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Mammalian calcitonin receptor-like receptor/receptor activity modifying protein complexes define calcitonin gene-related peptide and adrenomedullin receptors in *Drosophila* Schneider 2 cells

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Abstract Differential glycosylation of human and rat (r) calcitonin (CT) receptor-like receptors (CRLR) as a result of interactions with accessory receptor activity-modifying proteins (RAMP)1 or -2 was considered to define CT gene-related peptide (CGRP) or adrenomedullin (ADM) receptors in mammalian cells. Here, *Drosophila* Schneider (S2) cells stably co-expressed rCRLR and RAMP1 or -2 as functional CGRP or ADM receptors. Different from mammalian cells, rCRLR expressed in S2 cells are uniformly glycosylated proteins independent of RAMP1 or RAMP2. Bis(sulfosuccinimidyl)suberate cross-linking revealed receptor components with the size of rCRLR, increased by the molecular weights of the corresponding RAMPs and [^{125}I]CGRP or [^{125}I]ADM. In conclusion, [^{125}I]CGRP/rCRLR/RAMP1 and [^{125}I]ADM/rCRLR/RAMP2 complexes have been recognized in *Drosophila* S2 cells.

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

1. Introduction

Calcitonin (CT) gene-related peptide (CGRP), adrenomedullin (ADM), CT and amylin are homologous peptides with 6- or 7-amino acid ring structures and amidated carboxy-termini required for biological activity [1]. CGRP and ADM are potent vasorelaxant and hypotensive peptides with positive inotropic action on the heart. Along these lines, CGRP was shown to have therapeutic potential in cardiovascular disorders [1]. An enhanced survival rate of ADM-overexpressing transgenic mice in response to lipopolysaccharide administration suggested a protective action of the peptide in septic shock preventing severe hypotension [2]. Therapeutic use of CGRP and ADM awaits the development of corresponding low molecular weight non-peptide mimics with suitable bioavailability. This requires so far non-existing high throughput in vitro screening systems, using cell lines that express a high density of CGRP and ADM receptors.

Initially orphan human (h) and rat (r) CT receptor-like receptors (CRLR) of the B family of G protein-coupled receptors are CGRP or ADM receptors when co-expressed with human receptor activity modifying protein RAMP1 or RAMP2, respectively [3,4]. RAMP1 and RAMP2 have been shown to direct CRLR to the cell surface. Mature glycosylation of CRLR occurred in the presence of RAMP1 and core-glycosylation was observed when CRLR was expressed alone or together with RAMP2. Differential glycosylation was considered as a mechanism to define the specificity of CGRP and ADM receptors.

Drosophila cell lines, in particular the baculovirus/Sf9 cell system, are powerful and versatile expression systems for heterologous proteins that require posttranslational modification for proper function. But Sf9 cells die from baculovirus infection which is not the case with *Drosophila* Schneider 2 (S2) cells [5]. The latter are suitable for the continuous production in suspension culture at high cell density and room temperature of a variety of stably transfected heterologous proteins such as the glucagon receptor [6].

Here, *Drosophila* S2 cell lines have been developed that stably express CGRP or ADM receptors as rCRLR/RAMP1 or rCRLR/RAMP2 complexes, recognized for the first time, at the cell surface.

2. Materials and methods

2.1. Materials

Rat and human αCGRP(1–37) and rat amylin were purchased from Bachem AG (Bubendorf, Switzerland); rβCGRP(1–37), rαCGRP(8–37) and rCT from Peninsula Laboratories (Belmont, CA, USA); rADM(1–50) from Peptide Institute (Osaka, Japan); and rADM(20–50) from Phoenix Pharmaceuticals (Mountain View, CA, USA). Culture media and FCS were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Hygromycin B was from Life Technologies (Gaithersburg, MD, USA). *N*-glycosidase F was supplied by Boehringer Mannheim (Mannheim, Germany). ^{125}I-Na and ECL Western blot detection reagents were obtained from Amersham International (Little Chalfont, UK), and anti-V5 horseradish peroxidase (HRP)-labeled antibody was from Invitrogen (Carlsbad, CA, USA). The membrane impermeable, water-soluble crosslinker bis(sulfosuccinimidyl)suberate (BS3) was supplied by Pierce (Rockford, IL, USA). Chemicals and other reagents were purchased from Sigma (St. Louis, MO, USA) and E. Merck (Darmstadt, Germany) at the highest grade available.

2.2. Expression of rCRLR and RAMP1 and RAMP2 in Drosophila S2 cells

The vector pAc5.1/V5-His (Invitrogen, Carlsbad, CA, USA) was used for constitutive expression of rCRLR and RAMP1 and RAMP2 fused to carboxy-terminal V5- and His_6-epitopes in *Drosophila* S2 cells. Briefly, coding sequences of RAMP1 and RAMP2 with

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Abbreviations: ADM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; S2 cells, *Drosophila* Schneider 2 cells; RAMP, receptor activity modifying protein

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the translational stop sites removed, were amplified by PCR from cloned cDNA (provided by S. Foord, Glaxo Wellcome, Stevenage, UK). Oligonucleotide primers were designed with 5′ *Kpn*I and 3′ *Xba*I (RAMP1) and with 5′ *Eco*RI and 3′ *Not*I (RAMP2) restriction sites for unidirectional cloning into pAc5.1/V5-His in frame with vector-encoded V5-His_6-epitopes. Similarly, a DNA fragment spanning the coding sequence of rCRLR between a unique internal *Hin*dIII restriction site and the translation stop codon was amplified from cloned rCRLR encoding cDNA (provided by M.G. Rosenfeld, University of California, San Diego, CA, USA). Cloning of this PCR product carrying 5′ *Hin*dIII and 3′ *Xho*I restriction sites, and of a *Not*I/*Hin*dIII restriction fragment encoding the remaining amino-terminal portion of rCRLR into *Not*I/*Xho*I digested pAc5.1/V5-His yielded the expression construct for a rCRLR-V5-His_6 fusion protein. All constructs were verified by sequencing before the transformation of insect cells.

The cells were maintained in suspension culture at 23°C in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal calf serum. 10^7 S2 cells were co-transfected by $CaPO_4$ precipitation (Invitrogen, Carlsbad, CA, USA) with 19 μg of the vector pAc5.1/V5-His (mock transfections) or rCRLR, hRAMP1 or -2 expression constructs in pAc5.1/V5-His or combinations thereof, and with 1 μg of the hygromycin resistance plasmid pCoHYGRO (Invitrogen, Carlsbad, CA, USA). 24 h after transfection 300 μg/ml hygromycin-B was added to the medium. The medium was changed every 4 days. Stably transfected, polyclonal hygromycin-resistant cell lines were obtained after 3–4 weeks and subcultured (1:5 dilution) every 3–4 days.

2.3. Radioligand binding and cAMP accumulation

^{125}I-labeled hαCGRP and [^{125}I]rADM were prepared as previously described [4]. Aliquots of 0.5×10^6 S2 cells were incubated for 1 h at 4°C with 1700 Bq [^{125}I]hαCGRP or [^{125}I]rADM in the absence and presence of non-labeled peptides in a total volume of 200 μl DMEM/Ham F12 (1:1) supplemented with 0.1% BSA (binding medium). Subsequently, the cells were collected by centrifugation at $14\,000\times g$ and 4°C for 5 min, and the supernatant was aspirated. The tip of the tube containing the cell pellet was cut off and bound radioligand was estimated in a γ-counter (MR252, Kontron, Zurich, Switzerland). Radioactivity in the pellet of cells that were incubated in the presence of 0.1 μM non-labeled peptides was considered as non-specific binding. Cyclic AMP accumulation was measured in 2×10^6 S2 cells incubated at room temperature for 15 min in 200 μl Schneider's *Drosophila* medium containing 1 mM isobutylmethylxanthine (IBMX) in the absence and presence of the indicated peptides. The cells were collected by centrifugation at $1000\times g$ and room temperature for 5 min and cAMP was measured as described [4].

2.4. Cross-linking of cell surface proteins, deglycosylation and Western blot analysis

For the cross-linking analysis 2.5×10^6 S2 cells, stably expressing CGRP or ADM receptors, were incubated for 1 h at 4°C with 6700 Bq [^{125}I]hαCGRP or [^{125}I]rADM in the absence and presence of non-labeled CGRP and ADM in 400 μl binding medium. Subsequently, the cells were collected by centrifugation at $14\,000\times g$ and 4°C for 5 min. The cells were resuspended in 400 μl 0.1 M PBS and incubated for 40 min with 1 mM membrane-impermeable cross-linker BS3. The reaction was quenched with 1 M Tris–HCl, pH 7.5. The cells were collected by centrifugation at $14\,000\times g$ at room temperature for 5 min and resuspended in 50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40. The cell lysates were cleared by centrifugation at $14\,000\times g$ at room temperature for 5 min and the supernatants were kept at −20°C for further analysis. Deglycosylation of proteins in lysates of S2 cells was carried out with *N*-glycosidase F. Aliquots, equivalent to $2–2.5\times10^6$ cells, were incubated for 18 h at 37°C in 50 μl 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 0.5% octylglucopyranoside, 1% β-mercaptoethanol in the absence and presence of 4 U *N*-glycosidase F. The incubations were stopped with protein gel loading buffer (0.25 M Tris–HCl, pH 6.8, 0.4% bromophenol blue, 8% β-mercaptoethanol, 8% SDS, 40% glycerol). The proteins were separated by 15% Tris–glycine SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose Hybond™ ECL™ (Amersham International, Little Chalfont, UK) in 14.4 g/l glycine, 3.03 g/l Tris, 20% methanol in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA, USA) at 25 V and 4°C overnight. V5- and His_6-tagged rCRLR and RAMPs were visualized with peroxidase-labeled anti-V5 (1:5000 final dilution) monoclonal antibodies according to the protocol of the ECL Kit (Amersham International, Little Chalfont, UK). Biotinylated protein size markers were visualized by ECL according to the manufacturer's instructions. Cross-linked [^{125}I]hαCGRP and [^{125}I]rADM were detected with Hyperfilm™ MP film (Amersham International, Little Chalfont, UK).

2.5. Statistics

The values for half-maximal inhibitory concentrations (IC_{50}) and for half-maximal effective concentrations (EC_{50}) were calculated by non-linear regression analysis using Fig. P 6.0 software (Biosoft, Cambridge, UK).

3. Results and discussion

3.1. CGRP and ADM receptor function in Drosophila S2 cells

Rat CRLR and RAMP1 and RAMP2 with carboxy-terminal V5- and His_6-epitope tags were stably expressed in S2 cells. In control transfected cells and in cells transfected with individual RAMP1, RAMP2 and rCRLR encoding cDNA alone, specific binding of [^{125} I]hαCGRP and [^{125} I]rADM was not

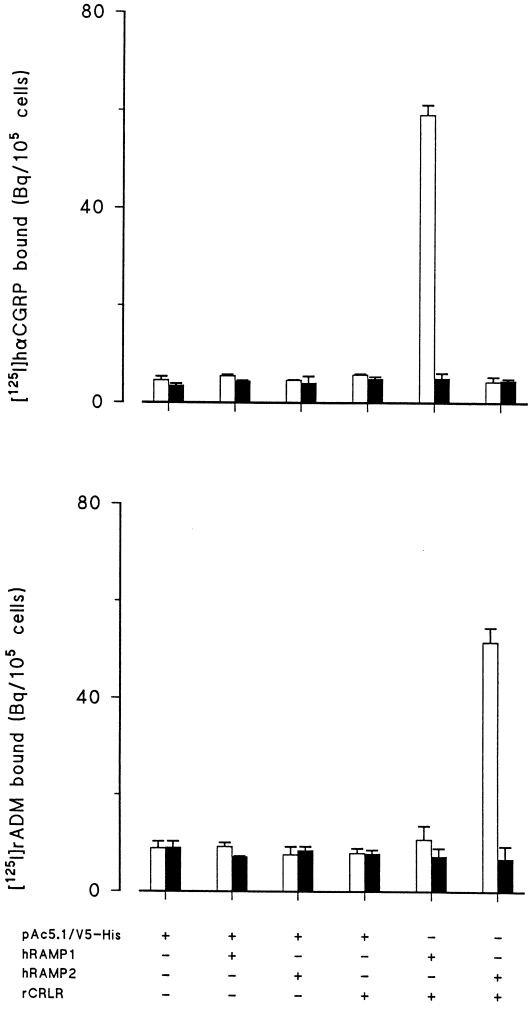

Fig. 1. Binding of [^{125}I]hαCGRP (top) and [^{125}I]rADM (bottom) to *Drosophila* S2 cells stably expressing combinations of rCRLR and RAMPs. Open bars indicate total binding and closed bars non-specific binding in the presence of 10^{-6} M non-labeled corresponding peptides. The empty *Drosophila* expression vector pAc5.1/V5-His was used for mock transfections and to equalize the total amounts of DNA in individual transfections. Binding conditions are described in Section 2. The results are mean values ± S.E.M. of triplicate determinations of experiments carried out at least three times.

detected (Fig. 1). Specific [^{125}I]hαCGRP binding was 17% of added radioligand in the cells co-expressing rCRLR and RAMP1, but not with RAMP2. Rat CRLR and RAMP2 co-expressing cells, on the other hand, exhibited 16% specific [^{125}I]rADM, but no [^{125}I]hαCGRP binding. In cells co-expressing rCRLR and RAMP1 [^{125}I]hαCGRP binding was displaced by rαCGRP(1–37), rβCGRP(1–37) and the CGRP antagonist rαCGRP(8–37) with similar half-maximal inhibitory concentrations (IC_{50}), and rADM(1–50) was 18-fold less potent than rαCGRP(1–37) (Table 1). The ADM antagonist rADM(20–50), rat amylin and rCT did not affect [^{125}I]hαCGRP binding at up to 0.5 μM. In the S2 cells co-expressing rCRLR and RAMP2 [^{125}I]rADM binding was most potently inhibited by rADM(1–50), and rADM(20–50), rαCGRP(1–37), rβCGRP(1–37) and rαCGRP(8–37) were 16- and 260-fold less effective than rADM(1–50). [^{125}I]rADM binding was unaffected by up to 1 μM rat amylin and rCT. The IC_{50} of rαCGRP(1–37), rβCGRP(1–37) and of rADM(1–50) of [^{125}I]hαCGRP and -rADM receptor binding corresponded to the half-maximal effective concentration (EC_{50}) of cAMP accumulation. Taken together, CGRP and ADM receptors expressed in S2 cells co-transfected with rCRLR and RAMP1 or RAMP2 encoding cDNA exhibited the pharmacological profile and coupling to cAMP production indistinguishable from that of the receptors expressed in mammalian cells [3,4]. The results also indicate that *Drosophila* S2 cells do not express recognizable amounts of endogenous homologues of rCRLR, RAMP1 and RAMP2 interfering with the functional expression of mammalian CGRP or ADM receptors.

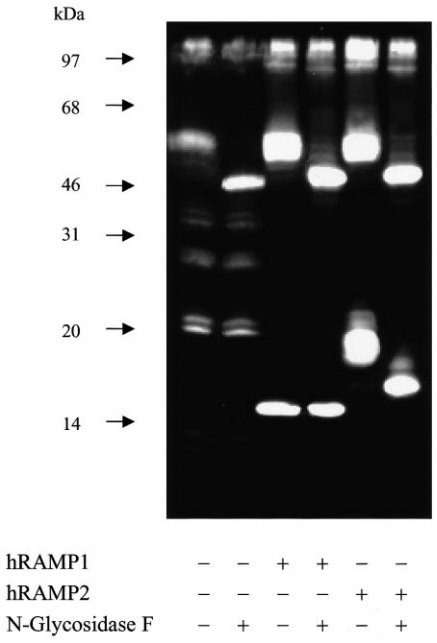

Fig. 2. Glycosylation of rCRLR and RAMP1 and RAMP2 revealed by Western blot analysis. Extracts of S2 cells, stably expressing V5- and His_6-epitope tagged rCRLR and RAMP1 or RAMP2 in indicated combinations, were subjected to SDS polyacrylamide (15%) gel electrophoresis with and without *N*-glycosidase F treatment. The proteins were visualized on Western blots with monoclonal V5-antibody and the ECL technique (see Section 2). Representative experiment carried out at least three times.

3.2. Glycosylation of rCRLR and RAMPs in Drosophila S2 cells

Glycosylation of V5- and His_6-epitope tagged rCRLR and RAMP1 and RAMP2, stably expressed in S2 cells, was analyzed on Western blots in the absence and presence of *N*-glycosidase F (Fig. 2). Rat CRLR alone or together with RAMP1 or RAMP2 had an indistinguishable apparent M_r of 55–59 kDa reduced to 48 kDa through treatment with *N*-glycosidase F. The latter corresponds to the protein backbone of rCRLR. In S2 cells glycosylated rCRLR is similar in size to the hCRLR [3] and rCRLR (not shown) in human embryonic kidney (HEK) cells alone and with RAMP2. In HEK cells RAMP1 brought about more extensive glycosylation of hCRLR [3] and rCRLR (not shown) to 66 and 74 kDa components, respectively, not recognized in *Drosophila* S2 cells. RAMP1 expressed in *Drosophila* S2 cells had an apparent M_r of 15 kDa, as predicted from the amino acid sequence, unaltered by *N*-glycosidase F. RAMP2, on the other hand, had a predominant 18 kDa M_r reduced by *N*-glycosidase F to a major 16 kDa protein in agreement with the calculated M_r of non-glycosylated RAMP2. This demonstrates glycosylation of RAMP2, but not of RAMP1. The findings are consistent with a single *N*-glycosylation site predicted from the amino acid sequence of RAMP2 not conserved in RAMP1 [3]. Taken together, the results demonstrate glycosylation of rCRLR and RAMP2 in *Drosophila* S2 cells. Moreover, glycosylation of the rCRLR in S2 cells is independent of the presence of RAMP1 or RAMP2 and does not indicate CGRP or ADM specificity.

3.3. Characterization of [^{125}I]hαCGRP and [^{125}I]rADM-binding protein components in S2 cells

The [^{125}I]hαCGRP was cross-linked with BS3 to *Drosophila* S2 cells co-expressing V5- and His_6-epitope tagged rCRLR

Table 1
[^{125}I]hαCGRP and [^{125}I]rADM binding inhibition (IC_{50}) and cAMP accumulation (EC_{50}) in *Drosophila* S2 cells transfected with rCRLR together with RAMP1 and RAMP2

	rCRLR+RAMP1		rCRLR+RAMP2	
	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)
rαCGRP(1–37)	6.5 ± 2.2	10.7 ± 3.2	225 ± 35	> 1000
rβCGRP(1–37)	4.7 ± 0.72	5.1 ± 0.3	190 ± 46	> 1000
rαCGRP(8–37)	1.7 ± 0.36	> 1000	97 ± 19	> 1000
rADM(1–50)	118 ± 19	> 1000	0.87 ± 0.2	7.7 ± 3.6
rADM(20–50)	> 1000	> 1000	14 ± 8.6	> 1000
Rat amylin	> 500	> 1000	> 1000	> 1000
rCT	> 1000	> 1000	> 1000	> 1000

Results are means ± S.E.M. of three independent experiments

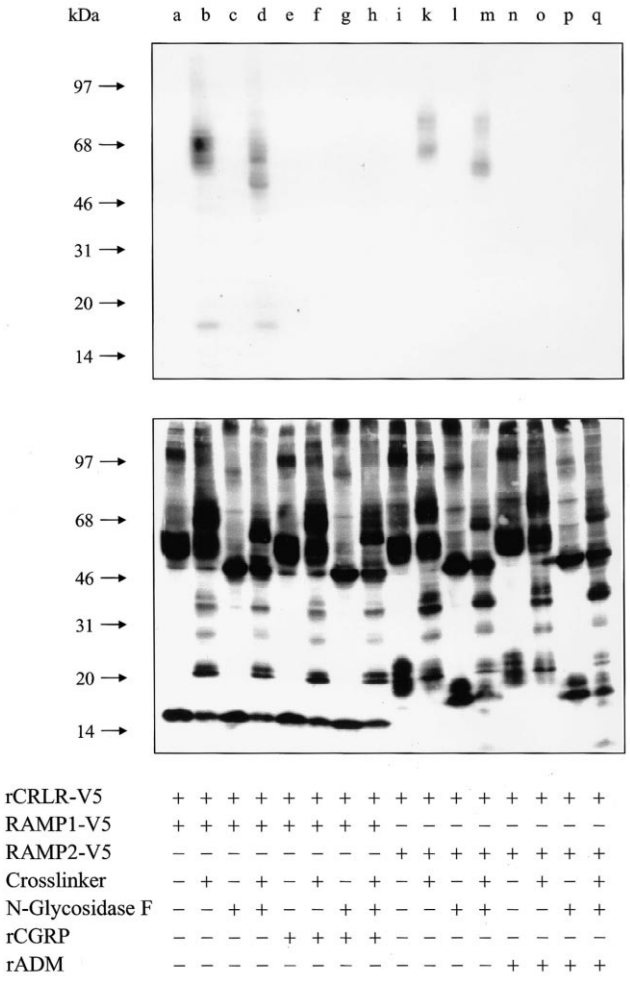

Fig. 3. BS3-cross-linking of [^{125}I]hαCGRP to *Drosophila* S2 cells that express rCRLR and together with RAMP1 (lanes a–h) or of [^{125}I]rADM to cells that co-express rCRLR and RAMP2 (lanes i–q). Cross-linker and non-labeled peptides were added and the cell extracts treated with *N*-glycosidase F as indicated. After SDS polyacrylamide gel electrophoresis of cell extracts, Western blots were autoradiographed (top) and analyzed with V5-antibody (bottom). Representative experiment carried out at least three times.

and RAMP1. Polyacrylamide gel electrophoresis of cell extracts and autoradiography of Western blots revealed major specifically ^{125}I-labeled proteins between 63 and 71 kDa and a minor 18 kDa component (Fig. 3, top). The radiolabeled proteins were not observed when the cross-linker was omitted. Treatment of cross-linked samples with *N*-glycosidase F reduced the size of the large 63–71 to 51–68 kDa proteins, but the 18 kDa radiolabeled component remained unchanged. Similarly, cross-linking of [^{125}I]rADM to S2 cells co-expressing V5- and His_6-epitope tagged rCRLR and RAMP2 revealed two specifically labeled components with apparent M_r of 72–76 and 64–67 kDa. The latter were reduced with *N*-glycosidase F to 54–58 kDa.

Subsequent analysis of the same Western blot with V5-epitope specific antibodies was carried out to further characterize [^{125}I]hαCGRP- and [^{125}I]rADM-binding proteins. In extracts of rCRLR and RAMP1 or RAMP2 co-expressing S2 cells not treated with cross-linker, glycosylated rCRLR and RAMP2, and RAMP1 were observed (Fig. 3, bottom). In cell extracts treated with BS3 additional major protein components were detected in the position of [^{125}I]hαCGRP- and [^{125}I]rADM-binding components, identified by autoradiography. They were only recognized when RAMP1 or RAMP2 were co-expressed with rCRLR and the M_r were consistent with those of glycosylated rCRLR cross-linked to RAMP1 or to glycosylated RAMP2. Moreover, decreased amounts of free RAMP1 and RAMP2 were observed in cross-linked as compared to non-cross-linked samples, consistent with a shift of a fraction of the two RAMPs into larger molecular weight cross-linking products like rCRLR/RAMP complexes. Additional minor non-identified components, recognized by the V5-antibody, with apparent M_r of 20–46 kDa were also observed. These minor cross-linking products in extracts of rCRLR/RAMP1 co-expressing cells had not a M_r of 18 kDa expected for [^{125}I]hαCGRP cross-linked to RAMP1. But the weak autoradiographic signal at 18 kDa indicated that the amounts of this cross-linking product were likely below the detection limit of the V5-antibodies and represented a minor fraction of [^{125}I]hαCGRP cross-linked to RAMP1 alone slightly larger than RAMP1. Interaction on the cell surface of [^{125}I]hαCGRP with RAMP1 alone cannot be excluded. Evidence for RAMP2 cross-linked to [^{125}I]rADM has not been obtained.

Deglycosylated proteins were recognized in S2 cells following treatment with *N*-glycosidase F. In S2 cells co-expressing rCRLR and RAMP1 or RAMP2 and treated with cross-linked major protein components differed in size from the 48 kDa rCRLR by the M_r predicted for RAMP1 and non-glycosylated RAMP2, respectively. In the same samples receptor autoradiography indicated cross-linking of [^{125}I]hαCGRP to a deglycosylated rCRLR/RAMP1 complex and to rCRLR alone. In rCRLR and RAMP2 co-expressing cells corresponding [^{125}I]rADM/rCRLR/RAMP2 and [^{125}I]rADM/rCRLR complexes were also observed. But the larger [^{125}I]rADM-labeled component was unaltered following *N*-glycosidase F treatment, indicating that a rCRLR/RAMP2 complex was resistant to *N*-glycosidase-F when bound to [^{125}I]rADM.

In a parallel study carried out in mammalian cells (in preparation), myc-tagged RAMP1 was similarly cross-linked to [^{125}I]hαCGRP-binding V5- and His_6-tagged rCRLR. The results presented here and the observations obtained in mammalian cells demonstrate that the predicted co-transport of CRLR and RAMPs to the plasma membrane [3] results in the formation of CRLR/RAMP1 or CRLR/RAMP2 heterodimers on the cell surface that function as specific CGRP or ADM receptors in *Drosophila* and mammalian cells. This and the identification of amino-terminal putative extracellular domains of RAMP1/RAMP2 chimeras as the critical determinants of ligand specificity [7], imply direct interactions of extracellular domains of RAMP1 and RAMP2 with CRLR defining the ligand binding site. The here established polyclonal *Drosophila* S2 cell lines, stably co-expressing V5- and His_6-epitope tagged rCRLR and RAMP1 or RAMP2, are suitable for large scale suspension culture.

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