Biochemical characterization of G protein coupling to calcitonin gene–related peptide and adrenomedullin receptors using a native PAGE assay

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Calcitonin gene-related peptide (CGRP), adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2/IMD) have overlapping and unique functions in the nervous and circulatory systems including vasodilation, cardioprotection, and pain transmission. Their actions are mediated by the class B calcitonin–like G protein–coupled receptor (CLR), which heterodimerizes with three receptor activity–modifying proteins (RAMP1–3) that determine its peptide ligand selectivity. How the three agonists and RAMPs modulate CLR binding to transducer proteins remains poorly understood. Here, we biochemically characterized agonist-promoted G protein coupling to each CLR-RAMP complex. We adapted a native PAGE method to assess the formation and thermostabilities of detergent-solubilized fluorescent protein–tagged CLR-RAMP complexes expressed in mammalian cells. Addition of agonist and the purified G_s protein surrogate mini-G_s (mG_s) yielded a mobility-shifted agonist CLR-RAMP mG_s quaternary complex gel band that was sensitive to antagonists. Measuring the apparent affinities of the agonists for the mG_s-coupled receptors and of mG_s for the agonist-occupied receptors revealed that both ligand and RAMP control mG_s coupling and defined how agonist engagement of the CLR extracellular and transmembrane domains affects transducer recruitment. Using mini-G_sq and -G_us chimeras, we observed a coupling rank order of mG_s > mG_sq > mG_us for each receptor. Last, we demonstrated the physiological relevance of the native gel assays by showing that they can predict the cAMP-signaling potencies of AM and AM2/IMD chimeras. These results highlight the power of the native PAGE assay for membrane protein biochemistry and provide a biochemical foundation for understanding the molecular basis of shared and distinct signaling properties of CGRP, AM, and AM2/IMD.

Calcitonin gene-related peptides α and β (αCGRP and βCGRP), adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2/IMD) are related peptides that have overlapping and distinct functions in human physiology and disease that make them and their shared receptors important drug targets. Each of these peptides exhibits vasodilator activity and has cardioprotective capabilities (1–4). αCGRP is a neuropeptide with functions in pain transmission and neurogenic inflammation, and it has a key role in migraine headache pathogenesis (3). Monoclonal antibodies and small molecule drugs that antagonize CGRP signaling reached the market as migraine therapeutics in the last 2 years (5). AM has crucial developmental roles in blood and lymphatic vasculature and heart formation (6,7). It also has many functions in adults such as lymphatic maintenance and angiogenesis (8,9). AM2/IMD has angiogenic activity, stabilizes the endothelial barrier, and promotes vascular lumen enlargement in addition to having roles in metabolic regulation (1, 10–12). The two AM peptides hold promise as therapeutics for many diseases including heart attack, heart failure, and sepsis (8, 11, 13, 14).

These peptides signal through heterodimeric cell surface receptors comprising a shared class B G protein–coupled receptor (GPCR) subunit, the calcitonin receptor-like receptor (CLR), and one of three variable receptor activity–modifying protein (RAMP1–3) subunits. The RAMPs control CLR ligand binding and may modulate its signaling, but the latter remains poorly understood (15). Binding of the CGRP peptides is favored at the CGRP receptor (CLR-RAMP1), AM binding is favored at the AM1 (CLR-RAMP2) and AM2 (CLR-RAMP3) receptors, and AM2/IMD shows a slight preference for the AM2 receptor (16). When comparing cAMP-signaling potencies, the CGRP peptides exhibit a significant preference for the CGRP receptor, whereas the two AM peptides less stringently discriminate the three receptors. The complexity of this system with four peptide ligands activating one GPCR modulated by three accessory proteins makes deciphering the pharmacology and biology of these peptides and their receptors challenging. Moreover, there is growing evidence that the RAMPs interact with many different GPCRs (17, 18), which further complicates interpretation of cell-based and in vivo studies. There is a clear need for biochemical studies with defined components to advance our understanding of the signaling mechanisms of these complex receptors.

Peptide agonist binding to CLR follows the class B “two-domain” model in which the C-terminal half of the peptide binds to the CLR extracellular domain (ECD) and the N-terminal half binds the membrane-embedded CLR transmembrane domain (TMD). This is thought to occur in two steps with the first ECD-binding step bringing the N-terminal domain of the agonist in proximity to the TMD, thereby facilitating the second binding event and its resulting activation of the receptor (19). A
G protein coupling to the CGRP and AM receptors

We designed CLR and RAMP expression constructs with C-terminal EGFP and mCitrine tags, respectively (Fig. 1A). N-terminal maltose-binding protein (MBP) tags were originally included on both subunits for purification purposes, but these tags also seemed to promote more defined bands in native PAGE. We used polyethyleneimine (PEI)–mediated transient transfection to express the MBP–CLR–EGFP and MBP–RAMP–mCitrine constructs in HEK293S GnT1 cells (35). We reasoned that the homogenous N-glycosylation provided by this cell line would facilitate sharp bands on the native gels. After receptor expression in adherent cultures in 48-well plates, the cells were solubilized with lauryl maltose neopentyl glycol (LMNG)/cholesteryl hemisuccinate (CHS), the lysates were centrifuged at maximum speed in a bench-top microcentrifuge, and the supernatants were analyzed by hrCNE (36) with visualization of the detergent-solubilized receptors by gel fluorescence (Fig. 1B). Co-expression of the tagged CLR and RAMPs resulted in the appearance of intense sharp bands that migrated slower than the CLR and RAMP alone consistent with formation of the heterodimeric complexes. Analysis of the supernatants by denaturing SDS-PAGE revealed fluorescent bands of the expected molecular masses for the CLR and RAMP constructs (Fig. S1A). A control experiment with each of the RAMPs co-expressed with another class B GPCR, the parathyroid hormone receptor (PTH1R), showed no evidence for detergent-stable PTH1R-RAMP1, -2, or -3 complexes (Fig. 1C and Fig. S1B), demonstrating specificity. To prove that the starred bands in the native gel (Fig. 1B) were CLR-RAMP complexes, we excised these bands and placed the gel slices in the wells of a denaturing SDS-PAGE gel followed by electrophoresis. Fluorescent imaging revealed the presence of two bands of the correct sizes for the tagged CLR and RAMP constructs (Fig. 1D).
**G protein coupling to the CGRP and AM receptors**

![Diagram](image)

Figure 1. CLR and RAMPs form detergent-stable complexes visible by native PAGE. **A**, tagged receptor constructs used for expression in HEK293S GntT1 cells. B, 8% polyacrylamide hrCNE gel showing bands corresponding to LMNG/CHS-solubilized CLR (lanes marked with C) and each RAMP (lanes marked with R1, R2, and R3) expressed alone or co-expressed. Complex bands are starred. C, as in B except using the class B GPCR PTH1R (lanes marked with P) as the receptor. D, starred bands from B were excised, placed in the wells of a nonreducing 12% SDS-PAGE gel, and electrophoresed. Bands corresponding to the size of the CLR construct (~120 kDa) and the RAMP constructs (~83–88 kDa) are visible. B–D show representative images from two independent experiments, and the gels were imaged by in-gel fluorescence using a Chemidoc MP imager.

We were also able to use the native PAGE assay in a thermostability format to test the receptor stabilities in different detergents. Aliquots of the solubilized supernatants were incubated at various temperatures for 30 min, followed by centrifugation and analysis of the supernatant by hrCNE. Of the three detergent systems tested, the CLR-RAMP complexes were least stable in n-dodecyl-β-d-maltopyranoside (DDM)/CHS with the RAMP1 complex falling apart at 20–28 °C, the RAMP2 complex being unstable at all temperatures tested, and the RAMP3 complex breaking down above 28 °C (Fig. S2A). The three receptor complexes had similar thermostabilities in LMNG/CHS and LMNG/glyco-diosgenin (GDN)/CHS, with each complex being stable up to at least 37 °C (Fig. S2, B and C). All experiments hereafter used LMNG/CHS because it solubilized well and gave similar stabilities for the three CLR-RAMP complexes.

**Tagged receptor constructs used for hrCNE gel assays exhibit normal cAMP signaling**

We sought to use the native PAGE assay to characterize peptide ligand and mini-G protein binding to the CLR-RAMP complexes. Toward this end we generated new MBP–RAMP constructs with their native C termini to eliminate the possibility of steric hindrance of mG coupling from the mCitrine tag (Fig. 2A). Co-expression of these constructs with MBP–CLR–EGFP in HEK293S GntT1 cells, solubilization, and hrCNE analysis revealed formation of detergent-stable complexes visible by in-gel fluorescence from the EGFP (Fig. 2B). To ensure that these complexes maintained cAMP signaling properties comparable with the untagged receptors, we co-expressed each of the three tagged complexes in COS-7 cells and measured cAMP accumulation in response to the αCGRP, AM, and AM2/IMD agonists (Fig. 2, C–E). All three complexes exhibited agonist selectivity profiles comparable with those observed for the WT receptors (Fig. 2F), and AM2/IMD was a partial agonist at the RAMP2 complex as reported (37). Table S1 summarizes the potencies obtained in the signaling assays. The agonist potencies were slightly reduced at the tagged constructs as compared with WT receptors, but this effect was minor. The tagged constructs were thus good surrogates for use in experiments to define the ligand and mG-binding properties of the complexes using the hrCNE gel assay.

**Agonist-dependent coupling of CLR-RAMP complexes to mG₃ monitored by hrCNE gel mobility shift**

To measure peptide ligand and mG binding to the receptor complexes, we turned to the use of membrane preparations to enable uniform receptor addition across multiple reactions. Large-scale transfections of HEK293S GntT1 cells were performed for each of the three MBP–CLR–EGFP-MBP–RAMP complexes, the cells were harvested, and crude membranes were prepared. The heterodimer concentration in each preparation was estimated by LMNG/CHS solubilization followed by analysis on SDS-PAGE with comparison to known amounts of purified MBP–EGFP (Fig. S3A). The membrane preparations were each estimated to contain ~100 nM heterodimer enabling their use as 10× stocks to give ~10 nM receptor heterodimer final concentrations in the reactions. All hrCNE experiments hereafter used the membrane preparations. We expressed the mG₃, mG₄, and mG₅ proteins as N-terminally His₆-tagged SUMO–mG fusion proteins in *Escherichia coli* and purified them by immobilized metal affinity chromatography and gel-filtration chromatography (Fig. S3B). We originally intended to remove SUMO from the fusion proteins, but we found that the fusions worked better in the native PAGE assay, presumably because of their larger size (see below).

In the course of studies to assess peptide and mG binding to the receptor complexes by hrCNE assay, we noticed the tendency of the purified mG proteins to form disulfide-linked oligomers. The presence of multiple disulfide bonds in the CGRP and AM receptors and a disulfide bond in the peptide agonists prevented the use of DTT or TCEP to solve this problem. Fortunately, we found that a GSH/GSSG redox buffer could maintain the purified mG proteins in a largely reduced monomeric state without damaging the receptors and agonists. For the hrCNE experiments going forward, the purified mG proteins were preincubated in a GSH/GSSG redox buffer.
G protein coupling to the CGRP and AM receptors

Figure 2. Tagged CLR RAMP constructs used for hrCNE gel assays respond with the appropriate selectivity pattern to peptide agonists in a cell-based cAMP signaling assay. A, modified receptor constructs used hereafter have N-terminal MBP tags on the CLR and RAMPs and an EGFP tag on the CLR C terminus. B, 8% hrCNE gel with LMNG/CHS-solubilized CLR-RAMP complexes (lanes marked with R1, R2, and R3) that were co-expressed in HEK293S GnT1 cells shows a mobility shift (starred) relative to the fluorescent band showing CLR (lanes marked with C) expressed alone. C–E, representative concentration-response curves (n = 3) showing cAMP accumulation with each of the three CLR-RAMP constructs (tagged as in A) transiently expressed in COS-7 cells and stimulated with the indicated agonists. Error bars indicate S.D. for the duplicate technical replicates. F, scatter plot summarizing the cAMP-signaling potencies of three AMP antagonists from C–E obtained from three independent replicates with error bars showing S.E. Each CLR-RAMP complex is denoted as R1, R2, and R3. See Table S1 for a summary of the pEC50 values with error and statistical analyses.

Before addition to the binding reactions as described under “Experimental procedures.”

Exogenous synthetic peptide ligands (10 μM) and purified SUMO–mGα (50 μM) were added to the membrane preparations either alone or in combination in the presence of apyrase and incubated on ice for 30 min followed by solubilization with LMNG/CHS for 2 h at 4°C. The solubilized reactions were centrifuged, and the supernatants were analyzed by hrCNE to look for mobility shifts indicative of interactions (Fig. 3A). These experiments used three types of peptide ligands: short single-site ECD-binding high-affinity agonist variants (21, 25, 26), traditional dual site ECD/TMD-binding antagonists that lack the N-terminal disulfide-bonded loop, and the disulfide loop-containing agonists that bind both domains. αCGRP peptides were used for the CLR-RAMP1 complex, AM peptides for the CLR-RAMP2 complex, and AM2/IMD peptides for the CLR-RAMP3 complex as described in the Fig. 3 and Fig. S4 legends. No substantial change in mobility of the receptor heterodimer bands was observed in the presence of peptide antagonists, agonists, or SUMO–mGα alone or with the antagonists together with SUMO–mGα. In contrast, agonist and SUMO–mGα together yielded a prominent mobility shift for each of the three receptors consistent with formation of a stable agonist-CLR-RAMP-mGα quaternary complex (Fig. 3A).

To ensure that the shifted bands contained SUMO–mGα, SUMO protease was added to cleave the fusion protein, which should result in a smaller shift for the putative quaternary complex band. This was the case for the RAMP1 complex, whereas for the RAMP2/3 complexes, the protease treatment eliminated the shift altogether (Fig. 3A). Analysis of the reactions by denaturing SDS-PAGE with Coomassie Blue staining indicated that the SUMO–mGα fusion protein was properly cut by the protease (Fig. 4A). It is unclear whether the failure of the CLR-RAMP2/3 heterodimer bands to shift in the presence of SUMO protease–liberated mGα was because it did not couple to these receptors or because it bound but did not cause a mobility shift. Nonetheless, these data provided strong evidence for agonist-dependent coupling of the SUMO–mGα fusion protein to each receptor. We also performed the Fig. 3A experiments in the absence of apyrase and obtained similar results (Fig. 4B); however, we elected to include apyrase in all hrCNE assay experiments hereafter.

Next, we assessed the abilities of small molecule and peptide antagonists to antagonize the agonist- and SUMO–mGα-dependent mobility shift of the heterodimer bands. The small molecule antagonist telcagepant was developed as a potential migraine drug. It is specific for the CLR-RAMP1 complex in which it binds a pocket in the ECD complex to block anchoring of the peptide agonist C terminus (22, 23). High-affinity single-site ECD-binding (25, 26) and traditional dual site ECD/TMD-binding peptide antagonists appropriate for each receptor complex were used as described in the Fig. 3 legend. Each of the peptide antagonists prevented formation of the shifted bands, and telcagepant selectively prevented formation of the shifted band for the CLR-RAMP1 complex as expected (Fig. 3B). These results provided further evidence that the shifted bands were the quaternary complexes and demonstrated that the detergent-solubilized receptors responded appropriately to antagonists.
G protein coupling to the CGRP and AM receptors

**Figure 3.** Agonist-dependent coupling of detergent-solubilized CLR-RAMP complexes to mG, and inhibition of quaternary complex formation by small molecule and peptide antagonists assessed by the hrCNE gel shift assay. Fluorescently imaged 8% hrCNE gels using membrane preparations of MBP–CLR-EGFP and MBP-RAMP complexes (CLR/RAMP1, CLR/RAMP2, and CLR/RAMP3) that were solubilized in LMNG/CHS in the presence/absence of ligands and SUMO–mGs. A, quaternary complex formation. 10 µM agonist or antagonist, 50 µM SUMO–mGs, and 5 µM SUMO protease were added to the membrane preparations as indicated prior to solubilization. Shifted bands signifying formation of a quaternary complex are starred. For CLR/RAMP1, the agonist was αCGRP(1–37), the ECD antagonist was αCGRP(27–37) N31/D35/S34/P37/K35/S34/W36/S38, and the ECD/TMD antagonist was αCGRP(37–52) S38/G51/S52/W53/S54. For CLR/RAMP2 the agonist was AM(13–52), the ECD antagonist was AM(37–52) S48/Q51/S52/W53, and the ECD/TMD antagonist was AM(22–52). For CLR/RAMP3 the agonist was AM2/IMD(32–47) H45/S, and the ECD/TMD antagonist was AM2/IMD(16–47); B, antagonism of quaternary complex formation with simultaneous addition of 300 nM agonists, 10 µM competitive antagonists, and SUMO–mGs (50 µM for CLR/RAMP1 and CLR/RAMP2, and 25 µM for CLR/RAMP3) to the CLR/RAMP membrane preparations before LMNG/CHS solubilization. The agonists were αCGRP(1–37) for CLR/RAMP1, AM(13–52) for CLR/RAMP2, and AM(13–52) for CLR/RAMP3. Antagonists included the CLR/RAMP1-selective small molecule telcagepant and peptide antagonists. For CLR/RAMP1 the ECD antagonist was αCGRP(27–37) N31/D35/S34/P37/K35/S34/W36/S38, and the ECD/TMD antagonist was αCGRP(37–52). For CLR/RAMP2 the ECD antagonist was AM(37–52) S48/Q51/S52/W53, and the ECD/TMD antagonist was AM(22–52). For CLR/RAMP3 the ECD antagonist was AM2(37–52) S45/R49/K54/L58/S49/S50, and the ECD/TMD antagonist was AM2(IMD(16–47). For CLR/RAMP3 the reactions were solubilized overnight (13 h) because 2 h was insufficient to reach equilibrium with the ECD antagonist. A and B show representative images from two independent experiments.

**Quantitation of peptide agonist and mG, binding to CLR-RAMP complexes by hrCNE gel mobility shift assays**

The agonist apparent binding affinities for the CLR-RAMP complexes in the presence of an excess of SUMO–mGs were determined using the hrCNE gel mobility shift assay. SUMO–mGs was held constant at a high concentration (25 or 50 µM), and the peptide agonists were varied from ~1 nM to 10 µM (Fig. 4A). The appearance of the quaternary complex band (Fig. 4, B–D) and the disappearance of the heterodimer band (Fig. 5) were quantified by densitometry. Fig. 4 (E and F) shows scatter plots of the pEC$_{50}$ values resulting from these analyses applied to three independent replicates, and Table S2 summarizes the values. The two methods of quantitation gave similar results, but hereafter we focus on the values derived from the quaternary complex band appearance. At the CLR-RAMP1 complex, the strongest apparent affinity (EC$_{50}$) was observed for αCGRP (36 nM) followed by AM2/IMD (68 nM) and then AM with weaker binding (350 nM) (Fig. 4, B and E, and Table S2). At the CLR-RAMP2 complex, similar apparent affinities were observed for AM (45 nM) and AM2/IMD (70 nM), and αCGRP exhibited very weak binding with an unmeasurable apparent affinity greater than 1 µM (Fig. 4, C and E, and Table S2). At the CLR-RAMP3 complex, AM and AM2/IMD had similar apparent affinities of ~35 nM, and αCGRP binding was much weaker with an apparent affinity of 460 nM (Fig. 4, D and E, and Table S2).

Next, we performed the reverse experiment to determine the apparent binding affinities of SUMO–mGs, for each of the receptor complexes occupied by each of the peptide agonists. The agonist was held constant at a high concentration (10 µM), and SUMO–mGs was varied from ~1 nM to 75 µM (Fig. 5A). Densitometry was used to quantify the appearance of the quaternary complex band (Fig. 5, B–D) and the disappearance of the heterodimer band (Fig. 5) with increasing SUMO–mGs. Fig. 5 (E and F) shows scatter plots of the pEC$_{50}$ values resulting from these analyses applied to three independent replicates, and the values are summarized in Table S3. At the CLR-RAMP1 complex, αCGRP elicited an apparent mG, affinity (EC$_{50}$) of ~200 nM, whereas the AM and AM2/IMD peptides equally promoted mG, binding with apparent affinities of ~5 µM (Fig. 5, B and E, and Table S3). At the CLR-RAMP2 complex, the three peptides elicited weak apparent mG, affinities estimated to be ~70 µM for AM or AM2/IMD and ~120 µM for CGRP (Fig. 5, C and E, and Table S3). AM and AM2/IMD elicited equal apparent mG, affinities of ~10 µM at the CLR-RAMP3 complex, whereas αCGRP promoted mG, binding with an apparent affinity of ~40 µM (Fig. 5, D and E, and Table S3).

**Coupling of mG, and mG, chimeras to the CLR-RAMP complexes by hrCNE assay**

Coupling of purified SUMO–mG, and SUMO–mG, chimeras to each of the receptor complexes occupied by each of the three agonists was assessed in experiments like those presented in Fig. 5, but we did not quantitate these experiments by densitometry because weaker binding of these mG, proteins was observed (Fig. 6). The most notable coupling detected was that elicited by αCGRP at the CLR-RAMP1 complex where binding of the mG, and mG, proteins was observed with micromolar concentrations and the mG, exhibited greater apparent affinity than the mG, (Fig. 6, A and B). For the two AM peptides and the two AM receptors, there was evidence for weak mG, coupling, whereas coupling to mG, appeared to be limited. These results taken together with those in Fig. 5 were consistent with a coupling preference rank order for each receptor complex of mG, > mG, > mG, and there did not appear to be significant agonist-dependent alterations of this ranking. For completeness we also assessed the coupling of the three
SUMO–mG proteins to each of the receptors occupied with the βCGRP agonist, which differs from αCGRP at only three positions. The assays with βCGRP were very similar to those with αCGRP (Fig. S7).

The utility of hrCNE gel mobility shift assays to predict signaling behavior in cells: the ECD complex–binding segments of AM and AM2/IMD drive their cAMP-signaling potency differences at the CGRP receptor

Comparing the cAMP signaling results (Fig. 2) with the hrCNE gel shift assay results for agonist binding to SUMO–mGα–coupled receptors (Fig. 4) and SUMO–mGβ binding to the agonist-occupied receptors (Fig. 5) raised the question of how the hrCNE gel assay results related to the signaling outcomes observed in cells. We hypothesized that the apparent affinities of the agonists for the SUMO–mGα–coupled receptors were largely driven by the single-site interaction of the N-terminal half of the agonist with the CLR TMD. To test this we focused on the AM and AM2/IMD agonists at the CLR-RAMP1 complex. These agonists exhibited an ~8-fold difference in cAMP-signaling potencies (Fig. 2, C and F) that appeared to be reflected in their different apparent affinities for the SUMO–mGα–coupled receptor (Fig. 4, B and E). In contrast, these two agonists exhibited identical abilities to recruit SUMO–mGα to the agonist-occupied receptor (Fig. 5, B and E).

We reasoned that the different AM and AM2/IMD signaling potencies and apparent affinities for the SUMO–mGα–coupled receptor were largely due to the different affinities of these two peptides for the CLR-RAMP1 ECD complex that we previously reported (21). Thus, we predicted that chimeras of the AM and AM2/IMD agonists with their N-terminal TMD-binding and C-terminal ECD complex-binding segments swapped (Fig. 7, A and B) should exhibit swapped cAMP-signaling potencies with the stronger signaling potency arising from the stronger ECD complex–binding affinity observed for AM2/IMD at the CLR-RAMP1 ECD complex (Fig. 7, B and C). Indeed, as predicted

Figure 4. Measuring agonist apparent binding affinities for the detergent-solubilized CLR-RAMP complexes in the presence of excess SUMO–mGα by hrCNE gel shift assay. A, 8% hrCNE gels showing fluorescent bands corresponding to the MBP–CLR-EGFP (CLR) and MBP–RAMP (RAMP1, RAMP2, and RAMP3) heterodimers or quaternary complexes formed in the presence of an excess of SUMO–mGα (50 μM for CLR-RAMP1 and CLR-RAMP2, and 25 μM for CLR-RAMP3) and increasing concentrations of the indicated agonist peptides added to the membrane preparations prior to LMNG/CHS solubilization. Each gel is a representative from three independent experiments. B–D, binding curves with the quantitation of quaternary complex band appearance from A by densitometry. E, scatter plot showing the replicate pEC50 values for appearance of the quaternary complex band quantified by densitometry. Each CLR RAMP complex is denoted as R1/2/3. F, replicate pEC50 values for the disappearance of the heterodimer band quantified by densitometry. See Table S2 for a summary of the pEC50 values with error and statistical analyses.
the AM(13–33)–AM2/IMD(28–47) and AM2/IMD(8–27)–AM (34–52) agonist chimeras exhibited cAMP-signaling potencies at the untagged, WT CLR·RAMP1 complex expressed in COS-7 cells that were essentially equivalent to those of the WT agonists that corresponded to their C-terminal segment identity (Fig. 7, D and E, and Table S4). These results indicated that the hrCNE gel shift assays with detergent-solubilized tagged receptors and SUMO–mG₄s reported on physiologically relevant biochemistry of the CGRP receptor.

**Discussion**

The hrCNE method is a powerful technique for assessing the formation of membrane protein complexes in detergents (36, 39). Our results with the CLR·RAMP complexes using the HEK293S GnT1− cell line to eliminate N-glycan heterogeneity showed its capability to provide sharp defined bands even for dynamic membrane proteins such as GPCRs. The MBP tags and stabilizing effects of the RAMPs on CLR may also have contributed to the sharp bands we obtained. The CLR·RAMP complexes were specific because we saw no evidence for detergent-stable PTH1R·RAMP complexes (Fig. 1C). This was a nice control, but it was also a bit surprising because PTH1R has been reported to interact with RAMPs. Christopoulos et al. (40) showed an interaction of RAMP2, but not RAMP1/3, with PTH1R using an assay based on trafficking to the cell surface in HEK293 cells. More recently, using a bead-based immunoassay with DDM-solubilized HEK293 cells expressing epitope-tagged receptors, Lorenzen et al. (17) reported interaction of all three RAMPs with PTH1R. Interestingly, they also detected interactions of all three RAMPs with CLR in DDM. This appears to conflict with our observation that the CLR·RAMP2 complex was unstable in DDM/CHS (Fig. S2A). These discrepancies may reflect a requirement for fairly stable complexes to survive electrophoresis in the hrCNE method, or alternatively they may
Evidence that the shifted and SUMO could be observed using solubilized adherent cultures of the indicated agonist peptides and increasing concentrations of SUMO (as evidenced by the decreased intensities of the heterodimer bands observed at this concentration in the absence of agonist (Fig. 3A)). The tagged CLR and RAMP constructs used for the hrCNE assays with the membrane preparations were quite large (~180 kDa for the heterodimers), so it is not surprising that we did not observe mobility shifts in the presence of the much smaller peptide antagonists or agonists (2–5 kDa) alone, even though these were almost certainly bound to the receptors (Fig. 3A). In future work it should be possible to use peptide ligands labeled with various fluorophores to characterize their binding to the uncoupled receptors. We saw no evidence for agonist-promoted coupling of the tagged receptors to endogenous G proteins in the HEK293 cells, presumably because their levels were low relative to the overexpressed mG proteins N-terminally tagged with SUMO. It is unclear why the SUMO protease−liberated mG\(_s\) failed to generate a mobility shift with the agonist-occupied RAMP2 and -3 complexes. It may have bound without causing a mobility shift because of compensating conformational and/or charge changes, or alternatively SUMO may have provided nonspecific interactions with the detergent micelle that increased the fusion protein affinity for the detergent-solubilized receptors. Even if the latter were the case, it is unlikely to have altered the mG interactions with the receptors that are relevant to this study. mG proteins N-terminally tagged with...
fluorescent proteins have been used in live-cell studies with no apparent detriment to their function (33).

The binding experiments in Figs. 4 and 5 demonstrated that the hrCNE gel assays could provide quantitative information for the binding of peptide agonists to the mG<sub>s</sub>-coupled receptors and for mG<sub>s</sub> binding to the agonist-occupied receptors. The two binding events are cooperative and saturating the receptor with mG<sub>s</sub> in the absence of agonist was not feasible. In addition, we could not rule out the possibility of some perturbation of the equilibrium occurring during the electrophoresis. For these reasons we chose to conservatively fit pEC<sub>50</sub> values and described these as apparent binding affinities. Moreover, for some of the experiments where the agonist was varied, we were approaching ligand-depletion conditions because we had to use ~10 nM receptor to obtain a good fluorescence signal. This was reflected in the increased steepness of these curves, which were better fit with a four-parameter variable slope model (Fig. 4) than the fixed-slope three-parameter model that was used for the experiments in which SUMO–mG<sub>s</sub> was varied (Fig. 5). Nonetheless, the agonist apparent affinity values we obtained were reasonable considering their known pharmacology (16), and the apparent affinities determined for SUMO–mG<sub>s</sub> were in line with a prior study. Using a different binding assay, Nehmé <i>et al.</i> (31) measured apparent K<sub>D</sub> values of 200 and 430 nM for mG<sub>s</sub> binding the DDM-solubilized, agonist-occupied β<sub>1</sub>-adrenergic and adenosine 2A receptors, respectively. We obtained a similar apparent affinity value (EC<sub>50</sub>) of ~200 nM for SUMO–mG<sub>s</sub> binding to the CGRP-occupied CGRP receptor, but the other agonist-receptor pairings yielded lower mG<sub>s</sub> apparent affinities in the micromolar range.

Not surprisingly, the Fig. 4 and 5 experiments indicated agonist-dependent control of mG<sub>s</sub> coupling. More importantly,
however, the Fig. 5 experiments also provided evidence that the RAMPs modulate CLR G protein coupling in addition to their well-known function of modulating agonist binding. This is most evident from the data for the AM2/IMD agonist. It exhibited similar double-digit nanomolar apparent affinities for each SUMO–mG₄-coupled receptor (Fig. 4), but the apparent affinities of SUMO–mG₃ for the AM2/IMD-occupied receptors varied, with RAMP1 promoting the strongest apparent affinity (~5 μM), followed by RAMP3 (~10 μM) and then RAMP2 (~70 μM) (Fig. 5). These mG₃ affinity differences support differential modulation of CLR G protein coupling by the three RAMPs. Stronger RAMP1-mediated coupling was also evident in the experiments with the mG₃sq chimera (Fig. 6A). The recent cryo-EM studies of active state CLR RAMP complexes provide a possible molecular basis for these affinity differences. Analysis of dynamic information present in the cryo-EM data found evidence for coordinated motions between the ECD complexes and the G protein that appeared to be RAMP-dependent, with the ECD complex motion being most restricted by RAMP1, followed by RAMP3 and then RAMP2 (23).

In a previous study, Weston et al. (29) examined G protein coupling to CLR RAMP complexes expressed in yeast in which the endogenous G protein GPA1 was modified to contain the C-terminal five amino acids of eleven different human Ga subunits. They observed coupling of each CLR-RAMP complex to the chimeras with G3, Gsq, and G6 and reported dramatic agonist- and RAMP-dependent signaling bias. For each receptor complex, agonist potency rank order changes were observed with the three different G protein chimeras. Some of these findings appeared to translate to signaling studies in HEK293 cells. In the mG₃sq and mG₄sq chimeras we used here, all residues predicted to interact with the GPCR within the larger 19 amino acid segment nearest the C terminus were exchanged. Our Fig. 5 and 6 data were consistent with a coupling rank order of mG₃ > mG₄sq > mG₃sq for each receptor, and the different agonists did not appear to elicit substantial changes to this pattern. Thus, dramatic ligand- or RAMP-dependent biasing of G coupling preferences was not evident in our assays, although the weak mG₄sq and mG₃sq binding we observed could have prevented detection of bias. The native PAGE assay is likely less sensitive than the yeast signaling assay used by Weston et al. (29). Nonetheless, our data appear to be consistent with the study of Garelja et al. (28) in which activation of several signaling pathways downstream of the CLR-RAMP complexes was measured in COS-7 cells, and more balanced signaling was generally observed. In this study, agonist potency rank order changes were largely limited to experiments measuring ERK activation via the CLR-RAMP1 complex. Notably, they reported that only the CGRP agonist–CLR-RAMP1 pairing was able to elicit a measurable IP1 response (considered to be downstream of G3), and this is consistent with our findings that mG₃sq coupling was strongest for CGRP at CLR-RAMP1, and the two AM peptides more weakly promoted mG₃sq recruitment to the three receptors. Ultimately, obtaining a thorough understanding of signaling bias at the CLR-RAMP complexes will require additional studies with various methods.

Our results provide important insights into the two-domain ligand-binding mechanism and how agonist engagement of the CLR ECD and TMD contributes to G protein recruitment and ultimately the signaling response. The selectivity profiles obtained for agonist binding to the SUMO–mG₃-coupled receptors (Fig. 4, E and F) were similar to the agonist cAMP-signaling potency selectivity profiles (Fig. 2F). They were also similar to the peptide ECD complex-binding selectivity profiles previously reported (Fig. 7C) (21), with some deviations likely caused by additional selectivity-altering contacts of the agonists with the TMD. In contrast, the selectivity patterns obtained for SUMO–mG₆, binding to the agonist-occupied receptors (Fig. 5, E and F) were distinct from the cAMP-signaling potency and agonist-binding patterns. The two AM peptides equally recruited SUMO–mG₆ to the CGRP receptor despite having different signaling potencies and affinities for the SUMO–mG₆-coupled receptor. Also, CGRP was almost as good as the two AM peptides at recruiting SUMO–mG₆ to the two AM receptors despite its signaling potency and affinity for the SUMO–mG₆-coupled receptors being substantially weaker. Comparing the results in Figs. 2, 4, 5, and 7C strongly suggested that the affinities of the agonists for the SUMO–mG₃-coupled receptors were determined by their dual-site interactions with the CLR-RAMP ECD complex and the CLR TMD, whereas the recruitment of SUMO–mG₆, to the agonist-occupied receptors was largely driven by the single-site interaction of the N-terminal half of the agonist with the CLR TMD.

SUMO–mG₃ recruitment to the agonist-occupied receptors reflects agonist efficacy in part. mG₃ mimics the nucleotide-free heterotrimer, so it can be thought of as a surrogate for measuring G protein recruitment and GDP release. However, because GTP binding and G protein dissociation are not incorporated, mG₃ recruitment does not provide a full measure of efficacy. Indeed, different agonists can have different efficacies for a single G₃-coupled GPCR because they promote altered G-protein conformations with different sensitivities to GTP binding and ternary complex disruption (39). Nonetheless, for most of the agonist-receptor pairings studied here, our data appear to provide a reasonable explanation for how the agonist affinity and “efficacy,” as reflected by its SUMO–mG₃ recruitment ability, dictate its cAMP-signaling potency. The AM and AM2/IMD agonist chimeras experiments showed that their different cAMP-signaling potencies at CLR-RAMP1 were determined by their C-terminal ECD-binding segments, which have different ECD complex affinities (Fig. 7). These results were consistent with the N-terminal TMD-binding segment of the two AM peptides having equal efficacies for signaling through G3 at this receptor as suggested by the SUMO–mG₆ binding assay (Fig. 5, B and E).

Our data provide a plausible explanation for why CGRP signaling potency at CLR-RAMP1 and its binding affinity for the SUMO–mG₃-coupled receptor were stronger than AM2/IMD (Figs. 2, C and F, and 4, B and E), even though its ECD complex-binding affinity was weaker (Fig. 7C). This likely results from the much stronger SUMO–mG₃ recruitment promoted by the CGRP N-terminal TMD-binding segment as compared with AM2/IMD (Fig. 5, B and E), which may be due in part to CGRP residue Ala² in the disulfide-linked loop, which is a Gly at the equivalent positions in AM2/IMD (Gly¹³) and AM (Gly¹⁸). It was recently reported that AM2/IMD G13A and AM
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G19A exhibited increased cAMP-signaling potencies and CGRP A5G had decreased cAMP-signaling potencies at all three receptors (28, 37). Last, our data suggest that the differences in cAMP-signaling potencies of the agonists at the CLR-RAMP2 and CLR-RAMP3 complexes (Fig. 2, D–F) are determined in large part by their different ECD complexes—binding affinities (Fig. 7C) and to a lesser extent by their abilities to recruit SUMO–mG, which were similar (Fig. 5, C–E). One signaling aspect for which our native PAGE data did not provide an obvious explanation is the partial agonism of AM2/IMD at the CLR-RAMP2 complex.

In conclusion, our results revealed a role for RAMPs in modulating CLR G protein coupling and provided a framework for understanding how agonist engagement of the two domains of CLR affects transducer recruitment and cAMP signaling. Our data support a model in which the binding of the C-terminal half of the agonists to the ECD complexes is a primary determinant of receptor selectivity, and in several cases this also largely explains their differences in cAMP-signaling potencies. The binding of the N-terminal half of the agonists to the CLR TMD determines the strength of G protein recruitment, and for the CGRP agonist at CLR-RAMP1, this component is a significant contributor to its stronger cAMP-signaling potency. This information provides a valuable biochemical foundation for understanding the shared and distinct signaling properties of CGRP, AM, and AM2/IMD in human physiology and disease and guiding the development of therapeutic peptide analogs with desired signaling properties.

Experimental procedures

Reagents

Chemicals were purchased from Sigma–Aldrich. Restriction enzymes, NEBuilder HIFI Gibson assembly mastermix, T4 DNA ligase, Phusion polymerase, and apyrase were purchased from New England Biolabs. LMNG, DDM, GDN, and CHS were purchased from Anatrace (Maumee, OH). Branched PEI was from Sigma–Aldrich.

Cell culture

Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/liter glucose and l-glutamine) and nonessential amino acids (NEAAs) were from Lonza (Basel, Switzerland). Fetal bovine serum, trypsin–EDTA, and PBS were from Life Technologies. COS-7 cells (CRL-1651) and HEK293S GnT1– cells (CRL-3022) were from the American Type Culture Collection (Manassas, VA). COS-7 cells were grown in DMEM with 10% (v/v) FBS, and HEK293S GnT1– cells were grown in DMEM with 10% (v/v) FBS and 1× NEAA, both at 37°C, 5% CO2 in a humidified incubator unless otherwise noted.

Plasmids

Plasmids for expressing the tagged human receptors in mammalian cells used the pHLsec vector (45). The GPCRs were tagged at their N terminus with an HPC4 epitope tag and MBP and at their C terminus with monomeric EGFP and a 1D4 epitope tag. 3C protease cleavage sites flanked the receptors. The receptor constructs were as follows (with the receptor amino acid residue numbers indicated): HPC4-MBP-3C-CLR.29–403-3C-EGFP-1D4 (pMW084) and HPC4-MBP-3C-PTH1R.29–478-3C-EGFP-1D4 (pMW113). The RAMPs were tagged at their N termini with MBP followed by a 3C site, and their C termini were either tagged with mCitrine or left native. The RAMP constructs were as follows: MBP-3C-RAMP1.24–148-mCitrine (pMW051), MBP-3C-RAMP2.55–175-mCitrine (pMW070), MBP-3C-RAMP3.25–148-mCitrine (pMW069), MBP-3C-RAMP1.24–148 (pAMR026), MBP-3C-RAMP2.55–175 (pAMR027), and MBP-3C-RAMP3.25–148 (pAMR011). Cloning was performed by PCR/restriction enzyme digest/ligation or Gibson assembly methods. The fusion constructs were cloned between the Agel and KpnI sites of pHLsec, and EcoRV and NotI sites flanked the receptor/RAMP-encoding fragments. Oligonucleotides were synthetized by Integrated DNA Technologies (Coralville, IA) The plasmid for bacterial expression of MBP–TEV cleavage site-EGFP-His6 (pMW086) was made by PCR amplification of EGFP-His6, restriction digest with BamHI and NotI, and ligation into a PETDuet vector with MBP–TEV in the first multiple cloning site and Dsbc in the second multiple cloning site (46). DNA sequences for expression of the three mini-G proteins: mini-G, (construct 393), mini-Gsq (construct 70), and mini-Gsi (construct 43), were purchased as Gene art strings from Thermo Fisher Scientific using E. coli codon–optimized DNA sequences based on the published amino acid sequences (31). The Gene art strings included BamHI and NotI sites that were digested, and the inserts were ligated into the pSUMOT7Amp vector for bacterial expression (Lifesensors, Malvern, PA) to produce His6–SUMO–mG, (pMW101), His6–SUMO–mGsq (pMW099), and His6–SUMO–mGsi (pMW100). The University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research core facility was used to confirm the coding sequences of all plasmids by DNA sequencing. Amino acid sequences of the tagged CLR, PTH1R, RAMP, and mini-G constructs are listed in Figs. S8–S10. For protein expression in mammalian cells, the plasmids were purified using a Macherey–Nagel midi kit according to the manufacturer’s instructions. The pcDNA3.1 plasmids encoding untagged CLR and RAMP1 used for the cAMP signaling assays in Fig. 7 were previously described (26).

Peptides

Synthetic agonist peptides: αCGRP(1–37), βCGRP(1–37), AM(13–52), and AM2/IMD(1–47) were purchased from Bachem (Torrance, CA). Antagonist peptides and agonist chimeras were custom synthesized and HPLC-purified by RS Synthesis (Louisville, KY). The lyophilized powders were dissolved in sterile ultrapure water to 10 mg/ml and stored in aliquots at −80°C. Peptide concentrations were determined by UV absorbance at 280 nm following dilution of the peptides in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Extinction coefficients were calculated from the Tyr, Trp, and cystine content of each peptide. Because CGRP peptides lack Tyr and Trp residues, peptide concentration was calculated based on the peptide content reported by Bachem (78.5% w/w). The peptide sequences are listed in Table S5.
**Bacterial expression and purification of H$_6$–SUMO–mG fusion proteins**

The proteins were expressed in *E. coli* BL21 (DE3) with 6 liters of total culture volume. The cultures were induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside at mid-log phase, and the temperature was reduced to 16°C with expression overnight. The harvested cell pellets were resuspended in 100 ml of 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 25 mM imidazole, and stored at −80°C. All purification steps were carried out on ice or at 4°C, and the column steps used an AKTA purifier (GE Healthcare). 5 mM β-mercaptoethanol, 50 μM GDP, and 1 mM MgCl$_2$ were added to the thawed, resuspended pellets. The cells were lysed by sonication, and the total lysate was spun at 25,000 × g for 30 min. The supernatant was loaded on a 50 ml of nickel-chelating Sepharose column equilibrated in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 25 mM Imidazole, 5 mM β-mercaptoethanol, 50 μM GDP, and 1 mM MgCl$_2$). The column was washed with 200 ml of buffer, with the imidazole raised to 72.5 mM and then eluted with the buffer containing 262.5 mM imidazole. The protein was concentrated by precipitation with solid ammonium sulfate to 65% saturation, and the precipitated protein was pelleted at 15,000 × g and resuspended in a minimal volume of 25 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 2 mM DTT, 1 mM MgCl$_2$, 1 μM GDP. The SUMO–mG fusions were further purified by size-exclusion chromatography using a 520-ml Superdex200HR column (GE health care) and a buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 2 mM DTT, 1 mM MgCl$_2$, 1 μM GDP. The final pooled protein was concentrated using an Amicon Ultra, 10,000-Da molecular mass cutoff concentrator (Milipore Sigma) and dialyzed overnight against 1 liter of 25 mM HEPS, pH 7.5, 150 mM NaCl, 50% (v/v) glycerol, 0.5 mM DTT, 1 mM MgCl$_2$, 1 μM GDP for storage at −80°C. Protein concentrations were determined by Bradford assay with BSA standard and confirmed by UV absorbance at 280 nm. Final yields for each protein were 30 mg for H$_6$–SUMO–mG$_3$393, 47 mg for H$_6$–SUMO–mG$_{3}$q70, and 25 mg for H$_6$–SUMO–mG$_{4}$A3.

**Bacterial expression and purification of MBP–TEV cleavage site-EGFP-H$_8$**

Expression and purification by immobilized metal affinity chromatography were as above for the SUMO–mG fusion proteins except that the buffers lacked β-ME, GDP, and MgCl$_2$. Peak fractions from the nickel column were pooled and loaded onto an amylose high flow (NEB) column pre-equilibrated in buffer C (50 mM Tris-HCl, pH 7.5, 5% (v/v) glycerol, 150 mM NaCl). The column was washed with buffer C and then eluted using a linear gradient of buffer C to D (50 mM Tris-HCl, pH 7.5, 5% (v/v) glycerol, 150 mM NaCl, 10 mM maltose), dialyzed to storage buffer (25 mM sodium HEPS, pH 7.4, 50% (v/v) glycerol, 150 mM NaCl), and stored at −80°C. Protein concentration was determined by visible absorbance at 488 nm using the molar absorptivity of EGFP (55,000 M$^{-1}$ cm$^{-1}$ (47)) and was further verified by Bradford assay using a BSA standard curve.

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**Cell-based cAMP accumulation assays**

cAMP accumulation assays were performed as previously described (21), except that stimulation time was reduced to 15 min. In brief, COS-7 cells were transiently transfected with the expression constructs using PEI, and the cells were stimulated with agonist 48 h after transfection. The cells were lysed, and the cAMP content was determined using the LANCE cAMP kit (PerkinElmer) according to the manufacturer’s directions and a PolarSTAR Omega Plate Reader with an advanced optic head (BMG Labtech, Ortenberg, Germany).

**Crude membrane preparations**

HEK293S GnT1$^-$ cells were seeded at 2.3 million cells/dish into twenty 150-mm$^2$ Petri-style plastic culture dishes (Corning) and grown for 4 days (~90% confluency). The cells were co-transfected with the HPC4-MBP-3C-CLR.29–403-3C-EGFP-1D4 (MBP–CLR–EGFP) construct and one of the three RAMP constructs (MBP–RAMP1/2/3): MBP–3C-RAMP1.24–148, MBP–3C-RAMP2.55–175, or MBP–3C-RAMP3.24–148. DNA (43 μg/dish; 1:1 ratio of the CLR and RAMP constructs) was added to 4.3 ml of DMEM followed by the addition of PEI (64.5 μg/dish). The transfection mixture was incubated at room temperature for 10 min, and then valproic acid was added to 35 mM. Growth medium was removed from each of the dishes by aspiration and replaced with 26 ml of DMEM with 2% FBS, 1% NEAA, and 50 units/ml penicillin, 50 μg/ml streptomycin/dish. 4.3 ml of the transfection mixture was added to each plate (final concentration, 5 mM valproic acid), and the dishes were incubated at 30°C and 5% CO$_2$ for 3 days.

The medium was aspirated, the cells were washed with 8 ml of PBS/plate and harvested by adding 8 ml of PBS with 5 mM EDTA and scraping with a cell scraper. The cells were divided into four 50-ml conical tubes and pelleted by centrifugation at 1000 × g for 5 min at 20°C. The supernatants were discarded, and cell pellets were resuspended in 24 ml/conical ice-cold hypotonic buffer (25 mM HEPS, pH 7.5, 2 mM MgCl$_2$, 1 mM EDTA, with 1× EDTA-free Pierce protease inhibitor tablet (PI)) and homogenized using an Ultra Turrax T25 (IKA Works, Wilmington, NC) for 30 s at 10,000 rpm with the samples cooled in an ice-water bath. The samples were incubated for 10 min and then homogenized a second time followed by a low-speed spin of 800 × g at 4°C for 10 min to pellet cell debris. The supernatants from the low-speed spin were transferred to ultracentrifuge tubes and spun at 100,000 × g for 1 h at 4°C. The pelleted membranes from four tubes were combined in 6 ml of storage buffer (25 mM HEPS, pH 7.5, 25 mM NaCl, 2 mM MgCl$_2$, 10% (v/v) glycerol, 1× PI) and homogenized three times at 5,000 rpm for 30 s with cooling in between. Single-use aliquots were flash-frozen in liquid nitrogen and stored at −80°C.

To estimate the concentration of the CLR-RAMP heterodimer in the preparation, 80 μl was diluted to 400 μl in solubilizing buffer (25 mM HEPS, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 1× PI, 0.5% (w/v) LMNG, and 0.05% (w/v) CHS) and incubated 2 h on a tube tumbler at 4°C. The lysate was spun at 16,100 × g at 4°C for 10 min. 10 μl of the supernatant was combined with 5 μl of purified MBP–TEV–EGFP at known concentrations, run on a nonreducing 12%
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SDS-PAGE gel, and imaged by in-gel fluorescence using a Chemidoc MP (Bio-Rad). The protein concentration for the MBP–3C–CLR–3C–EGFP–1D4 was estimated by comparing the band intensity with that of the MBP–EGFP. We estimated ~100 nm heterodimer in each of the membrane preparations, and this allowed their use as 10× stocks for the native PAGE assays. Total protein content was determined for the membrane preparations using the DC protein assay (Bio-Rad), yielding concentrations for the MBP–CLR–EGFP MBP–RAMP1, -2, and -3 preparations of 4.56, 4.11, and 6.36 mg/ml, respectively.

Adherent cell expression of GPCR–RAMP heterodimers and detergent solubilization

HEK293S GnT1− cells were seeded at 120,000 cells/well into 48-well plates and grown to ~90% confluency in 250 µl growth media/well. The cells were transfected with 0.3 µg of total DNA and 0.45 µg of PEI/well. For co-transfections, 0.15 µg of each receptor component was used per well. The DNA and PEI were combined in 30 µl of DMEM, incubated at room temperature for 10 min, and then valproic acid was added (46.7 mM). The growth medium was removed by aspiration and replaced with 250 µl of DMEM with 2% FBS, 1× NEAA, 50 units/ml penicillin, 50 µg/ml streptomycin, and 30 µl of the transfection mixture. The plates were incubated for 3 days at 30°C, 5% CO2. For solubilization, the medium was removed by aspiration, the cells were washed with 250 µl of PBS/well, the PBS was aspirated, and the plates were placed on ice. 100 µl of solubilization buffer (25 mM HEPES, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 3% (w/v) glycerol, 0.625× PI, 0.5% (w/v) LMNG, and 0.05% (w/v) CHS) was added to each well, and the plate was incubated at 4°C with rocking for 2 h. The lysates were transferred to prechilled microcentrifuge tubes and centrifuged at 16,100 × g at 4°C for 10 min in a benchtop microcentrifuge, and the supernatants were used for hrCNE analysis.

Thermostability assay for CLR–RAMP complexes expressed in adherent cells

HEK293S GnT1− cells were seeded at 500,000 cells/well into 12-well plates and grown to ~90% confluency (24 h). The cells were transfected with 1.2 µg of total DNA/well (1:1 ratio of CLR and RAMP constructs) using PEI as described above and a 120-µl volume transfection mixture/well. The growth medium was removed from each well by aspiration and replaced with 1 ml of DMEM with 2% FBS, 1× NEAA, 50 units/ml penicillin, 50 µg/ml streptomycin, and 120 µl of the transfection mixture, and the plate was incubated for 3 days at 30°C and 5% CO2. The medium was aspirated, the cells were washed with 1 ml PBS/well, the PBS was aspirated, and the plates were placed on ice. 200 µl of solubilization buffer was added to each well, and the plate was incubated at 4°C for 2 h. The solubilization buffers were 25 mM HEPES, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 3% (w/v) glycerol, 0.625× PI, and one of three detergent systems: 1.5% (w/v) DDM, 0.15% (w/v) CHS, 0.5% (w/v) LMNG, 0.05% CHS; or 0.25% (w/v) LMNG, 0.25% (w/v) GDN, 0.05% (w/v) CHS. The lysates were transferred to cold microcentrifuge tubes and spun at 16,100 × g, 4°C for 10 min. The supernatant was aliquoted (22 µl) into cold microcentrifuge tubes, and each tube was incubated at the indicated temperature for 30 min. The tubes were cooled on ice for 5 min and spun at 16,100 × g at 4°C for 10 min, and the supernatants were used for hrCNE analysis.

**Binding and solubilization reactions using membrane preparations with exogenous addition of ligands and SUMO–mG proteins**

These reactions were assembled in microcentrifuge tubes with 30–50 µl of total assay volume. For all assay formats (Figs. 3–6 and Figs. S4 and S7), the final solubilized reactions contained ~10 nm receptor heterodimer from the membrane preparation and 0.05 unit/ml apyrase, ligands, and purified SUMO–mG proteins as indicated in binding buffer (25 mM HEPES, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM MgCl2, 2 mM CaCl2) supplemented with 0.075 mg/ml FAF–BSA, 1–2% (w/v) glycerol, 0.538× PI, 0.5% (w/v) LMNG, and 0.05% (w/v) CHS for solubilization. Where SUMO–mG proteins were used, they were preincubated with the redox pair GSH/GSSG in binding buffer supplemented with 0.1 mg/ml FAF–BSA and 0.25× PI on ice for 30 min, because the purified mG proteins tended to form intermolecular disulphide bonds. The GSH/GSSG was present at a fixed molar ratio with the SUMO–mG such that the GSH/GSSG was diluted out proportionally to the SUMO–mG. The GSH and GSSG were kept at a 5:1 molar ratio, and unless otherwise noted, the GSH was 3-fold higher molarity than SUMO–mG, e.g. for 50 µM SUMO–mG, 150 µM GSH and 30 µM GSSG were added. Prior to solubilization, the binding reactions contained the membrane preparation and other components (apyrase, ligands, and SUMO–mG as indicated) at 1.3× of the final concentrations in binding buffer supplemented with 0.1 mg/ml FAF–BSA, 0.25× PI. The binding reactions were incubated for 30 min on ice; then detergent buffer with the detergent at 4× (binding buffer supplemented with 1× PI, 2% (w/v) LMNG, 0.2% (w/v) CHS) was added; and the reactions were tumbled at 4°C for 2 h to solubilize followed by centrifugation at 16,100 × g at 4°C for 10 min and analysis of the supernatants by hrCNE. The preincubation and set up of binding reactions differed slightly based on the assay format as described below.

For the assays investigating peptide ligand and SUMO–mG interactions with the CLR–RAMP complexes (Fig. 3A and Fig. S4), the binding reactions were assembled by first combing buffer components as a mastermix (binding buffer, with or without apyrase for Fig. 3A versus S4, FAF–BSA, and PI) followed by addition of SUMO–mG, and GSH/GSSG to indicated reaction tubes for the preincubation. Then the ligands, membrane preparation, and SUMO protease were added to the reactions as indicated.

For experiments with competing agonists and antagonists (Fig. 3B), SUMO–mG, and the GSH/GSSG redox pair were diluted to 2.67× of the final concentrations for the 30-min preincubation. Ligands, apyrase, and the membrane preparation were combined as indicated at 2.67× of final concentrations (15 µl volume) in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI. The membrane preparation was added after the ligands for simultaneous exposure to agonists and antagonists. 15 µl of the SUMO–mG with GSH/GSSG or binding buffer
supplemented with 0.1 mg/ml FAF–BSA and 0.25× PI for wells without SUMO–mG, was then added to the reaction tubes containing ligands, apyrase, and the membrane preparation.

To measure agonist binding to CLR-RAMP complexes in the presence of excess SUMO–mG (Fig. 4), the preincubation was assembled with apyrase, SUMO–mG, and GSH/GSSG at 2.67× of the final concentration in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI and incubated for 30 min on ice. The membrane preparation was then added to the mastermix. For the CLR RAMP3 reactions, the redox pair concentration was 3-fold lower (133.3 μM SUMO–mG, 133.3 μM GSH, 26.67 μM GSSG) because this receptor complex seemed to be more sensitive to the reducing agent in this assay format. The agonist was diluted to 2.67× of the final highest concentration in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI and then 3-fold serially diluted in the same buffer. The binding reactions were assembled by combining equal volumes of the master mix and agonist dilutions (or binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI for the reaction lacking agonist).

For measuring SUMO–mG binding to agonist-occupied CLR-RAMP complexes (Figs. 5 and 6 and Fig. S7), the SUMO–mG and GSH/GSSG were diluted to 2.67× of the highest final concentration (200 μM SUMO–mG with 600 μM GSH and 120 μM GSSG except in the case of SUMO–mGFP for which GSH/GSSG was 3-fold higher because it has more cysteines) in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI for the preincubation and then 3-fold serially diluted in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI. Agonists, apyrase, and the membrane preparation were combined in a mastermix at 2.67× of final concentrations in binding buffer with 0.1 mg/ml FAF–BSA and 0.25× PI, and the binding reactions were assembled by combining equal volumes of the master mix with the SUMO–mG dilution or with binding buffer supplemented with 0.1 mg/ml FAF–BSA and 0.25× PI for the reaction lacking SUMO–mG.

hrCNE and SDS-PAGE

The native gels were set up with cold buffers and run at 4 °C. The hrCNE protocols, gel, and buffer recipes were based on those described (36, 39). 8% resolving polyacrylamide hrCNE gels were used with cathode buffer: (~175 ml) 50 mM Tricine, 7.5 mM imidazole, pH 7.0, supplemented with the detergent used for solubilization at the following concentrations: 0.01% (w/v) LMNG, 0.001% (w/v) CHS; 0.02% (w/v) DDM, 0.002% (w/v) CHS; or 0.005% (w/v) LMNG, 0.005% (w/v) GDN, 0.001% (w/v) CHS; anode buffer (1 liter): 25 mM imidazole, pH 7.0. The gels were prerun at 100 V for 20 min, 20 μl of solubilized lysate supernatant was loaded, and the gels were run at 200 V for 2.5 h in experiments examining heterodimer formation or thermostatlability and for 3.5 h in experiments examining peptide and SUMO–mG interactions. The gels were rinsed in ddH₂O and imaged by in-gel fluorescence using the ProQEmerald488 preset on a Chemidoc MP (Bio-Rad). This channel detects both EGFP and mCitrine.

For SDS-PAGE gels, 15 μl of the solubilized lysate supernatants was combined with 5 μl of 4× nonreducing SDS loading dye, run on 12% polyacrylamide gels, and visualized by in-gel fluorescence as above. To excise the hrCNE gel heterodimer bands for analysis by SDS-PAGE, the fluorescent images of the gels were printed at actual size, the hrCNE gel was placed over the printed image, and a gel fragment corresponding to the dimer band was excised with a razor blade. Excised fragments were placed in the wells of a 12% SDS-PAGE gel and covered with 1× nonreducing SDS loading dye. The SDS-PAGE gels were run at 50V until the loading dye passed through the stacking gel and then at 200 V for 1.5 h. 5 μl of the WesternBio (Bio-Rad) protein standard was loaded in lane 1, and fluorescent marker bands were imaged using the Cy3 and Cy5.5 channels on the Chemidoc MP.

Densitometry analysis

All gels that were used for quantifying dimer or quaternary complex bands were imaged at similar exposure times (~80 s), ensuring that there were no saturated pixels. Densitometry analysis was conducted using the ImageLab software (Bio-Rad). Using the Lane Profile tool, the distance from the top of the gel to the boundaries of the fluorescent bands was determined. Lane 1 was used to determine the boundaries of the dimer band, and lane 10 was used to determine the boundaries of the quaternary complex band. These measurements were used to manually determine the quaternary complex and dimer bands in the other lanes. The adjusted volume (Intensity) of the selected bands was plotted against agonist or SUMO–mG concentration in GraphPad Prism. The binding curves for SUMO–mG apparent affinities for the agonist-occupied receptors were fit to a three-parameter (fixed slope) dose-response model to determine pEC₅₀ values. The binding curves for agonist apparent affinities for the mGc coupled receptors were fit to a four-parameter (variable slope) dose-response model to determine pEC₅₀ values.

Data analysis and statistics

All experiments that involved quantifying results were done as three independent experiments on different days. Statistical analysis for the SUMO–mG, and agonist affinities for the CLR-RAMP complexes quantified by densitometry was done by comparison of pEC₅₀ values for appearance of the quaternary complex band or disappearance of the dimer band using an ordinary one-way analysis of variance with Tukey’s multiple comparisons test. For the cell-based assays measuring cAMP potencies at the tagged constructs (Fig. 2 and Table S1) and comparing WT to chimera peptides at the WT untagged receptors (Fig. 7 and Table S4), pEC₅₀ values were compared using an ordinary one-way analysis of variance with Tukey’s multiple comparisons test. Statistical significance was defined as p < 0.05.

Data availability

The data described in this article are available from the corresponding author (augen-pioszak@ouhsc.edu) upon reasonable request.

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M. R. and A. A. P. writing-review and editing; A. A. P. supervision; A. A. P. project administration.

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Abbreviations—The abbreviations used are: CGRP, calcitonin gene-related peptide; AM, adrenomedullin; IMD, intermedin; CLR, calcitonin receptor-like receptor; RAMP, receptor activity–modifying protein; mG, mini-G; GPCR, G protein–coupled receptor; ECD, extracellular domain; TMD, transmembrane domain; hrCNE, high-resolution clear native electrophoresis; MBP, maltose-binding protein; PEI, polyethyleneimine; LMNG, lauryl maltose neopentyl glycol; CHS, cholesteryl hemisuccinate; PTH1R, parathyroid hormone receptor; DDM, n-dodecyl-β-d-maltopyranoside; GDN, glyco-diosgen; DMEM, Dulbecco’s modified Eagle’s medium; NEAAs, non-essential amino acid; FBS, fetal bovine serum; PI, protease inhibitor tablet; EGF, enhanced GFP; FAF, fatty acid-free; FSEC, fluorescence-detection size-exclusion chromatography.

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