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Ligand-induced internalization, recycling, and resensitization of adrenomedullin receptors depend not on CLR or RAMP alone but on the receptor complex as a whole ☆

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ABSTRACT

Adrenomedullins (AM) is a multifaceted distinct subfamily of peptides that belongs to the calcitonin gene-related peptide (CGRP) superfamily. These peptides exert their functional activities *via* associations of calcitonin receptor-like receptors (CLRs) and receptor activity-modifying proteins (RAMPs) RAMP2 and RAMP3. Recent studies established that RAMPs and CLRs can modify biochemical properties such as trafficking and glycosylation of each other. However there is very little or no understanding regarding how RAMP or CLR influence ligand-induced events of AM-receptor complex. In this study, using pufferfish homologs of CLR (mfCLR1–3) and RAMP (mfRAMP2 and mfRAMP3), we revealed that all combinations of CLR and RAMP quickly underwent ligand-induced internalization; however, their recycling rates were different as follows: mfCLR1–mfRAMP3 > mfCLR2–mfRAMP3 > mfCLR3–mfRAMP3. Functional receptor assay confirmed that the recycled receptors were resensitized on the plasma membrane. In contrast, a negligible amount of mfCLR1–mfRAMP2 was recycled and reconstituted. Immunocytochemistry results indicated that the lower recovery rate of mfCLR3–mfRAMP3 and mfCLR1–mfRAMP2 was correlated with higher proportion of lysosomal localization of these receptor complexes compared to the other combinations. Collectively our results indicate, for the first time, that the ligand-induced internalization, recycling, and reconstitution properties of RAMP–CLR receptor complexes depend on the receptor-complex as a whole, and not on individual CLR or RAMP alone.

1. Introduction

Adrenomedullins (AMs) are a family of peptides belonging to a subfamily of calcitonin gene-related peptide (CGRP) superfamily. The other members of the CGRP superfamily include calcitonin (CT), CGRP, amylin (AMY), and calcitonin receptor stimulating peptide (CRSP) (Poyner et al., 2002; Katafuchi and Minamino, 2004). These peptides have a six/seven-membered disulfide ring structure in their N termini and amidated C termini. The AM subfamily is composed of 5 members in fish (AM1–AM5) and 3 members in mammals (AM1, AM2/intermedin, and AM5) (Roh et al., 2004; Ogoshi et al., 2006). These peptides signal through a unique receptor complex, which is composed of calcitonin receptor-like receptor (CLR) and a receptor activity modifying protein (RAMP) (McLatchie et al., 1998). CLR is a G protein-coupled receptor (GPCR) and RAMP is a single-transmembrane-span protein with a very short intracellular C terminus and a long N-terminal extracellular domain. The CLR family consists of 3 members (CLR1–CLR3) while the RAMP family has 5 members (RAMP1–RAMP5). It was found that when CLR heterodimerizes with RAMP1 it can act as CGRP1 receptor but when heterodimerizes with either RAMP2 or RAMP3 it can form a high affinity AM receptor (McLatchie et al., 1998). Molecular imaging and biochemical analyses revealed that RAMPs form a stable heteromeric complex with CLR in the endoplasmic reticulum/Golgi, which is maintained throughout their life cycle including trafficking to the cell surface, ligand binding, internalization, recycling, and degradation (Kuwasako et al., 2000; Bomberger et al., 2005a; Hay et al., 2006; Sexton et al., 2006). It has been established that the post-translational events like glycosylation

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and plasma membrane targeting of RAMP and CLR proteins are regulated interdependently (McLatchie et al., 1998; Nag et al., 2012).

Moreover, it was found that RAMPs are able to interact with the following members of the class II GPCR family: calcitonin receptor (CTR), VPAC1 vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor (VPAC1R), glucagon receptor, PTH1 parathyroid hormone receptor (PTH1R), and PTH2 parathyroid hormone receptor (PTH2R) (Christopoulos et al., 2003; Udawela et al., 2004; Hay et al., 2006; Magalhaes et al., 2012). A recent study further revealed that all the three RAMPs could heteromerize with calcium-sensing receptor (CaSR), a class III GPCR, promoting proper post-translational modification and efficient plasma membrane targeting of CaSR (Bouschet et al., 2005). These studies indicated that RAMPs might play roles to modify a wide range of members of GPCR superfamily in different ways, some of which include post-translational modification, plasma membrane targeting, ligand binding, and alteration of ligand-stimulated intracellular signaling pathways.

Recently, we have identified 3 CLR (mfCLR1–3) and 5 RAMP (mfRAMP1–5) genes in mefugu (mf), an euryhaline pufferfish, and characterized their functional combinations, tissue distributions, and ligand specificities (Nag et al., 2006). We found that these mfRAMP isoforms distinctly modify the pharmacological properties of mfCLRs (Nag et al., 2006, 2007). Specific receptors for AM1 (the ortholog of mammalian AM), were generated by the following combinations of mfCLRs and mfRAMPs: mfCLR1–mfRAMP2/3/5, mfCLR2–mfRAMP2, and mfCLR3–mfRAMP3. Although much information has been accumulated related to the effects on the biochemical properties of CLRs and RAMPs, little is known about their fate namely whether they are internalized and recycled together or separately and how their fate is affected by the combination of CLRs and RAMPs. Here, using fluorescence-activated cell sorting (FACS) analyses, second messenger assay, Western blotting, and immunocytochemistry, we revealed that the ligand-induced intracellular events of AM1 receptor is the composite property of the complex as a whole.

2. Materials and methods

2.1. Peptides and plasmids

Pufferfish AM1, AM2, and AM5 were custom synthesized by Peptide Institute (Osaka, Japan). N-terminally FLAG-tagged mfRAMP3 and HA-tagged mfCLRs (mfCLR1, mfCLR2, and mfCLR3) were constructed in pSecTag A vector (Invitrogen) by replacing endogenous putative pre-sequences of the relevant genes as described earlier (Nag et al., 2006). For this study we have used full-length mfRAMP2a isoform, and for simplicity throughout this paper we have mentioned it as mfRAMP2.

2.2. Cell culture, plasmid transfection and receptor assay

COS7 cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) at 37 °C under 5% CO_2. Cells were seeded in culture dishes, and at ~90% confluency they were transiently transfected with the mentioned constructs or mock vector using Lipofectamine 2000 according to the manufacturer's instructions. Culture medium was replenished every 24 h. At 48 h post-transfection, cells were either allowed for specific treatment or processed for immunocytochemistry or Western blot experiments. Ligand-induced intracellular cAMP assay was performed as described previously (Nag et al., 2006).

2.3. Cell surface biotinylation and Western blotting

COS7 cells, in 6-well plates, were transiently transfected with the mentioned vectors. Cell surface proteins were biotinylated with 0.5 mg/ml membrane-impermeable biotinylating reagent EZ-link Sulfo-NHS-SS-biotin (Pierce) in PBS, and samples were prepared and subjected to Western blot analysis as described before (Nag et al., 2006, 2012).

2.4. Biochemical treatments

Cells were plated into 6-well plates, transfected with the indicated constructs, and at 46 h post transfection allowed for the following chemical treatments. Cells were either treated with cycloheximide (Sigma) for the indicated periods at 37 °C or used directly for necessary experiments. Cells were then either exposed to AM1 at the indicated doses for 30 min at 37 °C or processed directly. After the treatments, cells were washed twice with PBS containing 10% FBS and 50 mg/ml cycloheximide, and either processed for FACS analysis or processed for biotinylation and Western blotting.

2.5. Flow cytometry

Cells were dissociated by treatment with 0.25% trypsin, fixed with 4% PFA, and immediately washed twice with buffer (PBS containing 3% FBS), and single cell suspensions were obtained by passing through 40-μm cell-strainer. They were treated with anti-FLAG mouse monoclonal antibody (Sigma, 1000×), followed by treatment with goat anti-mouse secondary IgG conjugated with FITC and 5 μg/ml propidium iodide and washed with the same buffer. Approximately 5×10^5 cells per tube were subjected for FACS analysis. Cell viability was assessed by exclusion of propidium iodide-positive population. Data were collected using FACSCalibur (BD Bioscience) and CellQuest Pro software.

2.6. Immunocytochemistry

Cells were grown on coverslips and at 48 h post-transfection, were fixed with ice-cold 4% paraformaldehyde (PFA) for 20 min, then washed twice with ice-cold PBS, and permeabilized with 0.1% (vol/vol) Triton X-100 (Acros Organics) in PBS for 10 min. After blocking with 3% FBS in PBS, cells were incubated with the indicated primary antibodies: rabbit anti-FLAG (Sigma); mouse anti-Rab7 (Abcam); and mouse anti-LAMP1 (Abcam) overnight at 4 °C. Samples were prepared, and images were taken as described previously (Nag et al., 2006, 2012).

3. Results

3.1. Tag attachment in N-terminil of RAMPs and CLRs does not alter AM1 receptor function

Since we inserted FLAG and HA tags into the N-terminus of mfRAMP and mfCLR proteins, respectively, it was necessary to determine whether tag insertion influenced the receptor function. We, therefore, transfected COS7 cells with a variety of combinations of FLAG-tagged mfRAMP2 and mfRAMP3, and HA-tagged mfCLR1–3, and measured intracellular cAMP levels of the transfected cells using a standard protocol on stimulation with AM1. No significant difference was observed between the cAMP responses of the tagged receptor characterized here (Fig. 1) and the wild-type receptor characterized previously (Nag et al., 2006, 2012). This observation suggested similar behaviors of the tagged and untagged mfAM-receptors regarding ligand-induced cAMP