Activation of Calcitonin Receptor and Calcitonin Receptor-like Receptor by Membrane-anchored Ligands*

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G protein-coupled receptors (GPCRs) are the most important pharmacological targets, and more than 40% of drugs in use today modulate GPCR signaling. A major hurdle in the development of therapies targeting GPCRs is the drug candidate’s nonspecific actions in multiple tissues. The ability to spatially control GPCR signaling would provide a venue for developing therapies that require targeted GPCR signaling. Here, we show that the fusion of a RAMP1 co-receptor with the calcitonin gene-related peptide (CGRP), or calcitonin, transforms the RAMP1 from a co-receptor to bona fide membrane-anchored ligands (CGRP-RAMP1 and CAL-RAMP1). The CAL-RAMP1 selectively activates the calcitonin receptor (CR), whereas, the CGRP-RAMP1 activates both the calcitonin receptor-like receptor (CLR) and CR. Unlike a free peptide, which moves freely in the extracellular space and differentiates targets based on molecular affinity, the anchored CGRP-RAMP1 and CAL-RAMP1 ligands confine their activities to individual cells. In addition, our study showed that a CGRP8–37-RAMP1 chimera, but not RAMP1, functions as an antagonist for CGRP-RAMP1-mediated signaling, suggesting that the activation of CLR by CGRP-RAMP1 shares similar molecular mechanisms with the CGRP-mediated activation of CLR/RAMP1 receptor complexes. Taken together, our finding thus provides a novel class of ligands that activate CR and CLR exclusively in an autocrine manner and a proof-of-concept demonstration for future development of targeted therapies aimed at these receptors in specific cell populations.

G protein-coupled receptors (GPCRs) represent the largest family of cell surface receptors and interact with free ligands ranging from photons, ions, derivatives of lipids, carbohydrates, amino acids, or nucleosides to gene-encoded polypeptides. More than 40% of drugs in use today target GPCR activities (2–4), and these drugs include the traditional small compounds and large polypeptide analogs. However, thus far there is no pharmacological means to activate GPCR signaling in select cell populations (5). Therefore, the ability to spatially control GPCR signaling could open new therapeutic opportunities precluded from consideration in the past.

One approach to modulate GPCR activities in a cell-specific manner is to derive the pharmacological agent within the cell and limit its mobility to the boundary of cell surface membranes. Although this approach would handicap the endowed benefit of a free moving drug, it eliminates side effects derived from uncontrolled distribution in the extracellular space. Conceptually, this approach would be most applicable to GPCRs with a polypeptide ligand because a peptide can be specifically expressed within a potential target cell. To investigate whether it is feasible to drive GPCR signaling in a cell-specific manner, we explored the class B GPCRs that are exclusively regulated by polypeptide hormones, and are important targets for the development of drugs for dwarfism, diabetes, osteoporosis, Paget disease, myocardial infarction, and inflammatory diseases (1, 6–10). Among this group of GPCRs, the calcitonin receptor-like receptor (CLR) and calcitonin receptor (CR) are indispensable for calcium homeostasis and vasculogenesis, respectively (11–16). Interestingly, both CLR and CR require receptor activity-modifying proteins (RAMP1, -2, and -3) for efficient signaling with their cognate ligands including, calcitonin gene-related peptides (CGRP), amylin, adrenomedullin and intermedin (12, 17–19). RAMPs are type I transmembrane proteins that regulate receptor trafficking and ligand binding (20–24). Because membrane-anchored RAMPs are devoid of signaling capability, we hypothesized that RAMPs could be useful as a vehicle to restrict the mobility and bioactivity of peptide ligands for CLR and CR to select cell populations.

To test this hypothesis, we generated and analyzed the bioactivity of a variety of tethered ligand-RAMP1 fusion proteins consisting of a ligand (calcitonin, CGRP, amylin, vasoactive inhibitory peptide (VIP), or pituitary adenylate cyclase-activating peptide (PACAP)) and RAMP1, in transfected HEK293T cells (Fig. 1A)(6).

EXPERIMENTAL PROCEDURES

Generation of Wild Type and Mutant Constructs for CLR, CR, and RAMP Co-receptors—Expression constructs for chimeric proteins, including CGRP-RAMP1, CGRP-RAMP2, CGRP8–37-RAMP1, CGRPAA-RAMP1, CAL-RAMP1, AMY-RAMP1, VIP-RAMP1, PACAP-RAMP1, CGRP-CLR, and CGRP-CLR-RAMP1, were generated by overlapping PCR with primers that...
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cover the selected regions from two separate genes, and were subcloned into the expression vector, pcDNA3.1 Zeo (Invitrogen, San Diego, CA). All cDNA constructs were appended with a signal peptide for secretion of the prolactin precursor at the N terminus followed by a Myc or a FLAG epitope (Fig. 1A). In all tethered ligand-(co)receptor constructs, the mature ligand sequence was linked directly to the mature RAMP or CLR protein sequences via a glycine residue that is normally processed to become an amidated terminus in mature peptide hormones (6).

Activation of Receptor and cAMP Assay—To assay the signal transduction of receptors, expression vectors encoding human CLR, CR, or a chimeric receptor were transiently transfected together with expression constructs encoding a wild-type RAMP or a tethered ligand-RAMP chimera into HEK293T cells using Lipofectamine 2000 or the calcium phosphate precipitation method. At 24 h after transfection, cells were seeded at a density of 2 × 10⁵ per well in 48-well plates with 500 µl of Dulbecco’s modified Eagle’s medium/F12 medium containing 0.1% bovine serum albumin and 0.25 mM 3-isobutyl-1-methylxanthine (IBMX). Replicates of transfected cells were collected at 3, 6, or 12 h after incubation, and frozen at −80 °C before cAMP assay. Culture medium was reacted with acetic anhydride triethylamine, and total cAMP content was measured using a radioimmunoassay or a homogeneous time-resolved fluorescence assay (HTRF, Cisbio International, France) (25). For the HTRF assay, 10–15 µl aliquots of lysates or diluents from each sample were plated in 384-white plates. Following a 30-min incubation at room temperature, 5 µl of labeled d2 cAMP and 5 µl of anti-cAMP antibody were added to each well using a multichannel pipette. The plates were incubated at 37 °C for 1 h. The fluorescent signals were read with an Analyst GT device (Molecular Devices, Inc., Sunnyvale, CA) at 665 nm and 620 nm. The ratio of 665 nm/620 nm fluorescence signal was then calculated and curves fitted using Graphpad Prism 4 Software. All experiments were repeated at least three times.

Immunoprecipitation of Recombinant CLR and RAMP1 Proteins—To characterize the association between CLR and RAMP1, HEK293T cells were transiently transfected with expression constructs for CLR- and a RAMP1-derived protein. At 48 h after transfection, cells were harvested and lysed with immunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, and 1× protease inhibitor mixture (Roche). To immunoprecipitate RAMP1-associated protein complexes, cell lysates were incubated with an anti-Myc antibody (Sigma-Aldrich) for 3 h, followed by incubation with protein A/G-agarose beads (Amersham Biosciences) for 1 h at room temperature. The agarose beads were washed three times with lysis buffer to remove unbound proteins and then boiled in a loading buffer under reducing conditions (100 mM dithiothreitol and 5% 2-mercaptoethanol) for 10 min. The samples were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences) in a Trans-Blot® S.D. semi-dry transfer cell (Bio-Rad). The membranes were then blocked with 5% low fat milk before immunoblotting using an anti-Myc or an anti-FLAG monoclonal antibody as primary antibody. Following incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000 final dilutions) for 20 min, immunofluorescent imaging was detected using the ECL system (Amersham Biosciences).

RESULTS AND DISCUSSION

Stimulation of CLR and CR by Membrane-anchored Ligands—Analysis of cAMP production in transfected cells showed that co-expression of CLR with RAMP1 has no effect on the adenylate cyclase activity in the absence of a cognate ligand, similar to the transfection with an empty pcDNA3.1 vector (Fig. 1A). In contrast, co-transfection with an increasing amount of an expression vector encoding a tethered CGRP-RAMP1 fusion protein resulted in a dose-dependent increase of cAMP production in transfected cells (Fig. 1A). Importantly, we found that co-expression of CGRP-RAMP1 and CLR increases cAMP production to levels comparable to that observed in CLR/RAMP1-expressing cells following treatments with a 30 nM dose of the CGRP peptide (Fig. 1B), suggesting that CGRP-RAMP1 is capable of fully activating CLR in transfected cells. In contrast, co-expression of fusion proteins with VIP, PACAP, or amylin at the N terminus of RAMP1 (VIP-RAMP1, PACAP-RAMP1, and AMY-RAMP1) had no effect on the adenylate cyclase activity of transfected cells (Fig. 1A)(6, 26). Similar to studies of CLR, co-expression of CR with a tethered CAL-RAMP1 or CGRP-RAMP1 fusion protein led to significant increases of cAMP production in transfected HEK293T cells (Fig. 1C).

Importantly, cell surface expression and Western blotting analyses showed that VIP-RAMP1, PACAP-RAMP1, and AMY-RAMP1 chimeras of expected sizes are expressed on the surface of transfected cells (Fig. 1, D and E). Therefore, the activation of CLR and CR by anchored ligands is ligand-specific whereas the lack of agonistic activity of VIP-RAMP1, PACAP-RAMP1, and AMY-RAMP1 chimeras is not likely due to defective processing or expression. Furthermore, to exclude the possibility that CGRP-RAMP1- and CAL-RAMP1-mediated signaling is a result of activation by free peptides generated from the degradation of fusion proteins, we analyzed the bioac-
tivity of conditioned medium from cells expressing CGRP-RAMP1 or CAL-RAMP1. Functional analysis showed that concentrated conditioned medium from cells expressing CGRP-RAMP1/CLR or CAL-RAMP1/CR do not exhibit any agonistic activity on cAMP production in cells expressing CLR/RAMP1 or CR/RAMP1 receptor complexes (data not shown). Collectively, these results demonstrated that fusion of RAMP1 with CGRP, or calcitonin, effectively transforms the RAMP1 co-receptor into anchored ligands that stimulate CLR and/or CR signaling in a stochastic manner. Unlike a typical hormone which activates receptors based on molecular affinity, the membrane-anchored CGRP-RAMP1 and CAL-RAMP1 differentiate targets by molecular affinities plus the locality of the receptor (6, 26). Thus, CGRP-RAMP1 and CAL-RAMP1 rep-
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**Figure 2.** Stringent structural requirements for CGRP-RAMP1-mediated CLR signaling. A. Except for the combination of CGRP-RAMP1 and CLR (blank square), co-expression of other chimeric receptor/coreceptor pairs including, RAMP1/CLR, CGRP-RAMP2/CLR, CGRP-CLR-RAMP1/CLR, CGRP8–37-RAMP1/CLR, and CGRPAA-RAMP1/CLR, was without effect on the adenylate cyclase activity of transfected HEK293T cells. B, stimulation of cAMP production in cells co-expressing CGRP-CLR and RAMP1 by CGRP and intermedin. In the absence of a free ligand, CGRP-CLR fusion protein has negligible bioactivity with or without the RAMP1 co-receptor. Each data point represents the mean ± S.E. of quadruplicate samples. Similar results were observed in at least four separate experiments.

CGRP-RAMP1 and CGRP Peptide-mediated CLR Signaling Requires Similar Structural Components—To elucidate the fidelity of CGRP-RAMP1-mediated CLR signaling, we studied five additional combinations of tethered fusion proteins with different spatial arrangements of the three structural components (the ligand, the RAMP ectodomain, and the CLR ectodomain) for CLR signaling. These receptor combinations included, 1) CGRP-CLR+RAMP1, 2) CGRP-RAMP2+CLR, 3) CGRP-CLR-RAMP1+CLR, 4) CGRP8–37-RAMP1+CLR, and 5) CGRPAA-RAMP1+CLR (Fig. 2A). Analysis of cAMP production in transfected cells showed that co-expression of a tethered CGRP-CLR fusion protein, in which the mature CGRP sequence is anchored at the N terminus of CLR, has no effect on cAMP production of transfected cells in the absence or presence of RAMP1 (Fig. 2A). Because treatments of cells expressing CGRP-CLR and RAMP1 with CGRP or intermedin result in dose-dependent increases of cAMP production, these data indicate that the anchoring of a CGRP peptide on CLR ectodomain is not sufficient to activate CLR signaling efficiently. In addition, we found that co-expression of tethered ligand-RAMP proteins containing a RAMP2 anchor (CGRP-RAMP2), or the ectodomain of CLR (CGRP-CLR-RAMP1), has negligible effect on CLR signaling (Fig. 2A), consistent with earlier observations that CGRP exhibits a negligible agonistic activity on CLR signaling in the absence of a RAMP, or in the presence of RAMP2 (12, 20). Furthermore, analysis of cells expressing CGRP-RAMP1 mutants with a truncation (CGRP8–37-RAMP1 in which residues 1–7 of the N-terminal receptor-activation domain of CGRP are deleted), or point mutations (CGRPAA-RAMP1 in which the disulfide bond-forming Cys-2 and Cys-7 are substituted with alanines) showed that alterations of the activation domain in CGRP abolish the agonistic activity of CGRP-RAMP1 (Fig. 2A) (6, 26, 28). The combined results thus suggested that the activation of CLR by CGRP-RAMP1 or CGRP peptide requires similar structural components, even though the dynamics of signaling was switched from the binding of a free ligand with a receptor complex to the interaction between a membrane-anchored ligand and a seven-transmembrane receptor.

CGRP8–37-RAMP1, but Not RAMP1, Functions as a Competitive Antagonist to Block CGRP-RAMP1-mediated CLR Signaling—Taking advantage of our novel findings of CGRP-RAMP1, we further explored molecular mechanisms underlying the CGRP-mediated CLR signaling. Recent studies of CLR, dopamine D2 receptor, and several class C GPCRs (metabotropic glutamate, GABAB, and taste receptors) indicated that an important consequence of ligand binding to these GPCRs is the stabilization of receptor complexes (29, 30). To investigate whether this mechanism is applicable to CGRP-RAMP1-mediated CLR signaling, we compared the association between CGRP-RAMP1 and CLR to that of RAMP1 and CLR. Consistent with earlier studies of CLR, immunoprecipitation analyses showed that RAMP1 interacts with CLR in the absence of a free ligand (Fig. 3A) (31, 32). Likewise, CGRP-RAMP1 bound and co-precipitated with CLR. Importantly, quantitative analysis of Western blotting results showed that 6.13 ± 0.72 times more immunoreactive CLR is co-precipitated with a given unit of CGRP-RAMP1 as compared with RAMP1 (Fig. 3A). Although these data strengthened the concept that CGRP binding leads to the stabilization of CLR/RAMP1 receptor complexes, it is not clear whether CGRP-RAMP1 activates CLR via a process identical to that occurs after CGRP interacts with the CLR/RAMP1 receptor complex. We reasoned that if the interaction between CGRP-RAMP1 and CLR is similar to that of CGRP-activated CLR/RAMP1 signaling, the CGRP-RAMP1-mediated signaling would be efficiently blocked by the tethered CGRP8–37-RAMP1 fusion protein, but not RAMP1.
or the competitive antagonist CGRP8–37 (28). Consistent with our hypothesis, co-expression of an increasing amount of the tethered CGRP8–37-RAMP1 fusion protein, but not RAMP1, significantly reduced CGRP-RAMP1-activated cAMP production in CLR-expressing cells (Fig. 3C). Likewise, we found that treatments with a high concentration of CGRP8–37 have a negligible effect on CGRP-RAMP1-mediated CLR signaling in transfected cells (Fig. 3C). To investigate whether CGRP8–37-RAMP1 also functions as an antagonist for CGRP-mediated signaling, we analyzed cAMP production in cells expressing CGRP8–37-RAMP1 and CLR after CGRP treatments. Similar to cells expressing wild-type RAMP1 and CLR, CGRP treatments dose-dependently increased cAMP production in cells expressing CGRP8–37-RAMP1 and CLR (Fig. 3B), suggesting that the anchoring of CGRP8–37 at the N terminus of RAMP1 does not preclude the interaction between CGRP and the binding pocket within the CLR/CGRP8–37-RAMP1 complex. These observations thus support the notion that interactions of CGRP-RAMP1 and CLR partially resemble that of CGRPs and CLR/RAMP1 receptor complexes.

Taken together, our study provides a new class of ligands that activate CLR and CR independent of internal RAMPs. Importantly, these ligands act in a manner similar to a free ligand, but are restrained from random distribution outside the cell. These novel ligands not only satisfy criteria we envisioned as prerequisite for activating a GPCR is to properly present an agonistic molecular interface to the receptor. Moreover, we envision that this finding could also facilitate future design of large scale screenings of novel peptide hormone analogs based on recombinant technology in the yeast (27), instead of the traditional synthetic peptide methods.

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