Receptor-activity-modifying protein 1 forms heterodimers with two G-protein-coupled receptors to define ligand recognition

Kerstin LEUTHÄUSER*[1], Remo GUJER*[1], Amaya ALDECOA*, R. Anne McKINNEY†, Roman MUFF*, Jan A. FISCHER* and Walter BORN*[2]

*Research Laboratory for Calcium Metabolism, Departments of Orthopaedic Surgery and Medicine, University of Zurich, Klinik Balgrist, Forchstr. 340, CH-8008 Zurich, Switzerland, and †Brain Research Institute, University of Zurich, CH-8057 Zurich, Switzerland

Receptor-activity-modifying proteins (RAMPs) with single transmembrane domains define the function of two G-protein-coupled receptors of the B family. Cell-surface complexes of human RAMP1 (hRAMP1) and human calcitonin (CT) receptor isotype 2 (hCTR2) or rat CT-receptor-like receptor (rCRLR) have now been identified through protein cross-linking, co-immunoprecipitation and confocal microscopy. They are two distinct CT-gene-related peptide (CGRP) receptors coupled to cAMP production and pharmacologically distinguished by the CT and CGRP antagonists salmon CT(8-32) and human or rat CGRP(8-37). Thus direct molecular interactions of hRAMP1 with hCTR2 or rCRLR are required for CGRP recognition. hCTR2, moreover, adopts non-traditional functions through its association with hRAMP1.

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

INTRODUCTION

Calcitonin (CT), CT-gene-related peptide (CGRP), amylin and adrenomedullin (ADM) are peptides with limited amino acid sequence similarity [1]. N-terminal six- or seven-amino-acid ring structures linked by disulphide bonds between cysteine residues and amidated C-termini are required for biological activity. C-terminal fragments of CT, CGRP and ADM lacking the ring structures are antagonists. Cloned CT receptors (CTRs) [2] and initially orphan rat (r) and human (h) CTR-like receptors (CRLRs) [3,4] with 60% amino acid sequence similarity are class-B seven-transmembrane-domain G-protein-coupled receptors interacting with CT, CGRP, amylin and ADM. CRLRs require co-expression with receptor-activity-modifying protein (RAMP) 1 or 2, to yield CGRP and ADM receptors, respectively [5,6]. Mixed-type ADM/CGRP receptors were expressed in the presence of RAMP3 [7,8]. CTRs, on the other hand, do not require the three RAMPs identified so far for functional expression, but CGRP and ADM are not recognized. But, co-expression of the hCTR isotype 2 (hCTR2) [9] with hRAMP1 or 3 has led to the discovery of amylin receptors [10,11]. These observations imply RAMP-modulated conformational changes of two G-protein-coupled receptors to adopt binding epitopes for four peptides with limited common structural elements.

RAMP-controlled glycosylation and transport of CRLR to the plasma membrane have been observed [5]. But CRLR/RAMP association defining ligand specificity has so far not been demonstrated. Here chemical cross-linking, immunoprecipitation and confocal microscopy have been used to identify the hCTR2 or rCRLR associated with hRAMP1 at the cell surface as CGRP receptor isotypes.

EXPERIMENTAL

Materials

The membrane-impermeable cross-linker bis(sulphosuccinimidyl)suberate (BS^3) was purchased from Pierce (Rockford, IL, U.S.A.) and OptiMEM medium and LipofectAMINE were from Life Technologies (Gaithersburg, MD, U.S.A.). hCT was provided by E. Felder and salmon CT(8-32) [sCT(8-32)] by R. Gamse (both at Novartis, Basel, Switzerland). hαCGRP, hαCGRP(8-37) and rαCGRP were obtained from Bachem AG (Bubendorf, Switzerland), and rCT and rαCGRP(8-37) were from Peninsula Laboratories (Belmont, CA, U.S.A.). ^{125}I-labelled hαCGRP and hCT were prepared and purified as described in [6,12].

DNA constructs

The cDNA encoding hCTR2 with its 13 C-terminal amino acids replaced by a sequence containing a Myc-epitope tag [Ala-Tyr-Val-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Leu-Asn-Ser-Ala-Val-Asp-$(His)_6$] and integrated into pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) (hCTR2-Myc) was obtained from D. L. Galson (Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, MA, U.S.A.). Subcloning of a *Hin*dIII restriction fragment of hCTR2-Myc, encoding hCTR2 lacking the C-terminal 13 amino acids and the stop codon, into *Hin*dIII-digested enhanced green fluorescent protein (EGFP) encoding plasmid pEGFP-N3 (Clontech, Palo Alto, CA, U.S.A.) revealed hCTR2-EGFP. Plasmid rCRLR-V5 was created by subcloning the cDNA encoding rCRLR (obtained from M. G. Rosenfeld, University of California, San Diego, CA, U.S.A.)

Abbreviations used: ADM, adrenomedullin; CT, calcitonin; CTR, CT receptor; CGRP, CT-gene-related peptide; CRLR, CTR-like receptor; hCTR2, human CTR isotype 2; EGFP, enhanced green fluorescent protein; h, human; r, rat; RAMP, receptor-activity-modifying protein; BS^3, bis(sulphosuccinimidyl)suberate; sCT(8-32), salmon CT(8-32); TSA cells, simian virus 40 T-antigen-transformed human embryonic kidney cells.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail wborn@balgrist.unizh.ch).

with the stop codon removed by PCR into pcDNA3 in frame and upstream of a sequence encoding V5- and $(His)_6$-epitope tags provided by the *Drosophila* expression vector pAc5.1/V5-His (Invitrogen). rCRLR-EGFP was derived from rCRLR-V5 by replacing a *Xho*I–*Xba*I restriction fragment, encoding the V5- and $(His)_6$-epitope tags, by an EGFP-coding sequence designed with 5′ *Xho*I and 3′ *Xba*I ends and amplified from pEGFP-N3 by PCR. hRAMP1 and hRAMP1-Myc expression constructs in pcDNA3 were obtained from S. M. Foord (GlaxoWellcome, Stevenage, Herts., U.K.). Plasmid hRAMP1-V5 consisted of the coding sequence of hRAMP1 without the stop codon amplified by PCR and cloned into pcDNA3 upstream of and in frame with the pAc5.1/V5-His-derived V5-$(His)_6$ coding sequence. All constructs were verified by sequencing of amplified and recombined DNA in both directions.

Cell culture and transfection

COS-7 cells were cultured in Ham's F12/Dulbecco's modified Eagle's medium (1:1) medium supplemented with 10% fetal calf serum. The same medium supplemented with 400 μg/ml geneticin was used to culture simian virus 40 T-antigen-transformed human embryonic kidney (TSA) cells. COS-7 cells were grown in 24-well plates for cAMP-stimulation experiments and on poly-L-lysine-coated microscope slides for confocal microscopy. TSA cells used for cross-linking and immunoprecipitation experiments were cultured in 12-well plates and 100-mm dishes, respectively, coated with 0.1% gelatine. Transfections were carried out at 37 °C for 4 h in OptiMEM medium containing 2.4 μl/ml LIPOFECTAMINE™ and equal amounts of expression constructs or pcDNA3 in the indicated combinations at 800 ng/ml total DNA concentration. Experiments were performed 36–48 h after transfection.

cAMP production

COS-7 cells were incubated at 37 °C for 15 min with 1 mM 3-isobutyl-1-methylxanthine and indicated concentrations of peptides. Accumulated cAMP was measured by radioimmunoassay [6]. Half-maximal effective concentrations of h- and rαCGRP in the absence (EC_{50}) or presence (EC_{50}') of indicated constant concentrations of the antagonists (I) h- and rαCGRP-(8-37) and sCT(8-32) were determined in parallel experiments. The K_i values for h- and rαCGRP(8-37) and sCT(8-32) were calculated from the equation $EC_{50}'/EC_{50} = 1 + I/K_i$.

Cross-linking, immunoprecipitation and Western-blot analysis

TSA cells were incubated with 6500 Bq/well of the indicated ^{125}I-labelled peptides at 15 °C for 2 h and subsequently with 1 mM cross-linker BS^3 at room temperature for 1 h. Proteins in cell extracts were electrophoresed by SDS/PAGE (12% gels). For immunoprecipitation of Myc- and V5-tagged receptors the cells were solubilized in 50 mM Hepes, pH 7.5, 7 mM $MgCl_2$, 2 mM EDTA, 1 mM PMSF, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 1 mg/ml dodecyl β-D-maltoside and 0.2 mg/ml cholesterol hemisuccinate. The receptors were immunoprecipitated from cleared cell extracts at 4 °C by sequential incubation with 50 μl of ImmunoPure-immobilized Protein G (Pierce), 3 μg of Myc or V5 antibodies (Invitrogen) and 100 μl of ImmunoPure-immobilized Protein G. Retained proteins were eluted with protein gel sample buffer and subjected to SDS/PAGE (15% gel). For Western-blot analysis proteins were electrotransferred to nitrocellulose Hybond™ ECL™ membranes (Amersham, Little Chalfont, Bucks., U.K.) in a Trans-Blot cell (Bio-Rad, Richmond, CA, U.S.A.) at 40 V and 4 °C overnight. Immunoblots were blocked with 5% low-fat milk and epitope-tagged proteins visualized with the enhanced chemiluminescence (ECL) technique (Amersham) using horseradish peroxidase-labelled monoclonal antibodies (Invitrogen) to Myc (1:5000 final dilution) and V5 (1:2500 final dilution).

Confocal microscopy

After transfection (2 days) COS-7 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated sequentially with mouse monoclonal antibodies (1:500 dilution) to Myc (Invitrogen), biotin-labelled anti-mouse antibodies (1:250 dilution; Vector Laboratories, Burlingame, CA, U.S.A.) and with avidin-Neutrolite Texas Red (Molecular Probes, Eugene, OR, U.S.A.), and mounted with Flurostab™ (Bio-Science Products, Emmenbrücke, Switzerland). Confocal images were obtained with a laser scanning LSM 410 microscope (63 × 1.3 aperture objective; Zeiss, Jena, Germany) with sequential excitation by an external argon laser at 488 nm for EGFP and an internal He/Ne laser at 543 nm for Texas Red.

RESULTS AND DISCUSSION

Pharmacology of hCTR2 or rCRLR co-expressed with hRAMP1

Stimulation of cAMP production by CGRP and CT was examined in COS-7 cells transiently expressing hCTR2 or rCRLR in the absence and presence of hRAMP1. In cells transfected with the expression vector pcDNA3 or with expression constructs encoding hRAMP1 or rCRLR alone hCT and hαCGRP at up to 10^{-6} M did not affect basal cAMP levels of 0.8–1.5 pmol/10^5 cells. Expression of hCTR2 revealed maximal 16-fold stimulation of cAMP production by hCT in both the absence and presence of hRAMP1: the EC_{50} values were 0.05 ± 0.01 nM (mean ± S.E.M., $n = 8$) and 0.24 ± 0.04 nM ($n = 7$), respectively. The EC_{50} of hαCGRP was over 1000-fold higher in the absence of hRAMP1, but similar to that of hCT following co-expression of the hCTR2 and hRAMP1 (0.77 ± 0.37 nM, $n = 5$) (Figure 1A). The response to hαCGRP was attenuated by sCT(8-32) with a K_i of 2.9 ± 1.4 nM ($n = 5$), but was not affected by 100 nM hαCGRP(8-37). With hCT as agonist, sCT(8-32) had a similar K_i of 0.74 ± 0.12 nM ($n = 3$).

In the same cells co-expressing rCRLR and hRAMP1 maximal cAMP accumulation in response to rαCGRP was 10-fold with an EC_{50} of 0.1 ± 0.01 nM ($n = 4$; Figure 1B) and rCT at up to 10^{-6} M was ineffective. Consistent with a $CGRP_1$ receptor [13] rαCGRP(8-37) inhibited the response to rαCGRP with a K_i of 49 ± 22 nM ($n = 4$), but sCT(8-32) at up to 10^{-6} M was not an antagonist.

Taken together, the results indicate that the hCTR2 in the presence of hRAMP1 still recognizes a CT antagonist that inhibits hαCGRP action. Moreover, hCTR2 co-expressed with hRAMP1, unlike the rCRLR, interacts with intact hαCGRP, but not with the $CGRP_1$ receptor antagonist hαCGRP(8-37), implying distinct CGRP-binding mechanisms of the two receptors. These observations are also consistent with hCTR2/hRAMP1 mimicking some of the phenotypic properties of the $CGRP_2$ receptor subtype [13] in spite of a high affinity for amylin [11] not reported for $CGRP_2$.

[^{125}I]hαCGRP binding to hCTR2 or rCRLR associated with hRAMP1

Myc- and V5-epitope-tagged hCTR2, rCRLR and hRAMP1, functionally indistinguishable from non-tagged proteins, have

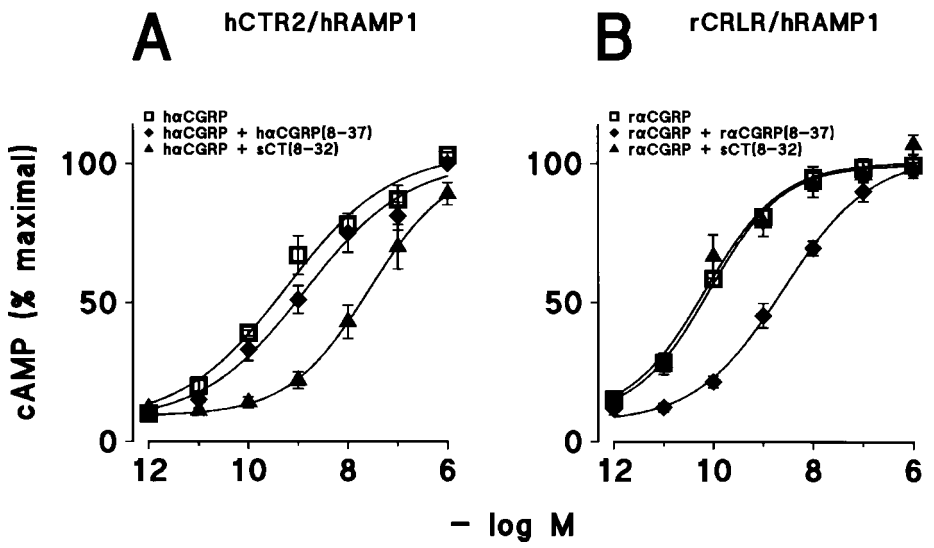

Figure 1 CGRP receptor isotypes distinguished by CT and CGRP antagonists

COS-7 cells were transiently co-transfected with hRAMP1 and hCTR2 (**A**) or rCRLR (**B**) expression constructs. Cellular cAMP was stimulated by hαCGRP in the absence and presence of 10^{-7} M hαCGRP(8-37) or sCT(8-32) (**A**), and by rαCGRP in the absence and presence of 10^{-6} M rαCGRP(8-37) or sCT(8-32) (**B**). Values are means ± S.E.M. of triplicate determinations of at least three experiments.

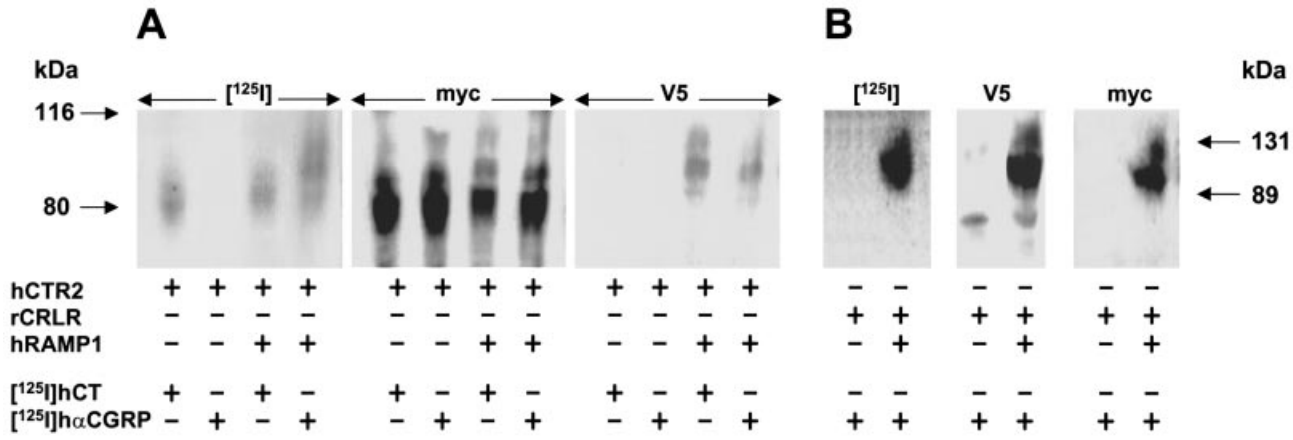

Figure 2 [^{125}I]CGRP binding to hCTR2/hRAMP1 or rCRLR/hRAMP1 complexes

TSA cells transiently expressed hCTR2-Myc in the absence and presence of hRAMP1-V5 (**A**) and rCRLR-V5 in the absence and presence of hRAMP1-Myc (**B**). The cells were incubated with the indicated radioligands and treated with the membrane-impermeable protein cross-linker BS^3. Cell extracts were subjected to SDS/PAGE (12% gels) and Western blots were analysed with Myc and V5 antibodies and by autoradiography.

been transiently expressed in TSA cells in the combinations indicated in Figure 2. Antibodies to Myc- and V5-epitope tags specifically recognized corresponding receptor components on Western blots of TSA cell extracts. Cross-reaction or non-specific immunoreactivity of Myc or V5 antibodies was not observed, as indicated by the empty lanes in Figure 2. Autoradiography and Western-blot analysis subsequent to cell-surface protein cross-linking with membrane-impermeable BS^3 and SDS/PAGE identified distinct [^{125}I]hCT- and [^{125}I]hαCGRP-binding proteins. Expression of hCTR2-Myc alone revealed a major 80-kDa protein, recognized with Myc antibodies, that specifically bound [^{125}I]hCT, but not [^{125}I]hαCGRP (Figure 2A). The binding was displaced by 10^{-6} M hCT, but not by 10^{-6} M hαCGRP (results not shown). Specific [^{125}I]hCT-binding to hCTR2-Myc also occurred in cells co-expressing hRAMP1-V5. But, additional non-identified [^{125}I]hαCGRP-binding components with larger apparent molecular masses were now recognized both with Myc and V5 antibodies, indicating [^{125}I]hαCGRP/hCTR2-Myc/hRAMP1-V5 cross-linking products. hαCGRP and hCT (10^{-6} M) displaced the [^{125}I]hαCGRP labelling. This corresponded to an IC_{50} of 14.5 ± 2.6 nM hαCGRP and 193 ± 40 nM hCT in intact TSA cells ($n = 8$).

In cells co-expressing rCRLR-V5 and hRAMP1-Myc, [^{125}I]hαCGRP was also cross-linked to a component detected with V5 and Myc antibodies (Figure 2B). In the absence of hRAMP1-Myc, [^{125}I]hαCGRP cross-linking was undetectable and rCRLR-V5 had a smaller size comparable with that reported for immature glycosylated hCRLR [5].

Therefore specific [^{125}I]hαCGRP binding to hCTR2 or rCRLR required the presence of hRAMP1 associated at the cell surface

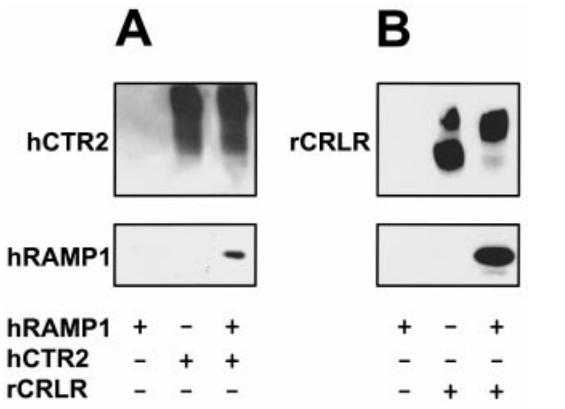

Figure 3 Co-immunoprecipitation of hRAMP1 with hCTR2 or rCRLR

hCTR2-Myc and hRAMP1-V5 (**A**) and rCRLR-V5 and hRAMP1-Myc (**B**) were transiently expressed in TSA cells. hCTR2-Myc was immunoprecipitated with antibodies to Myc and immunoblots were analysed with Myc (**A**, upper panel) and V5 antibodies (**A**, lower panel). rCRLR-V5 was immunoprecipitated with antibodies to V5 and immunoblots were analysed with V5 (**B**, upper panel) and Myc antibodies (**B**, lower panel). Shown is a representative experiment of three similar experiments.

within a distance that allowed cross-linking to receptors. Similarly, cross-linking experiments with rat [^{125}I]amylin in COS-7 cells co-expressing hCTR2 and hRAMP1 provided evidence for a close association of the two proteins defining amylin receptors [11].

The results presented here demonstrate for the first time that hRAMP1 interacts with hCTR2 or rCRLR, presumably with their extracellular domains, to reveal hαCGRP-binding epitopes. Direct contributions of hRAMP1 to CGRP-binding epitopes and/or hRAMP1-induced conformation of receptors required for CGRP binding have to be considered. Along these lines, [^{125}I]hαCGRP cross-linked to hRAMP1 was not revealed. This is consistent with the limited number of amino groups available for cross-linking in the extracellular domain of expressed RAMPs, two in hRAMP1-V5 and three in hRAMP1-Myc.

Co-immunoprecipitation of hRAMP1 with hCTR2 or rCRLR

Immunoprecipitation of hCTR2-Myc or rCRLR-V5 with the respective antibodies in cross-linked samples confirmed the identification of the [^{125}I]hαCGRP/hCTR2/hRAMP1 and [^{125}I]hαCGRP/rCRLR/hRAMP1 complexes (results not shown). Direct physical interaction of hRAMP1 with hCTR2 or rCRLR in TSA cells was substantiated through immunoprecipitation in the absence of protein cross-linker (Figure 3). In cells co-expressing hCTR2-Myc and hRAMP1-V5 Myc antibodies precipitated hCTR2-Myc together with hRAMP1-V5. Similarly, hRAMP1-Myc co-immunoprecipitated with rCRLR-V5. hRAMP1 migrated at its predicted size no longer associated with hCTR2-Myc or rCRLR-V5, under both reducing and non-reducing (results not shown) gel electrophoresis conditions. This indicates non-covalent interactions between hRAMP1 and the receptor proteins.

Co-localization of hRAMP1 and hCTR2 or rCRLR at the cell periphery

Subcellular localization of fully functional autofluorescent fusion hCTR2-EGFP or rCRLR-EGFP co-expressed with hRAMP1-Myc was investigated by confocal microscopy of paraformaldehyde-fixed and Triton X-100-permeabilized COS-7 cells (Figure 4). hCTR2-EGFP and hRAMP1-Myc were distributed throughout the cells. But superimposition of confocal images indicated co-localization of hCTR2-EGFP and hRAMP1-Myc

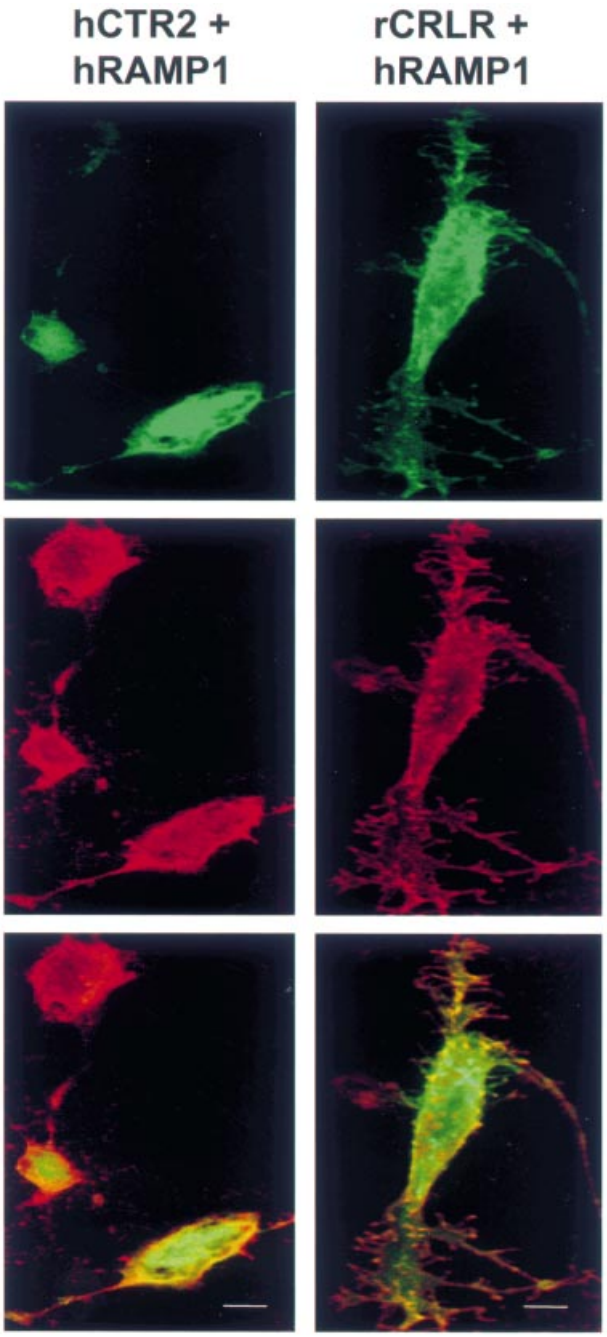

Figure 4 Co-localization of hRAMP1 with hCTR2 or rCRLR at the cell surface

COS-7 cells were transiently co-transfected with hCTR2-EGFP and hRAMP1-Myc (left-hand panels) or rCRLR-EGFP and hRAMP1-Myc (right-hand panels) expression constructs. Confocal microscopy revealed the distribution of EGFP-carrying receptors (top panels), Texas Red-immunostained hRAMP1-Myc (middle panels) and overlap of co-localized receptors and hRAMP1 in yellow (bottom panels). Staining was not observed in adjacent non-transfected cells. Scale bars, 10 μm. A representative experiment of three similar experiments is shown.

in the cell periphery and only minor punctate staining in the lumen. In cells co-expressing rCRLR-EGFP and hRAMP1-Myc clusters of both proteins were recognized mostly in peripheral parts. As a result, overlay of confocal images revealed distinct bright punctate spots of co-localized rCRLR-EGFP and hRAMP1-Myc almost exclusively at the cell periphery.

These observations support the chemical cross-linking and immunoprecipitation experiments. hCTR2 and rCRLR are associated with hRAMP1 to present hαCGRP-binding epitopes at the cell surface. Detailed topology of this association and amino acid sequences of receptors and hRAMP1 involved in these interactions remain to be elucidated.

The present report indicates novel biological functions for CTRs other than those in the classical target organs bone and kidney. Suitable antibodies to RAMP not available so far should reveal the CTR/RAMP complex *in vivo*. Along these lines, CTR-encoding mRNA, unlike CRLR mRNA, is expressed in the rat hypothalamus with well characterized high-affinity [^{125}I]sCT- and [^{125}I]hαCGRP-binding sites [14–16].

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