and -hAM were detected by autoradiography with Hyperfilm MP film (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.5. Immunostaining and confocal microscopy

CHO cells were detached with trypsin/EDTA 48 to 72 h after transfection. Staining was performed at room temperature. The cells were first incubated with 50 µg/ml TRITC-ConA (Molecular Probes, Eugene, OR, USA) in PBS for 20 min, washed with PBS and fixed with 4% formalin in PBS for 20 min, washed again with PBS and incubated with immunostaining medium (DMEM/Ham F12 (1:1), 0.1% BSA, 0.1% saponin) for 20 min. The cells were then incubated for 1 h in 200 µl immunostaining medium with rabbit antiserum to myc (Cell Signaling Technology, Beverley, MA, USA; 1:300) for receptor detection or with mouse antibodies to V5 (Invitrogen, Carlsbad, CA; 1:300) for hRAMP2 detec-
tion. Subsequently, the cells were washed twice and then incubated with Alexa488 anti-rabbit or Alexa488 anti-mouse antiserum (Molecular Probes, Eugene, OR, USA; 1:200) for 30 min. After 3 washes the cells were collected by centrifugation and 5 µl were dry-mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA). Confocal images were obtained with a laser scanning LSM410 microscope (100 x 1.3 aperture objective; Zeiss, Jena, Germany) by sequential excitation with an external argon laser at 488 nm for Alexa488 and an internal He/Ne laser at 543 nm for TRITC.

2.6. Statistical analysis
The values for half-maximal effective concentrations (EC$_{50}$) were calculated by sigmoidal regression analysis using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). The results are means ± standard error of the mean (SEM). Differences between mean values were analyzed by ANOVA. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Deletion of the 18 N-terminal amino acids of the hCL receptor results in loss of [^{125}I]h$\alpha$CGRP and -hAM binding and signalling
Binding and chemical cross-linking of [^{125}I]h$\alpha$CGRP and -hAM to corresponding receptors was carried out in CHO cells co-expressing the hCL or the mutant receptors together with hRAMP1 or -2 (Fig. 2). In cells transfected with hCL receptor and hRAMP1 expression constructs, [^{125}I]h$\alpha$CGRP was cross-linked in equal amounts to two protein components that, after deglycosylation, had apparent molecular weights of 50 and 65 kDa. They differed in size by the molecular weight calculated for hRAMP1. Both [^{125}I]h$\alpha$CGRP binding proteins remained undetectable in cells expressing hRAMP1 alone or hRAMP1 together with the $\Delta$18-hCL receptor. In cells expressing the hCL receptor together with hRAMP2 a predominant [^{125}I]hAM-cross-linked protein component had a size similar to the 50 kDa [^{125}I]h$\alpha$CGRP binding protein in hCL receptor/hRAMP1 co-expressing cells. A minor [^{125}I]hAM cross-linking product was slightly larger than the 65 kDa [^{125}I]h$\alpha$CGRP binding protein in hCL receptor/hRAMP1 expressing cells. [^{125}I]hAM-cross-linked proteins were undetectable in cells expressing hRAMP2 alone or together with the $\Delta$18-hCL receptor.
Fig. 2. Chemical cross-linking of $[^{125}]h\alphaCGRP$ and $-hAM$ to corresponding binding proteins. CHO cells were transfected with hRAMP1 (left panels) or hRAMP2 (right panels) expression constructs together with pcDNA3 or the indicated receptor expression constructs. Radioligand binding and cross-linking with BS$_3$, and immunoprecipitation of myc-tagged receptors with corresponding antibodies and subsequent deglycosylation was carried out as described in Materials and Methods. Immunoprecipitates were subjected to 10% SDS-PAGE and radioligand cross-linking products were visualized by autoradiography (top panels). The amount of $[^{125}]h\alphaCGRP$ and $-hAM$ co-immunoprecipitated with the myc-tagged receptors after cross-linking were measured in a $\gamma$-counter and reflected radioligand binding (bottom panels). $[^{125}]h\alphaCGRP$ and $-hAM$ in immunoprecipitates of cells transfected with hRAMP1 or -2 expression constructs together with pcDNA3 was considered as non-specifically bound. Binding of $[^{125}]h\alphaCGRP$ (4.7 ± 0.9 kBq/dish (n = 5)) and of $[^{125}]hAM$ (3.4 ± 1.6 kBq/dish (n = 4)) to hCL receptor/hRAMP1 and -2 expressing cells, respectively, was set to 100%.
Fig. 3. Stimulation of cAMP production. CHO cells were transfected with hCL (○), pCT-hCL (■) or Δ18-hCL (▲) receptor together with hRAMP1 (top panel) or hRAMP2 (bottom panel) and stimulated with indicated concentrations of hαCGRP (top panel) or hAM (bottom panel). Results are means ± SEM of four experiments.

In CHO cells transfected with the hCL or the Δ18-hCL receptor together with hRAMP1 or -2 mean basal cAMP levels ranged from 2.3 to 3.2 pmol/well (P > 0.05). In cells co-expressing the hCL receptor and hRAMP1 hαCGRP maximally stimulated cAMP formation 45 ± 10-fold with an EC$_{50}$ of 1.1 ± 0.4 nM (n = 4) (Fig. 3, top panel). In Δ18-hCL receptor and hRAMP1 co-expressing cells hαCGRP was about 1000-fold less potent and maximal cAMP levels were not obtained with up to 1 µM hαCGRP. In CHO-cells co-expressing the hCL receptor and hRAMP2 hAM stimulated cAMP formation 20 ± 7-fold with an EC$_{50}$ of 2.4 ± 1.3 nM (n = 4). (Fig. 3, bottom panel). In Δ18-hCL receptor and hRAMP2 co-expressing CHO-cells, on the other hand, 1 µM hAM only minimally stimulated cAMP formation. Taken together, truncation of the N-terminus of the hCL receptor by 18 amino acids impairs hRAMP1 dependent CGRP receptor function and abolishes hRAMP2 assisted hAM receptor activity.
3.2. Substitution of the 18 N-terminal amino acids of the hCL receptor by the corresponding N-terminal sequence of the pCT receptor maintains the CGRP receptor function, but impairs the interaction with hAM.

In CHO cells co-expressing the chimeric pCT-hCL receptor with hRAMP1 [\(^{125}\text{I}\)h\(\alpha\)CGRP binding and cross-linking to 50 and 65 kDa protein components was 150% of that in cells expressing the hCL receptor together with hRAMP1 (Fig. 2). The small increase in size of the [\(^{125}\text{I}\)h\(\alpha\)CGRP cross-linking products in pCT-hCL receptor/hRAMP1 as compared to hCL receptor/hRAMP1 expressing cells was likely caused by the 6 amino acid longer N-terminus of the pCT-hCL receptor as compared to the hCL receptor. But the size difference was comparable to that of [\(^{125}\text{I}\)h\(\alpha\)CGRP cross-linking products in the hCL receptor and hRAMP1 co-expressing cells and corresponded to the calculated molecular weight of hRAMP1. [\(^{125}\text{I}\)hAM receptor cross-linking was not observed in CHO cells expressing the pCT-hCL receptor together with hRAMP2. Altogether, the results indicate the importance of the 18 N-terminal amino acids of the hCL receptor for the hRAMP2-dependent [\(^{125}\text{I}\)hAM binding.

In CHO cells co-transfected with the pCT-hCL receptor and hRAMP1 or -2 expression constructs, mean basal cAMP levels ranged from 3.2 to 3.9 pmol/well (P > 0.05). In cells expressing the pCT-hCL receptor together with hRAMP1 the maximal cAMP response to h\(\alpha\)CGRP was 38 ± 11-fold with an EC\(_{50}\) of 0.5 ± 0.2 nM (n = 4), comparable to that in hCL receptor/hRAMP1 co-expressing cells (Fig. 3). A maximal 33 ± 22 fold stimulation of cAMP formation by hAM was observed in cells co-expressing the pCT-hCL receptor and hRAMP2, but the EC\(_{50}\) increased to 43 ± 14 nM (n = 4) as compared to 2.4 ± 1.3 nM (n = 4) in hCL receptor/hRAMP2 expressing cells (P < 0.05). Taken together, the CGRP receptor function of the pCT-hCL receptor co-expressed with hRAMP1 was that of the hCL receptor, but the 18 N-terminal amino acids of the hCL receptor were essential for the hRAMP2-dependent high affinity interaction with hAM.

3.3. Cell surface expression of receptor constructs and hRAMP2

The ∆18-hCL receptor co-transfected with hRAMP2 was expressed at the cell surface as revealed by co-localization with cell surface glycoproteins labeled with TRITC-ConA (Fig. 4). The pattern of co-localization (yellow) was indistinguishable from that of the hCL and pCT-hCL receptors co-transfected with hRAMP2. Similarly, hRAMP2 was expressed at the cell surface when co-expressed with the hCL, pCT-hCL and ∆18-hCL receptors. Thus, the impaired and lost function of pCT-hCL and ∆18-hCL receptors together with hRAMP2 with respect to [\(^{125}\text{I}\)hAM binding were not caused by defective cell surface delivery of the mutated receptors and hRAMP2.
Fig. 4. Immunochemical localization of receptors and hRAMP2. CHO cells were transfected with hCL (left panels), pCT-hCL (middle panels) or Δ18-hCL (right panels) receptors together with hRAMP2. The cells were detached and surface glycoproteins stained with TRITC-ConA (red), fixed, permeabilized and incubated with rabbit anti-myc antibodies for receptor recognition (upper panels) or with mouse anti-V5 antibodies for RAMP2 recognition (lower panels). Staining was carried out with Alexa488-labeled anti-rabbit or anti-mouse antibodies (green) and images were obtained by confocal laser scanning microscopy. The overlay of two out of six cells of two independent experiments is shown.

4. Discussion

The CL receptor requires RAMP1 or -2 for the recognition of CGRP and AM, respectively [5]. The closely related CT receptor recognizes CT without co-expressed RAMP, but RAMP1 is required for the binding of CGRP [8]. The overall homology of the amino acid sequences of the CL and CT receptors is 57%. But the N-terminal regions of the CL and the CT receptors differ widely. An N-terminal truncated CT receptor still recognized CT [13]. Here we have investigated the importance of the extreme N-terminus of the hCL receptor for the interaction with CGRP or AM. N-terminal truncation of the hCL receptor by 18 amino acids as shown here abolished hαCGRP and hAM binding and impaired stimulation of cAMP formation. With hαCGRP the EC$_{50}$ was increased by two orders of magnitude compared to the intact hCL receptor and hRAMP1. Near maximal cAMP levels were obtained at high concentrations. The interaction of hαCGRP with Δ18-hCL receptor/hRAMP1 rather than their expression was impaired. With hAM marginal cAMP stimulation was obtained at 1 μM as compared to 0.1 nM with the intact hCL receptor and hRAMP2. hAM was therefore four orders of magnitude less potent. Nonetheless, expression of the hCL and the Δ18-hCL receptor and hRAMP2 at the plasma membrane was comparable, as revealed by co-
localization with membrane glycoproteins examined by confocal scanning laser microscopy.

With the chimeric pCT-hCL receptor together with hRAMP1 a fully functional CGRP receptor was obtained. But in the presence of hRAMP2, hAM was two orders of magnitude less potent at the pCT-hCL receptor as compared to the wild-type hCL receptor.

The apparent molecular weights of $[^{125}\text{I}]h\alpha\text{CGRP}$ cross-linking products in hCL and pCT-hCL receptor/hRAMP1 expressing cells and their differences in size were consistent with deglycosylated $[^{125}\text{I}]h\alpha\text{CGRP}/hCL$ receptor, -/hCL receptor/hRAMP1, -/pCT-hCL receptor and -/pCT-hCL receptor/hRAMP1 complexes cross-linked at the cell surface. Similarly, in hCL receptor and hRAMP2 co-expressing cells, $[^{125}\text{I}]h\alpha\text{AM}/hCL$ receptor and -/hCL receptor/hRAMP2 complexes were recognized, but cross-linking of the latter appeared to occur with low efficiency.

A dibasic K/R-X-K/R motif adjacent to the N-terminus of the $\Delta 18$-CL receptor is the only common determinant of the extreme N-terminus in the CL and CT receptors. This motif may therefore be important for the recognition of CGRP by the CL receptor/RAMP1 complex. This also applies for the CL receptor/RAMP2 complex, but additional amino acids near the N-terminus of the CL receptor are important for AM receptor function.

Taken together, the 18 N-terminal amino acids of the hCL receptor are required for the recognition of CGRP and AM. Substitution of this extreme N-terminus of the hCL receptor by the N-terminal 24 amino acids of the pCT receptor reconstituted the CGRP receptor. Amino acids in the extreme N-terminus of the hCL receptor are essential for full recognition of hAM.

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References

Selective Inactivation of Adrenomedullin over Calcitonin Gene-related Peptide Receptor Function by the Deletion of Amino Acids 14 to 20 of the Mouse Calcitonin-Like Receptor *

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Abstract

The receptors for the neuropeptide calcitonin (CT) gene-related peptide (CGRP) and the multifunctional peptide hormone adrenomedullin (AM) are calcitonin-like receptor (CLR)/receptor-activity-modifying protein (RAMP)1 and CLR/RAMP2 heterodimers, respectively. Here, the amino acid sequence TRNKIMT, corresponding to the residues 14 to 20 of the N-terminus of the mouse (m) CLR, was found to be required for a functional mCLR/RAMP2 AM receptor. The deletion of the amino acids 14 to 20 (Δ14-20) or their substitution by alanine (14-20A) did not affect the heterodimerization of the mCLR with mRAMP1 or –2 neither the levels of expression altered at the surface of transiently transfected COS-7 cells. In mRAMP1/mCLR or -(Δ14-20) expressing cells CGRP stimulated cAMP formation with EC₅₀ of 0.12 ± 0.01 and 0.24 ± 0.12 nM, respectively. In mRAMP2/mCLR expressing cells the EC₅₀ of AM was 0.8 ± 0.2 nM. However, in cells expressing mRAMP2/mCLR(Δ14-20) up to 10⁻⁶ M AM failed to stimulate cAMP production. In mRAMP2/mCLR(14-20A) expressing cells the cAMP response to AM was minimally restored and the EC₅₀ was >100 nM.

In conclusion, the deletion of the amino acid sequence TRNKIMT of the extreme N-terminus of the mCLR maintained CGRP receptor function of mRAMP1/receptor heterodimers, but AM no longer activated the mutant mCLR(Δ14-20) in the presence of mRAMP2. The TRNKIMT sequence is required for normal mCLR/mRAMP2 association and as a consequence, high affinity AM binding signalling the activation of adenylyl cyclase.

¹ The abbreviations used are: CT, calcitonin; CGRP, CT gene-related peptide; AM, adrenomedullin; CLR, CT-like receptor; RAMP, receptor-activity-modifying protein; PTH, parathyroid hormone
Introduction

The neuropeptides α- and β-calcitonin (CT)\(^1\) gene-related peptide (CGRP)(1-37) and the multifunctional peptide hormone adrenomedullin (AM) of 52 amino acids in man and 50 amino acids in rat are potent vasodilators (1). CGRP and AM are only 20% identical, but have in common ring structures of six amino acids linked by disulfide bonds between cysteine residues and amidated C-termini, both required for biological activity.

Molecularly defined receptors for CGRP and AM are CT-like receptor (CLR)/receptor-activity-modifying protein (RAMP)1 and CLR/RAMP2 heterodimers, predominantly linked to cAMP production (2). The CLR belongs to the family B of G protein-coupled receptors with seven transmembrane domains that includes the 60% homologous CT receptor and receptors for secretin and parathyroid hormone (PTH). The CLR, unlike the other known members of this receptor family, requires as associated proteins the RAMP for the functional expression. RAMP1 and -2 and a third RAMP3 consist of between 147 and 189 amino acids. They are single transmembrane domain proteins with 30% amino acid sequence similarity. Their N-terminal extracellular domains of between 90 and 110 amino acids define in part CGRP and AM selectivity of the CLR (3). Substitution of the 18 N-terminal amino acids of the human CLR by the corresponding domain of the porcine CT receptor revealed a fully functional CGRP receptor in the presence of human RAMP1, but the RAMP2-dependent AM receptor function was impaired (4). This implies that subdomains of both the CLR and the RAMP determine distinct binding interaction sites for CGRP and AM in the corresponding heterodimers.

In other receptors of the B family of G protein-coupled receptors critical determinants for high affinity ligand binding are localized near the N-terminus. Photochemical cross-linking of PTH analogs and site directed mutagenesis identified Thr\(^{33}\) and Glu\(^{37}\) in the N-terminal extracellular domain of the rat PTH receptor as determinants for PTH binding (5). Similarly, \(^{125}\)I-CT analogues with photoreactive p-benzoyl-L-phenylamine in positions 16 and 26 labeled Thr\(^{30}\) close to the N-terminus and Phe\(^{137}\) adjacent to the first transmembrane domain of the human CT receptor (6). Similarly, in the secretin receptor the amino acids 1 to 10 were indispensable for high affinity ligand binding (7).

Here, the amino acids 14-20 of the mouse CLR with the sequence TRNKIMT were identified as a critical determinant for the functional interaction of the mCLR/mRAMP2 with AM. Importantly, the deletion of the TRNKIMT sequence in the mCLR maintained the CGRP receptor function in the presence of mRAMP1. Substitution of the amino acids 14 to 20 by alanine revealed a low affinity mCLR/mRAMP2 AM receptor.
Experimental procedures

Materials

Cell culture products were supplied by Invitrogen (Carlsbad, CA). Rat αCGRP (CGRP) and rat AM (AM) were obtained from Bachem AG (Bubendorf, Switzerland). Chemicals and other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and VWR International GmbH (Darmstadt, Germany).

Construction of mCLR mutants

A DNA fragment encoding the amino acids 1 to 292 of the mCLR was subcloned into the pBluescript SK- vector (Stratagene, La Jolla, CA). The amino acid deletions and substitutions in the extreme N-terminal region of the mCLR illustrated in Fig. 1 were introduced into the corresponding DNA sequence with the protocols of the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene). The primers carrying the mutations were designed according to the recommendations given by Stratagene. PCR was carried out in a final volume of 50µl, containing 100 ng plasmid template, 50 pmol of 5’ and 3’ primers, and 2.5 units of the PfuUltra™ High-Fidelity DNA Polymerase (Stratagene). The annealing temperature was set to 55°C for the first 5 cycles and to 62°C for the additional 25 cycles. The plasmid template was digested with DpnI and the PCR products were transformed into calcium competent E. coli XL-1 Blue cells. The DNA fragments encoding the N-terminal mCLR subregion with the introduced mutations were sequenced in both directions with ABI prism™ DNA Analyzer (Applied Biosystems, Foster City, CA). DNA fragments carrying the desired mutations were excised from pBluescript SK- and cloned into a pcDNA 3.1 construct that provided an in frame DNA fragment encoding the non-modified C-terminal region of the mCLR.

FIG. 1. Amino acid sequence alignment of wild-type and mutant V5-mCLR. The N-terminal 23 amino acids after the signal peptide and the V5 epitope are shown. The amino acids 14 – 20 (bold) were totally or partially deleted or substituted by alanine residues as indicated.
Cell culture and transfection

COS-7 cells were cultured in HamF12/DMEM (4.5 g/l glucose) medium (1:1) supplemented with 10% FCS and 2 mM glutamine (cell culture medium). For transfection the cells were seeded in 24- or 6-well plates, or in 100 mm culture dishes at a density of 15,000 cells per cm². 48 h later the cells were transfected with the indicated receptor and RAMP expression constructs and linear polyethylenimine (PEI; 25 kDa) (Polysciences, Warrington, PA). Briefly, 0.2 µg/cm² plasmid DNA was diluted with 6.25 µl/cm² cell culture medium. 0.5 µg/cm² PEI in 6.25 µl/cm² cell culture medium was prepared separately. The solutions were then combined and vortexed immediately. After incubation for 15 min at room temperature, the volume was adjusted to 125 µl/cm² with cell culture medium and the solution added to the cells. The cells were incubated at 37°C overnight. The volume was then increased to 250 µl/cm² with cell culture medium, and the cells incubated for 2 days.

Stimulation of cAMP production

2 days after transfection the COS-7 cells were incubated at 37°C for 15 min with the indicated concentrations of rCGRP and rAM in cell culture medium without FCS, but supplemented with 1 mM isobutylmethylxanthine and 0.1% bovine serum albumin. Subsequently, cAMP was extracted from the cells and measured by radioimmunoassay as described (8).

Immunofluorescent staining of intact and mutant V5-mCLR and of myc-mRAMP1 and –2 at the surface of COS-7 cells

The cells were grown and transfected on coverslides in 24-well plates. V5- and myc- immunofluorescence staining was carried out two days after transfection. The cells were fixed with 4% formalin in PBS for 20 min at room temperature, washed with PBS and preincubated in HamF12/DMEM containing 0.1% bovine serum albumin for 30 min at room temperature. The cells were then incubated for 2 h at room temperature in the same medium with mouse monoclonal antibodies to V5 (Invitrogen) and/or rabbit polyclonal antibodies to myc (Abcam, Cambridge, UK) (diluted 1:400). Subsequently the cells were stained for 30 min in the dark with Alexa488 goat anti-rabbit serum (Molecular Probes, Eugene, OR) and/or Cy3 conjugated sheep anti-mouse serum (Sigma-Aldrich, St. Louis, MO) (diluted 1:200). After mounting of the coverslips with Immu-Mount (Thermo Shandon, Inc., Pittsburgh, PA) the fluorescence was detected by a Kappa DX20 CCD camera connected to an Eclipse E600 Nikon microscope with a 0.45x projection lens through a Plan Fluor 20x/0.5 DLL objective using B-2A and G-2A filter blocks.
Protein cross-linking, immunoprecipitation, and deglycosylation

Cell surface protein cross-linking was carried out with 10 mM Bis(sulfosuccinimidyl) suberate (BS3; Pierce Biotechnology, Inc., Rockford, IL). The cells were lysed with 50 mM Hepes, pH 7.5, 7 mM MgCl₂, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml aprotinin, 3 µg/ml leupeptin, 1 mg/ml dodecyl-β-D-maltoside and 0.2 mg/ml cholesterol-hemisuccinate (cell lysis buffer). V5-tagged receptors were immunoprecipitated from the cleared lysates at 4°C by sequential incubation with 50 µl ImmunoPure® Immobilized Protein G (Pierce Biotechnology, Inc.) for 1 h, 3 µl of V5 antibodies for 2 h and 50 µl ImmunoPure® Immobilized Protein G overnight on an end-over-end rotator. The immobilized proteins were washed twice with cell lysis buffer and then deglycosylated in 45 µl 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 0.5% octyl-glucopyranoside, 1% β-mercaptoethanol supplemented with 2 U of N-glycosidase F (F. Hoffmann-La Roche, Ltd., Basel, Switzerland) at 37°C for 18 h. Proteins bound to ImmunoPure® Immobilized Protein G were eluted twice with 15 µl SDS-PAGE sample buffer and the eluates were pooled. Proteins in 50 µl total cell extracts were deglycosylated with 2 U of N-glycosidase F at 37°C for 18 h. The reactions were stopped with protein gel loading buffer.

Western blot analysis

Proteins in cell extracts obtained with lysis buffer or immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose Hybond™ ECL™ membranes (Amersham Biosciences UK Ltd., Buckinghamshire, UK) in a Trans-Blot® SD semi-dry transfer cell (BioRad Laboratories, Hercules, CA) at 20 V for 80 min. Immunoblots were washed with water for 5 min, 2 M NaOH for 10 min, and with water for 5 min. They were then blocked with 5% low fat milk. The epitope-tagged proteins were detected with alkaline phosphatase-labeled monoclonal V5- and myc antibodies (Invitrogen) (diluted 1:3,000 and 1:15,000 in 1% low fat milk, respectively), or with mouse monoclonal myc antibodies 9E10.2 (diluted 1:50 in 5% low fat milk) (9) and secondary alkaline phosphatase-conjugated goat antibodies to mouse IgG (1:15,000 final dilution in 1% low fat milk) (The Jackson Laboratory, Bar Harbour, ME). Actin as a reference for total protein loading was visualized with monoclonal antibodies to actin (diluted 1:5,000 in 5% low fat milk) (Chemicon International, Temecula, CA) and secondary alkaline phosphatase-labeled goat antibodies to mouse IgG (1:15,000 in 1% low fat milk). The alkaline phosphatase-conjugated antibodies were visualized by chemiluminescence with the Immun-Star® AP substrate Pack (BioRad) using a VersaDoc™ Imaging System (BioRad).
Data analysis

The values for halfmaximal effective concentrations (EC$_{50}$) were calculated by non-linear regression analysis using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). The results are means ± standard error of the mean (SEM). Differences between mean values were analyzed by ANOVA. P < 0.05 was considered statistically significant.

Results

Total and cell surface expression of intact and mutant V5-mCLR and of myc-mRAMP1 and –2

The relative expression levels of the V5-mCLR and of the N-terminal deletion mutants and of myc-mRAMP1 and –2 in transiently transfected COS-7 cells were estimated on Western blots of cell extracts (Fig. 2). The expression of the intact V5-mCLR was the same in the absence and presence of myc-mRAMP1. The expression levels of the V5-mCLR(Δ14-20) and -(Δ18-20) in the presence of myc-mRAMP1 were comparable and those of V5-mCLR(14-20A) and -(Δ14-17) slightly higher than that of the intact V5-mCLR. myc-mRAMP1 was expressed at equal levels with intact and mutant V5-mCLR.

The expression levels of the V5-mCLR in the absence and the presence of myc-mRAMP2 were similar and comparable to those of the V5-mCLR(Δ14-20) in the presence of myc-mRAMP2. The V5-mCLR-(Δ14-20) and -(Δ14-17) were expressed at lower and the V5-mCLR(14-20A) at higher levels than the intact V5-mCLR in the presence of myc-mRAMP2. myc-mRAMP2 was lower in the presence of the V5-mCLR(Δ14-17) than together with the V5-mCLR or the other mutants.

The expression of the intact and mutant V5-mCLR and of the myc-mRAMP1 and –2 at the cell surface was visualized by V5- and myc-immunofluorescence staining of intact COS-7 cells (Fig. 3). Cells transfected with the empty pcDNA3 expression vector or with myc-mRAMP1 alone did not stain (Fig. 3A), but myc-mRAMP1 was recognized in permeabilized COS-7 cells (not shown). Thus, myc-mRAMP1 in the absence of the V5-mCLR was not transported to the cell surface. But the V5-mCLR and myc-mRAMP2 expressed alone were both recognized at the cell surface. The cell surface expression of the intact V5-mCLR and of the mutants in the presence of myc-mRAMP1 or -2 was comparable (Fig. 3B). Moreover, the expression of myc-mRAMP1 and –2 at the cell surface was the same in cells co-expressing the intact V5-mCLR or the different mutants. Taken together, the mutations
introduced into the N-terminal extracellular domain of the V5-mCLR did not affect the expression of the receptors and the RAMP at the cell surface.

FIG. 2. Western blot analysis of total cell extracts. COS-7 cells were grown in 6-well plates and transfected with indicated combinations of myc-mRAMP1 or –2 and V5-mCLR or –mutant expression constructs. In transfections with the V5-mCLR alone, empty pcDNA3 expression vector was added to keep the DNA concentrations constant. Two days after transfection, the cells were harvested and the proteins were extracted with cell lysis buffer. Extracts were treated with N-glycosidase F and separated on a 10 – 20% gradient SDS polyacrylamide gel. Proteins were blotted on a nitrocellulose membrane, and V5-tagged receptors and myc-tagged RAMP were visualized with AP-conjugated V5- and myc antibodies. Actin as a reference for protein loading was detected with mouse monoclonal antibodies to actin and secondary AP-conjugated goat antibodies to mouse IgG. Representative experiment carried out three times.
FIG. 3. Cell surface immunofluorescence of wild-type and mutant V5-mCLR and of myc-mRAMP1 and -2. A, COS-7 cells were transfected with pcDNA3 or with V5-mCLR expression construct alone (upper panels) or with myc-mRAMP1 or -2 expression constructs alone (lower panels). After fixation the cells were incubated with mouse anti-V5- (upper panels) or rabbit anti-myc serum (lower panels) and subsequently stained with Cy3 conjugated sheep anti-mouse serum or with Alexa488 conjugated goat anti-rabbit antibodies, respectively. B, COS-7 cells were transfected with the constructs for the expression of intact V5-mCLR or the indicated mutants together with myc-mRAMP1 (left panels) or myc-mRAMP2 (right panels). The cells were simultaneously incubated with rabbit anti-myc- and mouse anti-V5 serum and stained with Alexa488 goat anti-rabbit- and Cy3 sheep anti-mouse serum. Photomicrographs were taken for the detection of V5- (red) and myc-epitopes (green) from the same field of inspection with identical exposure times using respective filter blocks as described in Experimental Procedures. Overlays in yellow indicate co-expression of the receptors and the RAMP. Scale bar 100 µm.

Stimulation of cAMP production
In COS-7 cells transiently co-transfected with myc-mRAMP1 and V5-mCLR expression constructs basal cAMP concentrations were 1.1 ± 0.2 pmol/100,000 cells. The stimulation of cAMP formation by 10^{-6} M CGRP was 48 ± 9-fold (100%) and indistinguishable in cells expressing myc-mRAMP1/V5-mCLR(Δ14-17), -(14-20A), -(Δ14-20) or (Δ18-20) (Fig. 4, Table I). The EC_{50} of CGRP in myc-RAMP1/V5-mCLR, -(Δ14-17), -(14-20A) and -(Δ14-20) expressing cells were 0.12 ± 0.01 nM, 0.24 ± 0.12 nM, 1.0 ± 0.2 nM and 1.5 ± 0.4 nM, respectively. In cells co-expressing myc-mRAMP1 and the V5-mCLR(Δ18-20), on the other hand, the stimulation of cAMP production by 10^{-6} M CGRP was 80% of that in myc-mRAMP1/V5-mCLR expressing cells and the EC_{50} of CGRP was > 100 nM.
FIG. 4. Stimulation of cyclic AMP production in COS-7 cells. COS-7 cells were transfected with myc-mRAMP1 (upper panel) or myc-mRAMP2 (bottom panel) expression constructs together with V5-mCLR (●), V5-mCLR(Δ14-20) (▲), V5-mCLR(14-20A) (▼), V5-mCLR(Δ14-17) (◊) or V5-mCLR(Δ18-20) (x) expression plasmids. The cells were incubated with the indicated concentrations of rCGRP (upper panel) or rAM (bottom panel). Cyclic AMP in cell extracts was measured as described in Experimental Procedures. The results are means ± SEM of three independent experiments.

In COS-7 cells co-expressing myc-mRAMP2 and the V5-mCLR or the indicated mutants basal cAMP levels were between 0.6 ± 0.1 and 2.0 ± 0.2 pmol/100,000 cells. In cells expressing myc-mRAMP2 together with the V5-mCLR or the -(Δ14-17), -(Δ18-20) and -(14-20A) mutants 10^{-6} M AM stimulated cAMP formation to comparable levels and the EC_{50} were 0.8 ± 0.2 nM, 9.3 ± 3.4 nM, 59 ± 7.2 nM and > 100 nM, respectively (Fig. 4, Table I). In contrast, in myc-mRAMP2/ V5-mCLR(Δ14-20) expressing cells the cAMP levels were not affected at up to 10^{-7} M AM. Taken together, the deletion of the amino acids 14-20 of the extreme N-terminus of the mCLR maintains CGRP receptor function in the presence of mRAMP1 but abolishes the mRAMP2-dependent AM receptor activity.
**TABLE I**

*CAMP stimulation in COS-7 cells*
Results are means ± S.E. of at least 4 independent experiments.

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<tr>
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<th>myc-mRAMP1</th>
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<th>myc-mRAMP2</th>
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<td>EC50 (nM)</td>
<td>maximal response (%)a</td>
<td>EC50 (nM)</td>
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<td>V5-mCLR</td>
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<td>0.80 ± 0.20</td>
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<tr>
<td>-(Δ14-20)</td>
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<td>99 ± 16</td>
<td>&gt; 1'000</td>
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<tr>
<td>-(Δ14-20A)</td>
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<td>122 ± 14</td>
<td>170 ± 54b</td>
<td>77 ± 7b</td>
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<tr>
<td>-(Δ14-17)</td>
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<td>106 ± 12</td>
<td>9.3 ± 3.4b</td>
<td>101 ± 21</td>
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<tr>
<td>-(Δ18-20)</td>
<td>152 ± 48b</td>
<td>76 ± 19</td>
<td>59 ± 7b</td>
<td>104 ± 33</td>
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a Maximal cAMP stimulation of V5-mCLR was set to 100%, b P < 0.05 vs. V5-mCLR, c P < 0.01 vs. V5-mCLR.

**Cell surface association of intact and mutant V5-mCLR with myc-mRAMP1 and –2**

The association at the cell surface of the V5-mCLR or its mutants with myc-mRAMP1 or –2 was assessed by chemical cross-linking of corresponding myc-mRAMP/V5-mCLR heterodimers in transiently transfected, intact COS-7 cells. Protein components with a V5-epitope tag were immunoprecipitated from cell homogenates and deglycosylated. The extracted proteins were then separated on SDS-PAGE and analyzed on Western blots with V5- and myc antibodies. Predominant V5-immunoreactive protein components with an apparent size of approximately 50 kDa represented the V5-mCLR and the indicated mutants (Fig. 5). The amounts of the V5-mCLR and of the mutants in the presence of myc-mRAMP1 were comparable and not different from V5-mCLR in the absence of myc-mRAMP1. In the presence of myc-mRAMP2 the amounts of the V5-mCLR and of the V5-mCLR(14-20A) and -(Δ18-20) were similar, but those of the V5-mCLR(Δ14-20) and of the V5-mCLR(Δ14-17) were lower. The amount of the V5-mCLR in the absence of myc-mRAMP2 was also lower than in its presence.

The extracts obtained with V5 antibodies from cells that expressed the V5-mCLR or its mutants together with myc-mRAMP1 contained myc-immunoreactive protein components of 16 kDa and approximately 66 kDa apparent size. They represented intact myc-mRAMP1 and myc-mRAMP1 cross-linked at the cell surface to the V5-mCLR and the mutants, respectively. This indicated that the deletions and substitutions introduced into the N-terminus of the mCLR did not affect the formation and cell surface expression of mCLR/mRAMP1 heterodimers.
FIG. 5. Western blot analysis of cell surface cross-linked- and V5-immunoprecipitated proteins. COS-7 cells were transfected with the indicated receptor and RAMP expression constructs. Control cells were transfected with the V5-mCLR construct together with pcDNA3 to keep the DNA concentration constant. Two days after transfection intact cells were incubated with BS3 protein cross-linker. V5 epitope-tagged receptors were immunoprecipitated from cell extracts, deglycosylated and separated on a 10 – 20 % gradient SDS-polyacrylamide gel. Myc-tagged RAMP and V5-tagged receptors were detected with corresponding antibodies as described in Experimental Procedures. Representative experiment carried out three times.

Immunoprecipitation with V5 antibodies of cells co-expressing myc-mRAMP2 together with the intact and mutant V5-mCLR revealed predominant myc-immunoreactive protein components of approximately 18 kDa apparent size. They represented deglycosylated myc-mRAMP2 co-precipitating with the intact and mutant V5-mCLR. Interestingly, receptor/myc-mRAMP2 cell surface cross-linking products of approximately 70 kDa were only recognized in extracts of cells that co-expressed myc-mRAMP2 together with the intact V5-mCLR or with the mutant V5-mCLR(14-20A) and -(Δ18-20). All together, this indicated that the deletions of the amino acids 14 to 17 or 14 to 20 of the extreme N-terminus of the mCLR did not affect co-immunoprecipitation of myc-mRAMP2, but prevented chemical cross-linking at the cell surface. Since the V5-mCLR(14-20A) and myc-mRAMP2 revealed a cross-linking product the Lys^{17} residue as the cross-linking site between the V5-mCLR and myc-mRAMP2 is excluded.
Discussion

The structures of CLR/RAMP1 or –2 defining CGRP or AM selectivity are largely unknown. The N-terminal extracellular domains of RAMP1 and -2 determine in part CGRP and AM specificity of the CLR (3). Along these lines, chemical cross-linking of $^{125}$I-CGRP or –AM to intact cells expressing the CLR with RAMP1 or –2 revealed $^{125}$I-CGRP/RAMP1 and $^{125}$I-AM/RAMP2 cross-linking products (10). This indicated that extracellular subdomains of the RAMP are presumably in close proximity, and contribute to the CGRP and AM binding sites of CLR/RAMP1 and –2 heterodimers. Accordingly, RAMP1, through association with the CTR, the closest homologue of the CLR in the family B of G protein-coupled receptors, brings about a CGRP receptor isotype (11). The CTR, on the other hand, unlike the CLR, does not recognize AM in the presence of RAMP2. This indicates that structural elements in the CLR, that are not present in the CTR, are required for the AM receptor function of RAMP2/CLR heterodimers. Interestingly, the overall homology of the amino acid sequences of the CLR and the CTR is 60%, but the extreme N-terminal regions of the receptors differ more widely. Here, targeted deletion and alanine substitution mutagenesis in the N-terminal region of the mCLR identified the amino acids 14 to 20 with the sequence TRNKIMT as a receptor domain that is required for the high affinity interaction with AM in the presence of mRAMP2. Deletion of the TRNKIMT sequence abolished the AM receptor function of the V5-mCLR without affecting the expression at the cell surface and heterodimerization with myc-mRAMP2. Chemical cross-linking of myc-mRAMP2 with the V5-mCLR at the surface of intact cells indicates the close proximity of the extracellular domains of the corresponding proteins. Cross-linking was no longer possible when the TRNKIMT sequence of the V5-mCLR was deleted. Cell-surface cross-linking of myc-mRAMP2 to the V5-mCLR with a TRNK deletion was also not observed, but here the AM receptor function in the presence of myc-mRAMP2 was maintained. The results may imply that the chemical cross-linker BS3 coupled myc-mRAMP2 to the Lys17 residue in the V5-mCLR. But this was ruled out by the substitution of the TRNKIMT sequence by 7 alanine residues revealing myc-mRAMP2/V5-mCLR(14-20A) cross-linking products indistinguishable from myc-mRAMP2/V5-mCLR. Taken together, the results demonstrate that structural alterations in the N-terminal extracellular domain of the V5-mCLR brought about by the deletion of the TRNKIMT sequence, inhibit the formation of a high affinity AM recognition site in myc-mRAMP2/V5-mCLR(Δ14-20) heterodimers. The structural alterations in the N-terminal extracellular domain of the V5-mCLR are less severe when the TRNKIMT sequence is replaced by 7 alanines.
Here, low affinity interaction with AM and chemical cross-linking of the myc-mRAMP2/V5-mCLR(14-20A) complex at the cell surface was maintained.

In an attempt to assign the defect in AM receptor function of the V5-mCLR(Δ14-20) to parts of the TRNKIMT motive, the TRNK and the IMT sequences were individually deleted. Interestingly, the CGRP and AM receptor functions of the V5-mCLR were minimally affected by the TRNK deletion. The deletion of the IMT sequence, on the other hand, increased the half-maximal concentrations of both CGRP and AM for the stimulation of cAMP formation by two orders of magnitude. Thus, minor changes in the amino acid sequence of the mCLR N-terminal extracellular domain impair high affinity interactions of mCLR/mRAMP1 and -2 heterodimers with CGRP and AM. Along these lines, the substitution of Asn\textsuperscript{117} in a consensus N-glycosylation site of the human CLR by amino acids other than Asp abolished the CGRP and AM receptor functions in the presence of RAMP1 and -2 (12).

In conclusion, minimal amino acid deletions or substitutions in the N-terminal extracellular domain of the CLR reveal distinct CGRP and/or AM receptor defects of the respective CLR/RAMP1 and -2 heterodimers. Here, the deletion of the sequence TRNKIMT, corresponding to amino acids 14 to 20 in the mCLR, selectively abolished its AM receptor function. This observation is important for the development of AM receptor deficient animals to reveal distinct biological functions of AM and CGRP in vivo.

References


The Aspartate$^{69}$ Residue of the Calcitonin-Like Receptor is Required for its Functional Expression together with Receptor-Activity-Modifying Proteins 1 and –2$^{+}$

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Abstract

The calcitonin-like receptor (CLR) associated with receptor-activity-modifying proteins (RAMP) -1 or -2 recognizes calcitonin gene-related peptide (CGRP) and adrenomedullin (AM), respectively. The amino acid sequence CNRTWDGWLCW corresponding to residues 64-74 in the extracellular N-terminus of the CLR is conserved. Aspartate\(^69\) (D\(^{69}\)), unlike asparagine\(^65\) (N\(^{65}\)) of a putative N-glycosylation site, is conserved in all family B1 G-protein-coupled receptors. Here the N\(^{65}\) and D\(^{69}\) of a V5-tagged mouse CLR have been mutated. The functions of the intact and the mutant CLR were investigated through co-expression with myc-tagged mouse RAMP1 or -2 in COS-7 cells. In CLR/RAMP1 expressing cells CGRP stimulated cAMP formation with an EC\(_{50}\) of 0.17 nM and the EC\(_{50}\) of AM in CLR/RAMP2 expressing cells was 0.50 nM. Indistinguishable receptors were obtained with the N65A and N65D mutants. The D69A, D69E and D69N mutants were expressed at the cell surface much like the intact CLR. cAMP stimulation by CGRP and AM was abolished in the D69A mutant. With the D69E mutant the EC\(_{50}\) of CGRP and AM were 1000-fold higher than those of the intact CLR. With the D69N mutant the EC\(_{50}\) of CGRP was 0.48 nM and that of AM 0.44 nM, but the maximal cAMP formation was reduced to 24% and to 12% of the intact CLR. Co-immunoprecipitation revealed reduced complex formation with RAMP1 together with the D69A, D69N and D69E mutants. In conclusion, mutation of the N\(^{65}\) putative N-glycosylation site does not affect CLR activity. But reduced complex formation of the D69A and D69E mutants with RAMP1 abolished and with the D69N mutant attenuated the functional interaction with CGRP.

\(^1\)Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; CRF, corticotropin releasing factor; GHRH, growth hormone releasing hormone; RAMP, receptor-activity-modifying protein; VIP, vasoactive intestinal polypeptide.

\(^2\)Gene Bank accession numbers: U17473 (man), L27487 (rat), AB015595 (mouse), AF419317 (pig), AR098168 (cow), BC044269 (xenopus), AB035314 (flounder), AJ508554 (salmon). The puffer fish sequence SINFRUP00000066454 was found with a protein BLAST search using the xenopus sequence.
Introduction

The family B1 of G protein-coupled receptors with seven transmembrane domains comprises the receptors for the hormones glucagon, glucagon-like peptide, gastric inhibitory peptide, secretin, vasoactive intestinal polypeptide (VIP)\(^1\), pituitary adenylate cyclase polypeptide, growth hormone releasing hormone (GHRH), corticotropin releasing factor (CRF), parathyroid hormone, calcitonin and calcitonin gene-related peptide (CGRP) \(^1\). The receptors have an N-terminal signal sequence required for targeting to the plasma membrane and an extracellular N-terminus of between 100 and 160 amino acids. Except for the CRF receptor 2 with five conserved cysteins all other members have six or seven conserved cysteins spread over the entire N-terminus. The aspartate (D) in a conserved C-x(4)-D-x(3,4)-C-W-x(11,12)-C-P motif in the N-terminal extra-cellular domain is present in 56 sequences from 15 receptors of the family B1. Otherwise, besides the conserved cysteins, less than 10 per cent of the amino acids in the extracellular domains of these receptors are identical.

The extracellular domain of family B1 receptors is involved in part in the interaction with different peptide ligands \(^1\). The substitution of the conserved aspartate in this domain abolished the function of the VIP and glucagon receptors in the face of unaltered cell surface expression \(^2;3\). Interestingly, a corresponding aspartate to glycine mutation in the GHRH receptor causes the little mouse phenotype associated with absent GHRH binding \(^4;5\). Substitution of five amino acids N-terminal of the conserved aspartate of the human CRF1 receptor by those of the secretin receptor increased the EC\(_{50}\) of CRF stimulated cAMP formation 100-fold in the face of unaltered maximal responses even though the aspartate was preserved \(^6\). The aspartate in the secretin receptor, on the other hand, when replaced by alanine, asparagine and glutamate did not affect receptor function in a major way \(^7\).

The calcitonin-like receptor (CLR) requires the receptor-activity-modifying proteins (RAMP) for the recognition of CGRP and adrenomedullin (AM) \(^8;10\). RAMP are single transmembrane domain proteins with an extracellular N-terminus of between 90 and 100 amino acids and a short cytoplasmatic tail of about 10 amino acids \(^11;12\). Together with RAMP1 or -2 the CLR recognizes the vasodilatory peptides CGRP and AM, respectively. The RAMP form non-covalent immunoprecipitable complexes with the CLR at the plasma membrane required for ligand recognition \(^10;13;14\).

The extreme N-terminus of the CLR determines the interaction with CGRP and AM in the presence of RAMP1 and -2 \(^15\). Moreover, an asparagine within a N-glycosylation consensus sequence near the first
transmembrane domain is required for CGRP binding independent of its glycosylation (16). The CLR from man, rat, mouse, pig, cow, xenopus, flounder, salmon and puffer fish have a conserved C-N-R-T-W-D-G-W-L-C-W sequence, corresponding to amino acid residues 64-74 in the central part of the extracellular domain2. This sequence is part of the conserved C-x(4)-D-x(3,4)-C-W-x(11,12)-C-P motif in family B1 receptors and includes besides the conserved D69 an N-glycosylation consensus sequence N65-X-[TS].

Here the role of the conserved D69 and the N-glycosylation site N65 has been investigated through point mutations of the mouse (m)CLR. Functional CGRP and AM receptors have been obtained with the N65A and N65D mutations. But D69A and D69E mutations abolished CGRP and AM receptor function. Residual activity was maintained with the D69N mutation.

![Figure 1](image1.png)

**FIGURE 1:** Representation of non-modified and mutant CLR. The CLR reveals a conserved CNRTWDGWLCW motif. The amino acids common to all family B1 receptors are black. The N-glycosylation consensus sequence is underlined. Deletions are indicated by dots and single amino acid substitutions are shown in bold.

**Materials and methods**

**Materials.**

Rat αCGRP(1-37) (αCGRP) and rAM(1-50) (AM) were purchased from Bachem. Restriction enzymes were obtained from Promega. 125I and Hybond ECL nitrocellulose membranes were from Amersham Pharmacia Biotech. Immun-Star™ AP substrate pack was from BioRad Laboratories. Cell culture products were supplied by Invitrogen and linear polyethyleneimine (PEI) (25 kDa) by Polysciences. Rabbit antibodies to myc were from Abcam and mouse monoclonal antibodies to V5 epitope were from Invitrogen. Alexa488-labelled goat anti-rabbit antibodies were from Molecular Probes and Cy3-labelled sheep anti-mouse antibodies
from Sigma. Other chemicals and reagents were purchased from Sigma and Merck at the highest grade available.

**DNA constructs.**

A DNA fragment encoding the amino acids 1 to 292 of the mCLR and a V5 epitope tag introduced after the signal sequence was subcloned into the pBluescript SK- vector (Stratagene). The amino acid deletions and substitutions in the extracellular region of the mCLR were introduced into the corresponding DNA sequence with the protocols of the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene) (Fig. 1). The primers carrying the mutations were designed according to recommendations of Stratagene. PCR was carried out in a final volume of 50 µl containing 100 ng plasmid template, 50 pmol of 5' and 3' primers, and 2.5 U PfuUltra™ High-Fidelity DNA Polymerase (Stratagene). The annealing temperature was set to 55°C for the first 5 cycles and to 62°C for additional 25 cycles. The plasmid template was digested with DpnI and the PCR products were transformed into calcium competent *E. coli* XL-1 Blue cells. The DNA fragments encoding the N-terminal mCLR subregion with the introduced mutations were sequenced in both directions with ABI prism™ DNA Analyzer (Applied Biosystems). DNA fragments carrying the desired mutations were excised from pBluescript SK- and cloned into a pcDNA 3.1 construct that provided an in frame DNA fragment encoding the non-modified C-terminal region of the mCLR. The constructs encoding mRAMP1 and -2 with N-terminal myc epitope tags were obtained as described (17).

**Cell culture, transfection and cAMP stimulation.**

COS-7 cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) (4.5 g/l glucose) and Ham’s F12 (1:1) medium containing 2 mM glutamine and 10% fetal calf serum. The cells were grown to 40-60% confluence and transfected with the indicated concentrations of the constructs encoding myc-tagged mRAMP1 and V5-tagged intact and mutant mCLR using linear PEI (14). Briefly, 0.2 µg/cm² plasmid DNA was diluted with 6.25 µl/cm² cell culture medium. This solution was then combined with 0.6 µg/cm² PEI in 6.25 µl/cm² cell culture medium, and vortexed immediately. After incubation for 15 min at room temperature the volume of the DNA/PEI mixture was adjusted to 125 µl/cm² with cell culture medium and added to the cells. The cells were incubated at 37°C for 16 to 24 h. The volume was then increased to 250 µl/cm² with cell culture medium, and the cells were incubated for another 48 h.

Cyclic AMP stimulation was performed in cell culture medium without fetal calf serum but supplemented with 0.1% bovine serum
albumin and 1 mM isobutylmethylxanthine for 15 min at 37°C. Cyclic AMP was extracted from the cells and measured by radioimmuno-assay as described (17).

**Cell extracts, cell surface immunoprecipitation and Western blot analysis.**

Proteins were extracted from cells with 50 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml aprotinin, 3 µg/ml leupeptin and 0.5% Triton X-100. Proteins in 50 µl extract were deglycosylated with 2 U of N-glycosidase F (F. Hoffmann-La Roche) at 37°C for 18 h. The reactions were stopped with protein gel loading buffer.

Immunoprecipitation of V5-tagged mCLR from the cell surface was carried out as described (13). Briefly, COS-7 cells transiently expressing the V5-mCLR/RAMP1 or -2 and cultured in 12.5 cm² flasks were incubated at 15°C with 400 µl of V5 antibodies (1:200) for 3 h. The cells were washed and lysed in 500 µl cell lysis buffer containing 50 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml aprotinin, 3 µg/ml leupeptin and 0.5% TritonX-100 or 25 mM octylglucoside. The lysates were cleared by centrifugation and the supernatants were incubated with Immuno-Pure Immobilized Protein G (Pierce) at 4°C for 2 h on an end-over-end rotator. The immobilized proteins were washed twice with cell lysis buffer and then deglycosylated at 37°C with 2 U of N-glycosidase F for 18 h. The reactions were stopped by adding protein gel loading buffer.

Proteins in cell extracts or immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose Hybond™ ECL™ membranes (Amersham Biosciences) in a Trans-Blot® SD semidry transfer cell (BioRad Laboratories) at 20 V for 60 min. The membranes were blocked with 5% low fat milk. The V5-tagged CLR and the myc-tagged RAMP1 and -2 were detected with AP-labeled monoclonal V5- and myc antibodies (Invitrogen) (diluted 1:3000 and 1:15000 in 1% low fat milk, respectively). Actin as a reference for protein loading was visualized with monoclonal antibodies to actin (diluted 1:5000 in 5% low fat milk) (Chemicon International) and secondary AP-labeled goat antibodies to mouse IgG (The Jackson Laboratory, 1:15000 in 1% low fat milk). The AP-conjugated antibodies were visualized by chemiluminescence with the Immun-Star™ AP substrate Pack (BioRad Laboratories) using a VersaDoc™ Imaging System (BioRad Laboratories). For reprobing the membranes with different antibodies the immunoblots were washed with water for 5 min, 2 M NaOH for 10 min, and with water for 5 min.
**Immunocytochemistry.**

Immunostaining of formalin fixed (4%) COS-7 cells was performed as described (17). Briefly, the cells were simultaneously incubated with rabbit anti-myc (1:500) and mouse anti-V5 (1:500) antisera for 2 h at room temperature. After washing, the cells were simultaneously incubated with Alexa488-labelled goat anti-rabbit (1:200) and Cy3-labelled sheep anti-mouse antisera (1:200) for 30 min at room temperature. After washing, the cells were mounted with Immu-Mount (Shandon Scientific). The cells were viewed with an Eclipse E600 Nikon microscope equipped with a Plan Fluor 20x/0.5 DLL objective, a G-2A filter and a Kappa DX20 CCD camera connected to the microscope with a Nikon 0.45x projection lens. Immunofluorescence intensities were recorded with a Nikon U-III multipoint sensor system and respective reciprocal exposure times were taken as a measure for cell surface expression of the myc- and the V5-tagged proteins.

**Data analysis.**

In cAMP stimulation experiments half-maximal effective concentrations (EC$_{50}$) were calculated by non-linear regression analysis using the FigP 6.0 software (Biosoft). Results are means ± standard error of the means. Comparison to controls was carried out using the Student’s t-test. $P$ values of <0.05 were considered statistically significant.

**Results**

**CGRP and AM receptor function of intact and mutant mouse CLR.**

The CLR was N-terminally tagged with a V5 epitope and mouse RAMP1 and -2 were N-terminally tagged with a myc epitope. Here they are abbreviated CLR, RAMP1 and -2. The sequence CNRTWDGWLCW is conserved in all known CLR (Fig. 1). Numbering refers to the native sequence of the mouse CLR (Swiss-Prot Q9R1W5). In the ∆66-71 deletion mutant T$_{67}$ in the N-glycosylation consensus site and D$_{69}$ were removed. The WDGW deletion in the ∆68-71 mutant left intact the putative glycosylation site but removed D$_{69}$. With both mutants co-transfected with RAMP1 and -2 CGRP and AM failed to stimulate cAMP formation (Fig. 2; Table 1). But, selective elimination of the potential N-glycosylation site by N65A and N65D substitutions did not affect CGRP and AM receptor function of the CLR.

The functional role of the conserved D$_{69}$ was investigated with D69A, D69E and D69N substitutions. The D69A mutant was inactive (Fig. 2, Table 1). In D69E/RAMP1 and -2 expressing cells cAMP formation was marginally stimulated by $\alpha$CGRP and AM at $\geq$100 nM. With
D69N/RAMP1 and -2 maximal levels of cAMP formation were reduced by 76% and 88% ($P < 0.001$), respectively, compared to CLR/ RAMP1 and -2. But the EC$_{50}$ of $\alpha$CGRP and AM were maintained. Taken together, mutation of the putative N-glycosylation site N$^{65}$ of the CLR had no functional consequences on $\alpha$CGRP and AM stimulated cAMP accumulation. Substitution of asparagine$^{69}$ by alanine and glutamate abolished receptor function. The aspartate to asparagine substitution reduced maximal cAMP stimulation with unaltered EC$_{50}$ of $\alpha$CGRP and AM.

![Figure 2: Stimulation of cAMP accumulation in COS-7 cells](image)

Table 1. cAMP stimulation by $\alpha$CGRP and AM in COS-7 cells transfected with the mouse CLR and its mutants together with mouse RAMP1 or –2.

<table>
<thead>
<tr>
<th></th>
<th>RAMP1</th>
<th>RAMP2</th>
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<tbody>
<tr>
<td></td>
<td>Maximal (%)$^a$</td>
<td>EC$_{50}$ (nM)</td>
</tr>
<tr>
<td>CLR</td>
<td>100$^b$</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>$\Delta 66-71$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$\Delta 68-71$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>N65A</td>
<td>131 ± 22</td>
<td>0.41 ± 0.15</td>
</tr>
<tr>
<td>N65D</td>
<td>130 ± 7</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>D69A</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>D69N</td>
<td>24 ± 4$^c$</td>
<td>0.48 ± 0.06$^c$</td>
</tr>
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</table>

Results are means ± standard error of the means of 3 to 6 independent experiments. n.s.; no cAMP stimulation. n.d.; not determinable. $^a$Maximal cAMP stimulation of CLR was set to 100%. $^b$cAMP stimulation by $\alpha$CGRP was 31 ± 7-fold and by AM 10 ± 0.4-fold. $^cP < 0.05$ vs. CLR.
Expression and glycosylation of the CLR mutants.

Protein expression was assessed in cell extracts after N-deglycosylation. Actin was a measure for protein loading. The CLR and its mutants of 45-50 kDa, and the 15 and 18 kDa RAMP1 and -2 were expressed at similar levels (Fig. 3). The apparent size of the deglycosylated proteins corresponded to those calculated for the protein backbones. Western blot analysis of non-deglycosylated samples revealed a major component of the CLR together with RAMP1 and -2 with an apparent Mr of 65 kDa and several larger components with 70 to 100 kDa apparent size (Fig. 4). A similar pattern was observed with the N65D mutant. A major component of the N65A presented a slightly lower molecular weight of 60 kDa in the presence of RAMP1 and -2. After N-glycosidase F treatment all the receptors had apparent sizes of between 45-50 kDa. Taken together, the expected glycosylated components of the CLR were observed in the presence of RAMP1 and -2 (10). Substitution of asparagine by alanine or aspartate only minimally affected the glycosylation pattern.

FIGURE 3: Expression levels of the V5-mCLR and its mutants and of myc-mRAMP1 and -2 in transiently transfected COS-7 cells. Cell extracts were treated with 0.04 U/µl N-glycosidase F, and the proteins were separated on 10-20% SDS-PAGE and analyzed on Western blots with mouse monoclonal AP-conjugated antibodies to V5 or myc. Actin was detected with mouse monoclonal antibodies to actin and secondary AP-conjugated sheep antiserum to mouse immunoglobulins. The representative experiment was carried out three times.
Figure 4: Glycosylation of the CLR and of its mutants N65A and N65D. COS-7 cells expressed the V5-mCLR and the indicated mutants together with myc-mRAMP-1 or -2. Cell extracts incubated in the absence and presence of N-glycosidase F (0.04 U/µl) were subjected to 10-20% SDS-PAGE, and the proteins were analyzed on Western blots with mouse monoclonal AP-conjugated antibodies to V5. The representative experiment was carried out three times.

Cell surface expression of the CLR mutants and of RAMP1 and -2.

The CLR was expressed at the cell surface independently of coexpressed RAMP1 or -2 (Fig. 5A). RAMP1, unlike RAMP2 was not detected at the cell surface in the absence of the CLR. The levels of expression of the CLR alone were 74 ± 10% (P < 0.05) and 54 ± 1% (P < 0.001) of those in the presence of RAMP1 or -2, respectively (Fig. 5 B, C). With RAMP1 all the mutant receptors were expressed at the levels of the intact CLR, and they facilitated the transport of RAMP1 to the cell surface to a similar extent. The cell surface expression of the glycosylated RAMP2 was not affected by the presence of the CLR, and the expression of the mutant receptors was similar to that of the intact CLR. The coexpression of the D69A, D69E and D69N with RAMP1 and -2 was indistinguishable from that of the intact CLR. The co-expression of the Δ66-71, Δ68-71 deletion and the N65E and N65A substitution mutants in the presence of the RAMP (not shown). Taken together, the intact CLR and all the mutant receptors were expressed at comparable levels at the cell surface together with RAMP1 and -2.
FIGURE 5: Cell surface expression of epitope tagged intact and mutant CLR and RAMP1 and -2 in COS-7 cells. A: cells were transfected with pcDNA3 (mock) or with V5-CLR, or with myc-RAMP1 or -2 expression constructs together with pcDNA3 to correct for the total amounts of transfected DNA. B: cells were co-transfected with V5-CLR, V5-D69A, V5-D69N or V5-D69E together with myc-RAMP1 or -2 expressing constructs. Formalin fixed cells were stained simultaneously with mouse monoclonal anti-V5 and rabbit anti-myc antibodies and visualized with Cy3-labeled sheep anti-mouse (red) and Alexa 488-labeled goat anti-rabbit (green) antisera. Co-expression is shown in the overlays in yellow. The scale bar represents 200 µm. C: Exposure times were measured using filterblocks for the detection of Cy3 (V5; open columns) or Alexa 488 (myc; closed columns) of cells stained as in Fig. 3B. Inverse exposure times of cells transfected with V5-CLR and myc-RAMP1 or -2 expressing constructs were set to 100%. Results are representative (A) or means ± standard error of the means of 3 independent experiments (B).
Cell surface co-immunoprecipitation of RAMP1 and -2 with CLR mutants.

The CLR and D69A, D69E and D69N coexpressed with RAMP1 or -2 were immunoprecipitated from the cell surface with V5 antibodies. The deglycosylated immunoprecipitates were analyzed on Western blots. RAMP1 and -2 were co-immunoprecipitated when expressed with the intact CLR (Fig. 6). Co-immunoprecipitation of RAMP1 with the D69A, D69E and D69N mutants was reduced as compared to the intact CLR. RAMP2 was co-immunoprecipitated with no evident difference between the intact CLR and the D69A, D69N and D69E mutants.

![Cell surface co-immunoprecipitation of RAMP1 and -2 with CLR mutants.](image)

**FIGURE 6:** Cell surface co-immunoprecipitation of RAMP1 and -2 with the CLR and its mutants. COS-7 cells were transfected with V5-CLR, V5-D69A, V5-D69N or V5-D69E together with myc-mRAMP1 or -2 expression constructs. Intact cells were incubated with mouse monoclonal anti-V5 antibodies and the CLR and its mutants were immunoprecipitated from lysates. Immunoprecipitates were subjected to 10-20% SDS-PAGE, and Western blots were analyzed with mouse monoclonal AP-conjugated V5 and myc antibodies. The representative experiment was carried out three times.

Discussion

The VIP, GHRH and glucagon receptors in the family B1 of G protein-coupled receptors require the aspartate in a C-x(4)-D-x(3,4)-C-W-x(11,12)-C-P consensus sequence in the N-terminal extra-cellular domain for function (2-5). Within this consensus sequence the CLR and VIP, GHRH and glucagon receptors are 40% to 60% similar. The CLR requires associated RAMP1 or -2 for its expression as a CGRP or AM receptor. Interestingly, the VIP and glucagon receptor, unlike the GHRH receptor, also interact with the RAMP (18). The CLR and the glucagon receptors have a potential N-glycosylation site four amino acids N-terminal of the conserved aspartate. Here, the involvement of the N-glycosylation site and/or the conserved aspartate in the CLR for CLR/RAMP function have been investigated.

Substitution of the asparagine by alanine or aspartate did not affect N-glycosylation and the function of the mouse CLR as a CGRP and AM receptor. The human CLR, on the other hand, is glycosylated at
a corresponding site and minimal glycosylation is required for cell surface delivery (19;20). The human CLR has three N-glycosylation consensus sites compared to six in the mouse CLR studied here. In the mouse, the N- residue is therefore not important for glycosylation.

Interestingly, deletion of the N-glycosylation consensus site together with the conserved aspartate in the mutant abolished CLR activity. With the mutant the glycosylation site was preserved, but the aspartate was deleted. This mutant was also inactive. Both the and mutants were recognized at the cell surface.

Substitution of the negatively charged D by an A abolished the stimulation of cAMP formation by CGRP and AM. This was also observed in the D68A and D68G substituted VIP receptor, the D64G and D64K substituted glucagon receptor and the D60G mutation in the little mouse (2-5). Marginal stimulation was observed with CGRP and AM at concentrations over 100 nM with the mutant that had the negatively charged side chain extended by one methylene group. The correspondingly mutated D49E and D64E secretin and glucagon receptors were fully active and inactive, respectively (3;7). Interestingly, a residual maximal cAMP response to CGRP and AM of 24% and 12% in the presence of RAMP1 or -2, respectively, was obtained with the carboxylate group replaced by an amide group in the D69N mutant, and the EC was that of intact CLR. The corresponding D49N mutation of the secretin receptor had similar properties (7). The D64N mutated glucagon receptor was inactive (3). Thus, hydrogen bonds of the aspartate residue rather than the negative charge may contribute to the formation of biologically active CLR/RAMP complexes.

D69A, D69N and D69E substituted CLR were expressed in total cell extracts and delivered to the cell surface like the intact CLR. Moreover, all the mutants maintained the transport of the non-glycosylated RAMP1 to the periphery of the cell. But the functional interaction with CGRP and AM of the D69A-, D69N- and D69E/RAMP1 or -2 complexes at the cell surface was reduced or abolished. The reduced co-immunoprecipitation of RAMP1 with the modified CLR is consistent with the abolished or impaired stimulation of cAMP by CGRP.

In conclusion, a conserved aspartate in a consensus sequence C-x(4)-D-x(3,4)-C-W-x(11,12)-C-P in the N-terminal extracellular domain of family B1 G protein-coupled receptors is essential for the stable interaction of the CLR with RAMP1, and as a result CGRP receptor function.
REFERENCES

Outlook

Report I and II show, that besides the RAMP, the extreme N-terminus of the CLR contribute to CGRP or AM selectivity. An additional sequence important for receptor/RAMP/ligand interactions, comparable to the receptors for VIP, GHRH and glucagon was found for the CLR in report III [107, 108, 110, 112]. In current work, parts of the CLR, that enable the translocation of RAMP1 to the cell surface are identified with PTHR1/CLR chimeras (in preparation). These investigations of CLR/RAMP/ligand interactions through site directed mutagenesis give insight into their structure-function relationship. In combination with computer-based models non-peptidic low-molecular-weight receptor agonists or antagonists are screened for drug development.

An adenoviral construct of the chimeric pCTR-hCLR receptor was transfected into mouse iliac and pulmonary arteries. Relaxation of the arteries upon CGRP or AM treatment was obtained. With CGRP vaso-dilatation was that of untransfected arteries, but AM did not relax the transfected arteries (unpublished data). The results received in cell culture experiments were those in isolated mouse arteries.

Transgenic mice overexpressing RAMP2 and/or the CLR with an α-actin promoter are produced in our laboratory. The receptor protein carrying an epitope tag recognizable by specific antibodies have been localized to the hair follicles. Here they may be responsible for defective hair development [113].
References


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Curriculum vitae

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List of publications

Original reports


Abstracts

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