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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

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 [1]. 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 cross-reactivity 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 humans 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 cysteine 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 (Δ18-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.

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

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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 cysteines (*) 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 (Bubendorf, Switzerland) and hAM was from Peptide Institute (Osaka, Japan). Na[^{125}I] and Hybond ECL™ nitrocellulose membranes were from Amersham International (Little Chalfont, UK), and restriction enzymes were from Promega (Madison, WI, USA) and Roche Diagnostics (Rotkreuz, Switzerland). Tissue culture supplies, LipofectAMINE and OptiMEM medium for transfections were from Invitrogen (Carlsbad, CA, USA). Other reagents, unless otherwise 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$_6$ (hRAMP1 and hRAMP2) were obtained as previously described [8]. The DNA encoding a pCT–hCL fusion receptor with C-terminal myc and His$_6$ epitope tags was cloned into the mammalian expression vector pcDNA3 as follows. The cDNA encoding the hCL receptor was subcloned into the *Eco*RI restriction site of plasmid pIC19H (Gene Bank accession no. VB0091) [4]. An *Eco*RV/*Hpa*I 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 *Eco*RV/*Hpa*I DNA fragment encoding the Ile^{19} to Val^{107} sequence of the hCL receptor, amplified by PCR. This construct was digested with the *Eco*RV 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^{19} was introduced. This fragment was also obtained by PCR and had an *Eco*RV and an adjacent *Bam*HI restriction site at the 5′-end and a 3′ blunt end. The pCT–hCL receptor fusion gene was then digested with *Bam*HI and *Hin*dIII 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 *Bam*HI/*Hin*dIII digested pcDNA3 encoding myc and His$_6$ epitope tags downstream of the *Hin*dIII restriction site. The resulting plasmid was digested with the *Hin*dIII 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 *Hin*dIII restriction sites in frame with the upstream pCT–hCL receptor and the downstream myc-His$_6$ encoding sequences. The final pCT–hCL-myc-His$_6$ (pCT–hCL) receptor construct was verified by sequencing.

The Δ18-hCL-myc-His$_6$ receptor expression construct was derived from pCT–hCL-myc-His$_6$ receptor. pCT–hCL-myc-His$_6$ receptor construct was digested with *Bam*HI, *Rca*I and *Hpa*I restriction enzymes. The *Rca*I/*Hpa*I fragment encoding the Met^{20} to Asn^{139} domain of the hCL receptor was isolated. A *Bam*HI/*Rca*I DNA fragment encoding the 24 amino acids signal sequence of the pCT receptor in frame with the downstream hCL-myc-His$_6$ receptor sequence from Met^{20} to Asn^{139} was amplified with Pfu DNA Polymerase (Amersham Pharmacia Biotech, Little Chalfont, UK). The *Bam*HI/*Rca*I and *Rca*I/*Hpa*I fragments isolated from pCT–hCL-myc-His$_6$ receptor were cloned into the *Bam*HI/*Hpa*I digested pCT–hCL-myc-His$_6$, receptor revealing Δ18-hCL-myc-His$_6$ (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 2.6×10^4 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-His$_6$ 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, cross-linking 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 $200\times g$ for 5 min. [^{125}I]-labeled hαCGRP and -hAM (7.4 10^{13} Bq/mmol) were prepared as described [15,16]. The cells were incubated with 100 kBq [^{125}I]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, USA). 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 $200\times g$ 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, Hercules, CA, USA), 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin and 3 μg/ml leupeptin (lysis buffer). The cell lysates were cleared by centrifugation at $20\,000\times g$ 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) for 1 h to remove non-specifically adsorbing proteins, 3 μg myc antibodies (Invitrogen) 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 $2700\times g$ 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 γ-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) in 50 μl lysis buffer adjusted to 10 mM EDTA, 0.1% SDS, 0.5% octylglucopyranoside and 1% β-mercaptoethanol. The proteins were then eluted at 60°C for 5 min from the ImmunoPure immobilized protein G and the samples centrifuged at $10\,000\times g$ for 3 min. Proteins in the supernatant were subjected to 10% SDS–PAGE and electrotransferred to nitrocellulose Hybond ECL membranes (BioRad Laboratories) in a Trans-Blot cell (BioRad Laboratories) 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).

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; 1:300) for hRAMP2 detection. Subsequently, the cells were washed twice and then incubated with Alexa488 anti-rabbit or Alexa488 anti-mouse antiserum (Molecular Probes; 1:200) for 30 min. After three washes the cells were collected by centrifugation and 5 μl were dry-mounted with ImmuMount (Shandon, Pittsburgh, PA, USA). Confocal images were obtained on a Leica TCS SP2 (Leica Microsystems, Heidelberg, Ger-

many) (×63 1.32 N.A. HCX PL APO objective) by simultaneous excitation with an extended argon ion laser at 488 nm for the 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, San Diego, CA, USA). The results are means ± S.E.M. Differences between mean values were analyzed by ANOVA. P values < 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]hαCGRP and -hAM binding and signalling

Binding and chemical cross-linking of [^{125}I]hαCGRP 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 co-transfected with the hCL receptor and the hRAMP1 expression construct, [^{125}I]hα-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]hα-CGRP-binding proteins remained undetectable in cells expressing hRAMP1 alone or hRAMP1 together with the Δ18-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]hα-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]hαCGRP-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 Δ18-hCL receptor.

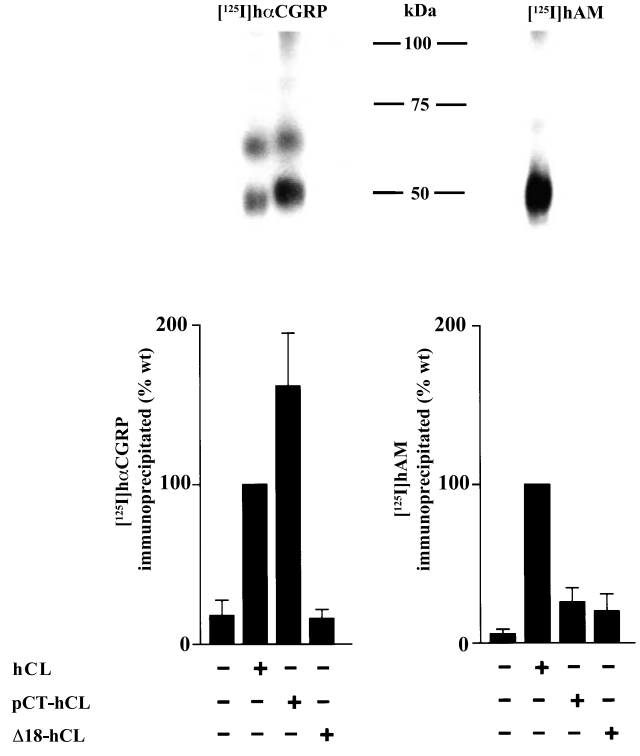

Fig. 2. Chemical cross-linking of [^{125}I]hαCGRP and -hAM to corresponding binding proteins. CHO cells were transfected with hRAMP1 (left panels) or hRAMP2 (right panels) expression constructs together with pcDNA3 or the indicated receptor expression constructs. Radioligand binding and cross-linking with BS^3, and immunoprecipitation of myc-tagged receptors with corresponding antibodies and subsequent deglycosylation was carried out as described in Section 2. Immunoextracts were subjected to 10% SDS–PAGE and radioligand cross-linking products were visualized by autoradiography (top panels). The amount of [^{125}I]hαCGRP and -hAM co-immunoprecipitated with the myc-tagged receptors after cross-linking were measured in a γ-counter and reflected radioligand binding (bottom panels). [^{125}I]hαCGRP and -hAM in immunoextracts of cells transfected with hRAMP1 or -2 expression constructs together with pcDNA3 was considered as non-specifically bound. Binding of [^{125}I]hαCGRP (4.7 ± 0.9 kBq/dish ($n = 5$)) and of [^{125}I]hAM (3.4 ± 1.6 kBq/dish ($n = 4$)) to hCL receptor/hRAMP1 and -2 expressing cells, respectively, was set to 100%.

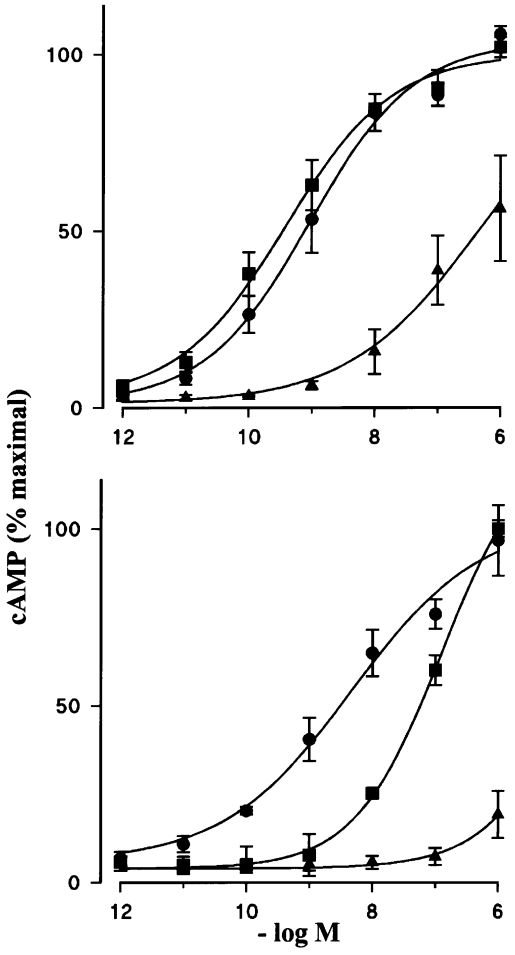

Fig. 3. Stimulation of cAMP production. CHO cells were transfected with hCL (●), pCT–hCL (■) or Δ18-hCL (▲) receptor together with hRAMP1 (top panel) or hRAMP2 (bottom panel) and stimulated with indicated concentrations of hαCGRP (top panel) or hAM (bottom panel). Results are means ± S.E.M. of four experiments.

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

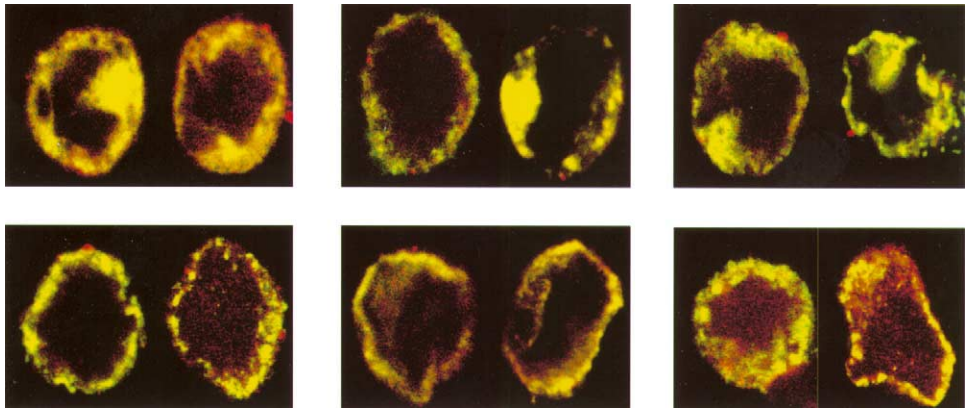

Fig. 4. Immunochemical localization of receptors and hRAMP2. CHO cells were transfected with hCL (left panels), pCT–hCL (middle panels) or Δ18-hCL (right panels) receptors together with hRAMP2. The cells were detached and surface glycoproteins stained with TRITC-ConA (red), fixed, permeabilized and incubated 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.

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}I]hαCGRP 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}I]hαCGRP 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}I]hαCGRP cross-linking products in the hCL receptor and hRAMP1 co-expressing cells and corresponded to the calculated molecular weight of hRAMP1. [^{125}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}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 hαCGRP 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 Δ18-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 Δ18-hCL receptors. Thus, the impaired and lost function of pCT–hCL and Δ18-hCL receptors together with hRAMP2 with respect to [^{125}I]AM binding were not caused by defective cell surface delivery of the mutated receptors and hRAMP2.

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 inter-

action 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}I]hαCGRP cross-linking products in hCL and pCT–hCL receptor/hRAMP1 expressing cells and their differences in size were consistent with deglycosylated [^{125}I]hαCGRP/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}I]hAM/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 Δ18-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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